AGE-ASSOCIATED INFLAMMATION IMPAIRS MYELOID DEVELOPMENT AND MONOCYTE AND MACROPHAGE FUNCTION

AGE-ASSOCIATED INFLAMMATION IMPAIRS MYELOID DEVELOPMENT AND MONOCYTE AND MACROPHAGE FUNCTION

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Doctor of Philosophy

McMaster University
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TITLE: Age-associated inflammation impairs myeloid development and monocyte and macrophage function

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LAY ABSTRACT

Inflammation in the absence of infection or injury naturally increases with age. Healthy aging is influenced by an individual's level of inflammation. The risk of developing chronic disease (e.g. heart disease, diabetes, arthritis) and mortality increases in people who have higher than age-average levels of inflammation. Monocytes and macrophages are cells of the immune system whose numbers and functions change with age, as well as in diseases that increase in prevalence with age. The goal of this thesis was to better understand how increased levels of inflammation affect monocyte and macrophage numbers and functions, which we believe increases susceptibility to chronic disease. We found that high levels of inflammation increase the numbers of these cells but they cannot properly perform their functions. By decreasing inflammation we were able to reverse some of these changes. These findings suggest that decreasing inflammation may prevent and slow the progression of chronic age-associated diseases.

ABSTRACT

As the population ages, the burden of chronic inflammatory disease increases and novel interventions to keep older adults healthier for longer are urgently required. Individuals with lower than age-average levels of circulating inflammatory cytokines (e.g. TNF, IL-6) appear to live longer, healthier lives. Our data suggest that chronic exposure to inflammation in the aging microenvironment alters myeloid cell production and function, which ultimately impacts health. In humans (20-100yo) we found that serum cytokines and health conditions were stronger predictors of myeloid cell numbers, and monocyte maturity and activation than age alone. Individuals with more chronic conditions had more myeloid derived suppressor cells and a higher ratio of $CD16^+$ to $CD14^+$ monocytes. Markers of monocyte activation (e.g. CCR2, CX_3CR_1, HLA-DR, CD16) were elevated in chronic inflammatory states (e.g. rheumatoid arthritis, RA; osteoarthritis, OA) when compared to healthy age- and sex-matched controls. CCR2 expression on monocytes correlated with pain in RA and OA. Following DMARD treatment in RA patients, inflammation, monocyte CCR2 expression, and disease activity decreased.

Myeloid cells expand with age in both mice and humans. In mice, we found that this resulted from increased monopoiesis in the bone marrow and the spleen. Using heterochronic bone marrow chimeras, we determined that this process is driven by tumour necrosis factor (TNF) from the aging microenvironment. Transcriptomic analysis of both circulating monocytes and alveolar macrophages revealed decreased expression of genes involved in myeloid lineage commitment and

maturation (i.e. *HDAC1, TET2, FOXO3*) in old WT mice, but not in in old TNF KOs. Although myeloid cells increase with age, their function is impaired. We found that both life-long and acute exposure to TNF can impair phagolysosomal fusion and decrease bacterial killing capacity of macrophages. Overall, these studies demonstrate that age-associated inflammation accelerates myeloid cell production at the expense of proper differentiation and function.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACPA	anti-citrullinated protein antibody
AIC	Akaike information criterion
AML	acute myeloid leukemia
ANOVA	analysis of variance
Arg-1	arginase-1
BMI	body mass index
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CDAI	clinical disease activity index
cDC	conventional dendritic cell
CDP	common DC progenitor
CEBP	CCAAT/enhancer-binding protein
CEBP	CCAAT/enhancer-binding protein
cMoP	committed monocyte progenitor
CMP	common myeloid progenitor
CMV	Cytomegalovirus
COPD	chronic obstructive pulmonary disorder
CRP	c-reactive protein
CSF-1	colony-stimulating factor 1
CX3CR1	CX3C chemokine receptor 1
DAMPs	danger associated molecular patterns
DC	dendritic cell
DMARD	disease-modifying anti-rheumatic drug

EMH	extramedullary hematopoiesis
EMP	erythromyeloid progenitor
ESR	erythroid sedimentation rate
FACS	Fluorescence-activation cell sorting
FL	FMS-related tyrosine kinase 3 ligand
Flt-3	FMS-related tyrosine kinase 3
G-CSF	granulocyte colony stimulating factor
GATA-6	GATA-binding protein 6
GM-CSF	granulocyte macrophage colony stimulating factor
gMDSC	granulocytic myeloid derived suppressor cell
GMP	granulocyte macrophage progenitor
GP	granulocyte progenitor
HLA-DR	human leukocyte antigen D related
HSC	hematopoietic stem cell
IDH1	isocitrate dehyrogenate 1
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IP-10	interferon gamma-induced protein 10
IRF	IFN regulatory factor
LPS	lipopolysaccharide
M-CSF	macrophage colony stimulating factor
M-CSFR	macrophage colony stimulating factor receptor
MAPK	Mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MDMs	monocyte-derived macrophages

MDP	macrophage dendritic cell progenitor
MDSC	myeloid derived suppressor cell
mMDSC	monocytic myeloid derived suppressor cell
MP	monocyte progenitor
MyD88	myeloid differentiation primary response gene 88
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NLRP3	NOD-like receptor pyrin domain-containing-3
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
PAMPs	pattern associated molecular patterns
PRR	Patter recognition receptor
pDC	plasmacytoid dendritic cell
PGE2	prostaglandin E_2
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PPARγ	peroxisome proliferator-activated receptor-γ
PTEN	phosphatase and tensin homolog
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end products
RF	rheumatoid factor
ROS	reactive oxygen species
SCF	stem cell factor
STAT	signal transducer and activator of transcription
TCR	T cell receptor
Tet2	Tet methylcytosine dioxygenase 2

TGFβ	transforming growth factor beta
TLR	toll-like receptor
TNF	tumour necrosis factor
TNFR	TNF receptor
VEGF(A)	vascular endothelial growth factor (A)
WT	wild type

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis document is an accurate representation of the experiments and novel findings that I have contributed to my field of study during my time as a doctoral candidate. The document itself and the two published manuscripts included within were written entirely by myself with editing input provided by my supervisor Dr. Dawn Bowdish. Some of the studies included collaborations with other scientists. As such my contribution and that of collaborators is described below:

Chapter 1: I designed the whole blood immunophenotyping panels and analyzed and interpreted all the data included in this chapter. Mohammad Malik and Dr. Dawn Bowdish recruited study participants. Acquiring patient consent, and sample collection and processing (e.g. immunophenotyping staining and data acquisition by FACS) was a joint effort between myself, Mohammad Malik, Grace Teskey, and Allison Kennedy. Quantification of serum cytokines was done by Mohammad Malik and myself.

Chapter 2: I processed patient samples, performed all the experiments and analyzed and interpreted the data. Drs. Maggie Larche and Raja Bobba recruited newly-referred RA patients and provided clinical care throughout the study. Barbara Baker, a clinical nurse, acquired consent from patients, collected blood samples and completed data collection on medication use, disease severity and clinical measures of CRP and ESR.

Chapter 3: I processed patient and healthy control samples, performed all the experiments and analyzed and interpreted the data. Dr. Monica Maly and Sarah

Karampatos recruited patients with knee-OA and collected data on disease activity and participant mobility. Dr. Dawn Bowdish collected blood samples from OA participants. I, along with Dr. Dawn Bowdish, recruited and acquired consent and blood samples from healthy age- and sex-matched controls.

Chapter 4.1: I performed all experiments and analyzed and interpreted all the data, with the following exceptions: Alicja Puchta performed animal work for experiments in Figure 4; I collected spleen tissue and performed flow analysis of monocytes. For Figure 5, Avee Naidoo prepared the heterochronic bone marrow chimeras, with my assistance in isolating and preparing donor bone marrow. I collected spleen tissue from these mice and performed flow analysis of the monocytes.

Chapter 4.2: I performed all experiments and analyzed and interpreted all the data, with the following exceptions: Christian Schulz collected lung tissue from which Alexander Misharin and his lab sorted single alveolar macrophages and performed RNA extraction, RNAsequencing and analysis (demultiplexed, trimmed and aligned reads to reference genome and generated gene counts). I performed statistical gests and generated figures using gene count data. Dr. Deborah Winter and her lab performed RNA extraction, RNAsequencing and data analysis in Figure 3.

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CHAPTER 1

Introduction

1.1 - Healthy aging and the immune system

Aging and Chronic Diseases

The oldest documented life expectancy is from the year 1543 when it was estimated to be 34 years[1]. Since then, life expectancy has more than doubled and is only projected to increase in the next 20 years[2]. Life expectancy at birth was 79.8 years for males and 83.9 years for females in Canada in 2017[3]. Much of the historical increase in life expectancy has been due to improved infant mortality with the advent of antibiotics and vaccines to treat and prevent previously fatal infections[4]. Global causes of mortality have shifted from communicable, infectious disease to non-communicable or chronic diseases including cardiovascular diseases (e.g. stroke, atherosclerosis), respiratory diseases (e.g. chronic obstructive pulmonary disease, asthma), diabetes and cancer[5]. Although life expectancy is increasing, the added years of life are not necessarily spent in good health. Health expectancy, which factors morbidity into life span, has not proportionally increased with the increase in life expectancy over the past 20 years. In fact, there has been little change to health expectancy between 1994 and 2010[6], and the majority of the population

will experience some sort of morbidity in their final 8-10 years of life[7]. From 2000 to 2011, the prevalence of heart disease, chronic obstructive pulmonary disorder (COPD) and diabetes increased in those over the age of 50[8]. Although our advancement in medical technology and the improved quality of health care have contributed to recent gains in life expectancy[2], non-communicable diseases are still a significant cause of mortality. Globally, 37.5% of all deaths attributed to non-communicable diseases occur in individuals aged 30-69[9], suggesting an earlier incidence of many of the diseases previously associated with advanced age. In fact, middle aged Canadians (aged 35-49) are at a higher risk of multimorbidity than those aged 50-64[10]. The 35-49 age group has the highest prevalence of obesity in Canada[11]. Elevated BMI is one of the biggest risk factors for developing chronic conditions and decreased life expectancy[12]. Reducing the number of years spent with morbidity not only improves health and quality of life, but it also reduces the costly care associated with managing illness and disability[13]. As such, there is a real need to understand the evolution of chronic diseases and the mechanisms driving them.

The Biology of Aging and Inflammation

There are many underlying processes that drive aging, however they all culminate in the reduced capacity for an individual to withstand and adapt to physical, social and psychological stressors. From studies in longevity we understand that our environment, lifestyle and behaviours influence our aging

trajectory[14]. Socioeconomic status is a key predictor of longevity as it is linked to our behaviours relating to physical activity, diet, substance abuse, smoking, and education, all of which impact healthy aging[15–17]. These environmental influences interact with our genetic predispositions to disease and help to explain the heterogeneity of the aging process between individuals[18]. One of our body's key sensors of the environment is the immune system. The innate immune system in particular acts as a thermostat for homeostasis. Monocytes and macrophages, cells of the innate immune system, are exquisitely equipped with an arsenal of pattern recognition receptors that can detect stress signals related to both endogenous (e.g. tissue damage, malignancy) and exogenous threats (e.g. infection). These sentinel cells initiate an inflammatory cascade meant to orchestrate the appropriate immune response to resolve the stress. An effective inflammatory response is acute and is promptly resolved, returning the system to its homeostatic state. If the immune response is insufficient in either the numbers or types of cells recruited, or their effector functions are impaired, the inflammatory response can be prolonged, leading to off-target host tissue damage[19]. If this break from the homeostatic state is maintained, it can lead to the initiation, development, or progression of other diseases[20,21]. Elevated inflammation contributes to malnutrition, decreased muscle mass and bone density, atherosclerosis and poor cardiovascular health, as well as cognitive decline, and this is independent of age[22].

As we age, the immune system's ability to respond to stress changes. This is marked by many cellular changes, to be discussed later, as well as an increase in the

basal inflammatory state. Aging can be described as a chronic inflammatory condition due to an increase in the steady-state levels of pro-inflammatory cytokines and mediators (i.e. TNF, IL-1β, IL-6, CRP) in the circulation and tissues, referred to as "inflammaging"[23]. This occurs in the absence of infection or injury and is thus sterile inflammation. Individuals with higher than age-average levels of inflammation are at increased risk of many age-related diseases (i.e. cancer, atherosclerosis, chronic lower respiratory disease) as well as infectious diseases (i.e. pneumonia[24]). Increased levels of IL-6 and TNF have been linked to increased frailty[25–27] as well as morbidity and mortality in the elderly[28–31], independent of other risk factors (i.e. tobacco use, diabetes, hypertension, hypercholesterolemia)[22], suggesting that changes in immune function and inflammation underlie the biological processes of aging. In particular, monocyte and macrophage dysfunction has been described in atherosclerosis[32,33], osteoporosis[34], obesity[35], diabetes[36], fibrosis[37] and cancer[38], all of which increase in incidence with age.

Although the source of increased inflammatory mediators remains unknown, it contributes to some of the age-associated changes in monocyte and macrophage function. Our lab has previously demonstrated that old mice that are deficient in TNF are protected from age-associated inflammation and fare better than their wild type counterparts in models of nasopharyngeal colonization with *Streptococcus pneumoniae*[39]. They are also protected from the age-associated expansion of inflammatory monocytes, which produce higher levels of inflammatory cytokines following stimulation with bacterial products. The NLRP3 inflammasome, which

senses host stress and tissue damage as well as bacterial and viral infections, has been implicated in aging as NLRP3 knock out mice are protected from age-associated inflammation, glucose intolerance, thymic involution, neurodegeneration, and osteoporosis[40]. These data suggest that inflammation plays an important role in driving aging. We hypothesize that monocytes and macrophages play a fundamental role in propagating age-associated inflammation and that their development and function changes in the presence of the inflammatory environment of an aging host.

1.2 –Macrophages as mediators of homeostasis

Elie Metchnikoff first discovered macrophages in marine invertebrates, and found that they were specially equipped to take up or phagocytose foreign particles and microbes[41]. Metchnikoff went on to discover macrophages' abilities to internalize and kill bacteria, clear senescent and damaged host cells, and recruit other leukocytes to sites of infection or tissue injury[41]. The dual roles of macrophages to protect the host from infection, but also restore tissue homeostasis is mediated by their ability to phagocytose particles in their surroundings and secrete soluble mediators of inflammation (e.g. chemokines, cytokines, growth factors, low molecular weight peptides, proteinases, reactive nitrogen and oxygen species, lipid metabolites)[42]. Macrophages can underpin the processes of inflammation or healing depending on their origin and their environment. Macrophages are seeded in organs and tissues during embryogenesis and are

derived from yolk sac and fetal liver precursors[43]. In the steady state, tissue macrophage populations are fairly steady as they are self-maintaining[44]. In response to infection and sterile inflammation, they are replenished by circulating monocytes derived from bone marrow hematopoietic progenitors. Following the resolution of inflammation, hematopoietic-derived macrophages die and embryonic-derived tissue macrophages repopulate the tissue niche[45]. Some populations of tissue macrophages with high turnover, like those in the gut[46], are continuously reseeded by monocytes.

Macrophages in tissue homeostasis, metabolism and immunity

Macrophages are involved in developmental processes like angiogenesis, bone remodelling, pruning of neurological synapses and are essential to tissue patterning in the mammary gland, kidney and pancreas[47,48]. In the brain they maintain neuron growth, viability and activity[48–50]. They are also involved in liver tissue regeneration by orchestrating hepatic progenitors via Wnt ligand secretion and inhibiting Notch signaling[51]. Macrophages are additionally involved in maintaining metabolic homeostasis, due to their presence in the liver, pancreas and adipose tissue. Macrophages additionally support brown adipose tissue thermogenesis by releasing fatty acids from white adipose tissue[52,53]. During times of infection macrophages promote glycolysis by secreting pro-inflammatory cytokines TNF, IL-6 and IL-1β that decrease nutrient storage by inducing peripheral insulin resistance[35,54].

This nutrient re-allocation helps to more efficiently fuel activated immune cells to fight the infection; however, prolonged inflammation can lead to metabolic dysfunction by maintaining an insulin resistant state[55,56]. In models of excessive caloric intake (i.e. obesity), macrophages also assume an activated phenotype and can lead to impaired beta islet cell function in the pancreas, and impaired lipolysis in adipose tissue and the liver[57,58].

Tissue and monocyte-derived macrophages are essential to driving the development of leukocytes as well as initiating immune responses. Stromal macrophages are key regulators of erythropoiesis[59] and may play a significant role in instructing hematopoiesis in the bone marrow, however their role in myelopoiesis remains unclear[60–62]. In the spleen and lymph nodes, macrophages present antigen to induce adaptive immunity. In the liver, macrophages work closely with hepatocytes in response to systemic inflammation, caused by infection, malignancy or chronic conditions, to mobilize acute phase proteins and complement[42]. Macrophages in the gut contribute to fluid balance, nutrient absorption and peristalsis. More importantly, they help to maintain a fine balance between tolerating the microbes that aid our digestion and nutrition[63], while protecting us against pathogens[64]. Macrophages in the lung clear inhaled particulates, regulate surfactant turnover, and internalize microbes while coordinating immune responses to respiratory infections[42]. Regulation of inflammation must be tightly controlled, especially at mucosal barriers, as prolonged

inflammation leads to damage and compromises the host to further inflammation and infection.

The local environment drives macrophage programming and polarization

The examples above illustrate the importance of macrophages in maintaining homeostasis. If macrophage function is altered or abrogated it often leads to infectious disease and pathologies like osteoporosis, neurodegenerative disorders, and inflammatory bowel disorders[42]. Tissue macrophages have diverse functions, which are specialized to the tissue they are found in. Unique transcriptional and epigenetic signatures are associated with each tissue macrophage population and they contribute to the functional diversity of these cells[65–68]. Fate-mapping experiments have allowed for a detailed study of the origins and development of macrophages. Tissue macrophages of the spleen, brain, liver, lung, pancreas and peritoneal cavity are derived from yolk sac erythromyeloid progenitors (EMPs)[69,70] that persist into adulthood and proliferate locally[70–74]. Erythrocytes, monocytes, granulocytes and mast cells are produced by EMPs as well, until embryonic day 16.5, after which point they are replaced by hematopoietic stem cell (HSC) derived cells[69]. HSC-derived myeloid cells rely on the transcription factor Myb, while EMP derived cells do not[70]. However once in a tissue, it is difficult to determine the origin of a macrophage from an EMP or HSC solely based on surface markers.

The differentiation from a myeloid progenitor to a macrophage requires certain transcriptional and epigenetic changes, which are thought to be irreversible;

however, since macrophages are master regulators of homeostasis, they must maintain some level of functional plasticity to adapt to the changing needs of their surrounding tissue. Macrophages respond to many signals or stimuli in their microenvironment such as the size, complexity and stiffness of the tissue, the oxygen and nutritional content, the inflammatory status, and the composition of local microbial communities[21]. The tissue signals a macrophage receives influence its transcriptional program and dictate its phenotype and function. Tissue specific transcription factors GATA-binding protein 6 (GATA-6), peroxisome proliferator-activated receptor-γ (PPARγ), and SPIC work with core myeloid transcription factors (PU.1, C/EBP, MAF and MAFB[75]) to regulate transcriptional programs in peritoneal[76], alveolar[77] and spleen red pulp macrophages[78], respectively. These adaptations are reversible as tissue-specific signatures are lost upon isolation and culture, which demonstrates the importance of the microenvironment in macrophage development and polarization[66]. As an example, when a peritoneal tissue macrophage is transplanted to the lung, it starts to resemble an alveolar macrophage, yet it retains parts of its original gene signature[65]. Thus the question remains as to how much of the macrophage program is hard-wired during differentiation and how much of it is modifiable by the environment.

As we age, the replenishment of tissue macrophages changes. Normally, some resident macrophage populations are maintained through self renewal of embryonic-derived macrophages, while others, like those in the gut, are derived from adult hematopoiesis and circulating monocytes[46]. With age, there is an increase

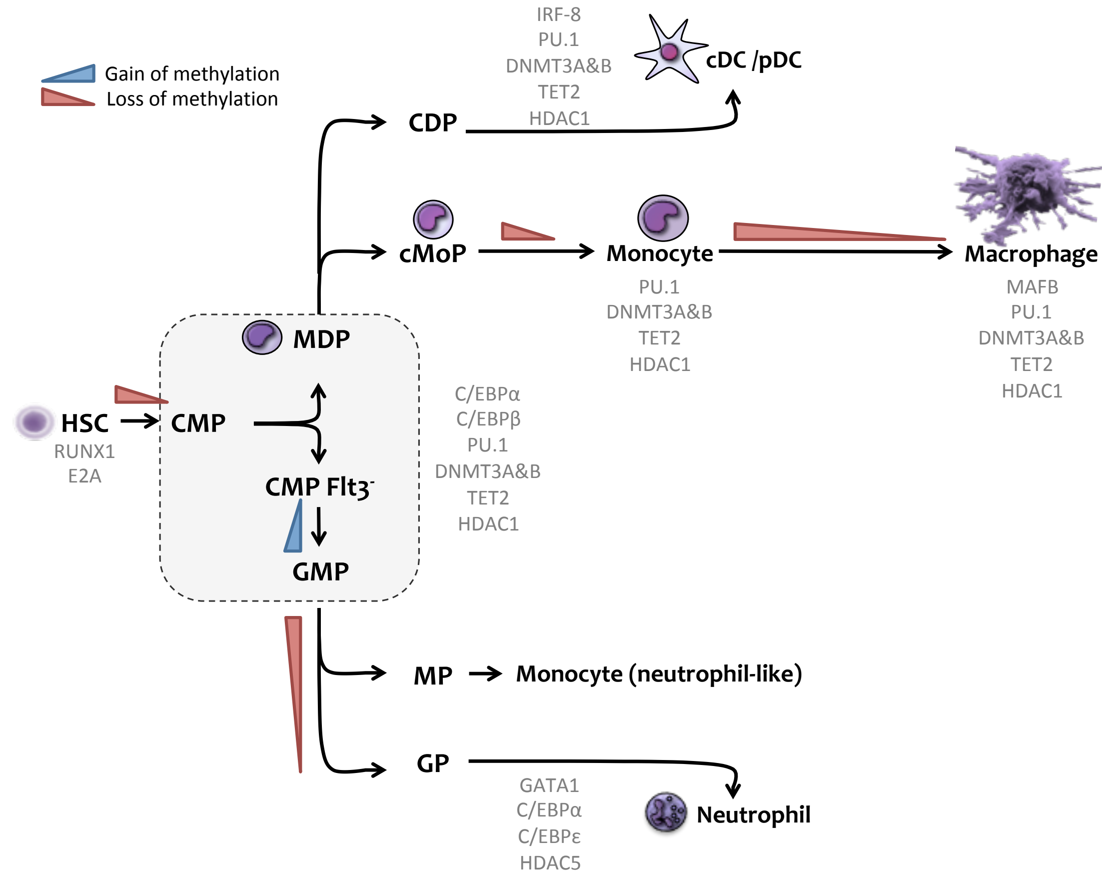
in the contribution of adult HSC-derived macrophages to resident populations in the heart, lungs, spleen and lamina propria[46,69,79]. What remains unknown is whether monocytes are directly recruited to re-seed tissue macrophages, or whether upstream progenitors take residence in the tissue and give rise to tissue macrophages. There is evidence of differences in functional potential between embryonic and adult-derived tissue macrophages. For example, retinoic acid can induce expression of GATA-6 in embryonic-derived peritoneal macrophages but not HSC-derived macrophages[76]. In helminth infection, pleural macrophages maintain an anti-inflammatory role, while adult-derived macrophages assume a pro-inflammatory role[80]. Embryonic and adult-derived cardiac macrophages both exist in the heart, but have different abilities to promote tissue repair following injury[81]. Thus the changes in the composition of tissue macrophage populations may contribute to the development of chronic conditions that occur with age.

1.3 – Monocytes: the reinforcements

Monocyte development

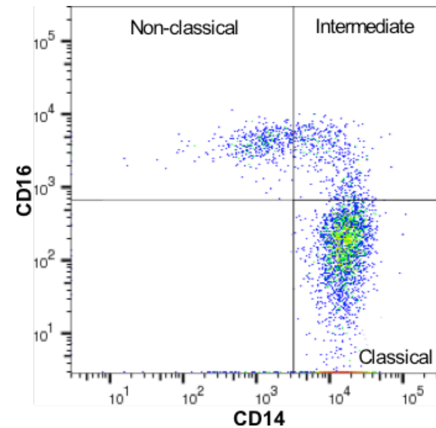
Under steady state conditions, monocytes are derived from hematopoietic progenitors in the bone marrow[82], but in inflammatory and pathological conditions, monopoiesis can occur in the spleen and other extramedullary sites (e.g. liver, kidneys)[83–86]. Hematopoietic stem cells are committed to the myeloid lineage by the transcription factor PU.1[82] to form the common myeloid progenitor (CMP)[87] which

further differentiates to the granulocyte macrophage progenitor (GMP). It was long thought that the GMP gave rise to the macrophage dendritic cell progenitor (MDP)[88], however new evidence in mice shows that MDPs are derived from CMPs[89]. Both GMPs and MDPs can give rise to monocytes, while MDPs cannot produce neutrophils and GMPs cannot produce dendritic cells[90]. The MDP can be driven to form the common DC progenitor (CDP) by signalling through the FMS-related tyrosine kinase 3 (Flt-3) by Flt3 ligand (FL) or it can be driven to form the committed monocyte progenitor (cMoP) by signalling through the macrophage colony stimulating factor (M-CSF) receptor (M-CSFR)[91–93]. M-CSFR is primarily expressed by myeloid and monocytic progenitors, monocytes and macrophages. M-CSF (colony-stimulating factor 1, CSF-1) is essential in driving the survival of both monocytes and macrophages by stimulating cells to enter the cell cycle and inhibiting apoptotic death[94]. Cells of mesenchymal origin (i.e. blood vessel endothelial cells, fibroblasts, bone marrow stromal cells), macrophages, and tumour cells produce M-CSF [95]. M-CSF is found at concentrations of 10 ng/mL in circulation and in the pg/mL range in tissues[96]. Mice deficient for M-CSF[97] or its receptor, M-CSFR (CD115)[98] have lower numbers of circulating monocytes in circulation. M-CSF is cleared by receptor-mediated endocytosis and degradation after binding to the M-CSFR, but this process does not rely on the kinase activity of the receptor[99]. When M-CSF is exogenously added, this clearance becomes saturated and macrophage numbers increase[100].



Myeloid development and differentiation. Myeloid lineage commitment and differentiation is orchestrated by transcription factors (e.g. PU.1,RUNX1, E2A, C/EBP(α/β)), DNA methyl-modifying enzymes (e.g. TET2), DNA methyltransferases (e.g. DNMT3A&B) and histone deacetylases (HDAC1) and other histone modifying enzymes that transform the epigenetic landscape, to modulate gene expression[101]. E2A is involved in maintaining self-renewal of pluripotent cells. A dose-dependent increase in PU.1 cooperates with C/EBPα to drive myeloid lineage commitment. PU.1 interacts with TET2[102] and DNMT3B to recruit them to their target sites, driving further lineage commitment. MAFB and IRF-8 orchestrate terminal differentiation of monocytes into macrophages or DC-like cells, respectively, in inflamed tissue. Global methylation status at certain stages of differentiation are included[103,104], but have not been studied since the recent discoveries of the MDP and cMoP[90,92]. cMoP, committed monocyte progenitor; cDC, conventional DC; CDP, common DC progenitor; CMP, common myeloid progenitor; GMP, granulocyte macrophage progenitor; GP, granulocyte progenitor; HSC, hematopoietic stem cell; MDP, macrophage dendritic cell progenitor; MP, monocyte progenitor; pDC, plasmacytoic DC.

Monocyte subsets and function



Circulating human monocyte subset discrimination by CD14 and CD16

Monocyte recruitment occurs in response to infectious, metabolic, and neoplastic stimuli. They can be sourced from monocyte reservoirs in the blood, spleen and bone marrow. In humans there are three monocyte subsets defined by their expression of surface antigens: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$)[105]. In mice there are two subsets of monocytes classified based on their expression of the Ly6C antigen: the $Ly6C^{high}$ and $Ly6C^{low}$ monocytes[106]. $Ly6C^{high}$ monocytes are functionally and phenotypically equivalent to classical and intermediate monocytes, while the $Ly6C^{low}$ monocytes are most similar to non-classical monocytes[107]. Monocyte maturation is influenced by M-CSF stimulation, which transitions them from the newly formed monocytes (classical and $Ly6C^{high}$) towards the non-classical/$Ly6C^{low}$ monocytes[107,108]. Each monocyte subsets is uniquely equipped to perform certain functions[109] and produces a unique combination of cytokines at different magnitudes following stimulation with lipopolysaccharide (LPS)[110]. Classical monocytes primarily function as phagocytes and have high antimicrobial capacity as they secrete reactive oxygen species (ROS), high levels of IL-10, CCL2, G-CSF and RANTES, and low levels of TNF following LPS

stimulation[109]. Intermediate monocytes express higher levels of HLA-DR and present antigen and activate T cells; however, they are less likely to initiate naïve T cell responses than conventional DCs[111]. They primarily produce IL-1β, IL-6 and TNF following LPS stimulation, but have low peroxidase activity[110]. Non-classical monocytes were only thought to respond to viral and nucleic acid stimuli via TLR-7 and -8 and producing of IL-1β and TNF[112]; however, they were then found to produce the highest levels of TNF and IL-1β following LPS stimulation when compared to the other monocyte subsets[110]. Intermediate, non-classical and Ly6C^{low} monocytes also express Fcγ receptors, which they use the mediate antibody dependent cytotoxicity[113].

In the steady state, monocytes recirculate between the blood and bone marrow. In the absence of infection and inflammation most monocytes return to the bone marrow and transition to non-classical or Ly6C^{low} monocytes that patrol the vascular endothelium[114,115]. In mice Ly6C^{high} monocytes have a half-life of around 19 hours before transitioning to Ly6C^{low} monocytes, which have a half-life of 2.2 days[115]. In humans, classical monocytes circulate for around one day before either dying or differentiating to an intermediate monocyte, which has a lifespan of 4.3 days. All intermediate monocytes eventually turn into a non-classical monocyte, which have the longest lifespan of the three subsets at 7.4 days[116]. In adoptive transfer experiments Ly6C^{low} monocytes were found in the blood, spleen, lung, liver and brain, but were absent from lymph nodes, thymus, peritoneum and the bronchoalveolar space[106]. In the steady state, Ly6C^{high} monocytes have been shown

to enter non-lymphoid tissues like the lung using CD62L, where they sample antigen that they then carry to draining lymph nodes[117]. This study was the first to demonstrate that monocytes can enter tissues without differentiating into macrophages or dendritic cells. These monocytes have a slightly different transcriptional profile when compared to circulating monocytes but are more similar to monocytes than to macrophages.

In the presence of inflammation, monocytes can tether to and invade the inflamed tissue, maturing into macrophages or inflammatory dendritic cells (DCs), depending on their surroundings. In inflamed tissues, Ly6C^{high} monocytes are the most abundantly recruited monocyte subset, while Ly6C^{low} monocytes are found in lower abundance and play a role in tissue repair and the resolution of inflammation. For example in models of *Listeria monocytogenes* (*Lm*) infection, Ly6C^{high} monocytes from the bone marrow are mobilized to fight the infection by secreting TNF and inducible nitric oxide synthase (iNOS)[118]. Although Ly6C^{low} monocytes help to initiate inflammation in the first 1-2 hours of infection, at 8 hours their transcriptional program transitions and they begin to express genes involved in tissue remodelling (e.g. arginase1, Fizz1, Mgl2, MR)[119]. After a myocardial infarct, Ly6C^{high} monocytes are the first to be recruited and they secrete TNF, matrix metalloproteases and cathepsins. They are followed by Ly6C^{low} monocytes which promote tissue repair by secreting vascular endothelial growth factor (VEGF)[119].

Monocyte maturation

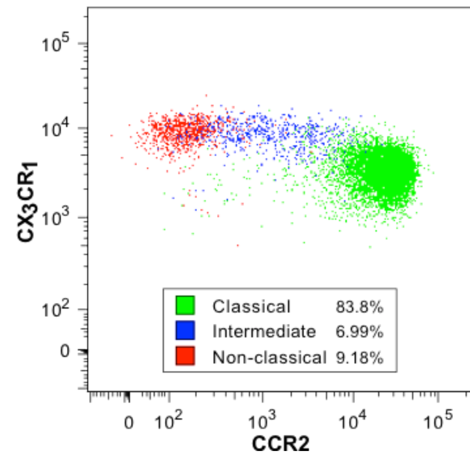
Using an arsenal of pattern recognition receptors (PRRs), monocytes can identify DAMPs and PAMPs to recognize both extracellular and intracellular threats. Cytokine receptors additionally influence their activation and maturation process. Newly formed monocytes (Ly6C^{high}, classical and intermediate) maintain a level of plasticity that allows them to adapt to their environment. They assume different functional characteristics and phenotypes based on the context they are studied in. For example in models of inflammation where high levels of T_H1 cytokines (e.g. GM-CSF, IFNγ) are present, monocytes differentiate into cells that have increased ability to phagocytose and kill intracellular pathogens, and secrete pro-inflammatory cytokines and chemokines[118,120–126]. When T_H2 cytokines are present (e.g. IL-4, IL-13), monocytes assume the role of clearing particulates, regulating inflammation and promoting tissue repair[125–128].

Although the intrinsic sensing of PRR-ligands does contribute to monocyte maturation, there is evidence that cytokines alone are sufficient[129–131]. In *Listeria monocytogenes* infection, the differentiation of Ly6C^{high} monocytes is driven by IFNγ produced by NK cells, but does not rely on MyD88 signaling[129]. Although cytokines may be sufficient, they are rarely present in isolation, therefore the entirety of the inflammatory environment must be considered. For example, human monocytes cultured *in vitro* with M-CSF adopt an anti-inflammatory phenotype, whereas those cultured in GM-CSF have a more activated phenotype[132,133]. When both populations

were stimulated with IFNγ and LPS, they upregulated many of the same genes and secreted cytokines similarly; however, monocytes cultured with M-CSF produced less TNF and more IL-10 than those cultured in GM-CSF, indicating that the environment during differentiation may set the tone of subsequent inflammatory responses[126]. In fact, a recent paradigm of innate immune memory has shown bacterial products and cytokines can influence monocyte maturation prior to arrival at a tissue during early hematopoiesis[134].

Monocyte trafficking

Monocytes use a host of different chemokine receptors and adhesion molecules to traffic to and from tissues. The differential expression between subsets of monocytes suggests they are regulated by different cues and serve different functions during homeostasis and inflammatory states[135]. CX_3CR_1 regulates monocyte retention in the bone marrow[136], while CCR2 expression is used by monocytes to leave the bone marrow in the steady state and in times of infectious or sterile inflammation. In acute inflammatory settings, myeloid progenitors have also been shown to use CCR2 to mobilize from the bone marrow to peripheral sites of inflammation[137]. CCL2(MCP-1) and CCL7 induce CCR2 signalling in Ly6C^{high} monocytes in the bone marrow leading to monocyte egress[138,139]. CCL2 is



Human monocyte subset expression of CX_3CR_1 and CCR2

primarily produced by mesenchymal stem cells in the bone marrow and is necessary for the release of monocytes in response to infectious or inflammatory stimuli[140]; however, the mobilization of monocytes does not strictly rely on CCR2. Angiotensin II drives recruitment of monocytes from the splenic reservoir following myocardial infarct[141], while CXCL1 recruits monocytes from the bone marrow and spleen in models of atherosclerosis[140]. CX_3CR_1 provides survival signals for Ly6C^{low} monocytes[119,142] and is required from recruitment of Ly6C^{low} monocytes to the spleen during bacterial infection[143] and for their patrolling behaviour of vascular endothelium and other tissues in the steady state[119,142]. CCR1 and CCR5 mediate inflammatory monocyte recruitment and have been shown to contribute to the progression of atherosclerosis[144], multiple sclerosis[145] and rheumatoid arthritis[146]. As newly formed monocytes mature, they lose expression of Ly6C and can gain expression of CCR7 and CCR8. These two chemokine receptors are used for monocyte trafficking from tissue to draining lymph nodes where they acquire DC-like characteristics and can present antigen[147].

Although monocytes are essential in orchestrating responses to infection[148–153] and tissue repair[154], their aberrant recruitment and function has been implicated in many pathologies (e.g. fibrosis, Crohn's disease, rheumatoid arthritis, atherosclerosis, coronary artery disease, and obesity, systemic lupus erythematosus)[155–162]. Most of the conditions are characterized by a heightened and or chronic inflammatory state and many of them increase in incidence with age. We hypothesize that age-associated inflammation may increase susceptibility to chronic

diseases by influencing monocyte development resulting in a higher number of monocytes, as well as polarizing them to be more inflammatory.

1.4 - Immunosenescence

Dysregulation of an immune response

Generally it is thought that aging results in a loss of function of the immune system and a decline in immune responses; however this is inaccurate, as certain parts of the immune system have inappropriately elevated responses, while others lose their ability to respond[19]. These changes in immune function culminate in the inability of the immune system to coordinate an efficient immune response and result in prolonged inflammation and collateral host tissue damage[19,21].

Age-associated changes to the lymphocytes

With age the adaptive immune system experiences an expansion of antigen-experienced B and T cells, while naïve populations decrease due to lower lymphopoiesis in the bone marrow. It is important to note that in mice naïve T cell output is dependent on the thymus, whereas in humans naïve T cells predominantly result from peripheral division after early life[163]. In mice, memory T cells formed during early adulthood maintain their function into old age[164–166]; however, in humans, their function declines in the second half of life. With age, human memory T

cells do not provide sufficient help to B cells[167], and their production of lytic molecules and cytokines becomes less diverse[168]. The diversity of the antigen receptor repertoire is decreased due to chronic viral infections, like cytomegalovirus, which expand existing memory populations, particularly CD8 T cells[169]. This is thought to decrease the CD4/CD8 T cell ratio with age. The formation of adaptive responses to novel antigens by naïve cells is also compromised with age, in part due to impaired T cell synapsis formation and TCR signalling[170]. Decreased production of IL-2 following T cell activation also leads to decreased proliferative potential for clonal expansion in mice[171]; however, this does not occur in humans, where naïve CD4 T cells are well maintained into the 7th decade of life[172]. This could be explained by their decreased expression of the pro-apoptotic protein Bim, which increases their survival[164]. There is some evidence that naïve CD4 T cells from old mice express markers of exhausted T cells (e.g. PD-1, LAG-3), and have been described as being pseudo-memory cells[173]. Senescent memory T cells have an increase in inhibitor receptors (e.g. KLRG1, CD85j, ILT-2) and a decrease in costimulatory receptors (e.g. CD28), which further contributes to their inability to be efficiently activated during recall responses[174]. Age-associated changes to T cells are intrinsic, as naïve CD4 T cells from an old mouse transferred to a young mouse maintain their functional defects[175], and similar findings have been reported for CD8 T cells[173].

New B cell responses are impaired with age due to intrinsic insufficiency of transcription factors and genes necessary for recombination and transition to pro-

and pre-B cells[176,177]. The decreased size of germinal centers and suboptimal T cell help via CD40-CD40L impairs B cell proliferation. This culminates in reduced quality of antibody production[178,179]. The production of autoantibodies specific for DNA, cardiolipin, and rheumatoid factor (anti-IgG antibodies) also increases with age. B cells do not experience the same rate of telomere shortening as T cells because they can increase their telomerase activity[180]. In fact, B cell aging seems to be driven more by the microenvironment as age-discordant bone marrow chimeras showed that cells from a young donor transplanted into an old host experienced decreased recombinase expression and activity and lower numbers of pre-B cells[177]. It is likely that decreased production of lymphopoiesis supporting factors by the stromal cells and age-associated changes to the T cells contribute to this phenomenon. Furthermore, TNF has been shown to induce B cell apoptosis and is elevated in the aging microenvironment[181,182].

It was thought for a long time that the age-associated changes to the lymphoid compartment were the most significant driver of immune aging and that inflammaging was the result of chronic viral infections activating effector T cells. Cytomegalovirus (CMV) was shown to correlate with an inversion in the ratio of CD4 to CD8 T cells, an individual's inflammatory status, and mortality in the elderly[183]; however, recent studies have found no difference in systemic inflammation between CMV positive and CMV negative elderly[184], but still found it was associated with mortality[185]. Another study found that inflammation was only increased in CMV positive individuals whose monocytes carried CMV DNA[186]. These findings suggest

that the myeloid compartment may be a more important contributor to inflammaging than previously appreciated.

Age-associated changes to myeloid cells

Age-related changes to the monocyte and macrophage compartment begin in the hematopoietic stem cell (HSC). With age, HSCs preferentially produce cells of the myeloid lineage (i.e. monocytes, macrophages, neutrophils and dendritic cells) in lieu of lymphoid cells (i.e. B cells, T cells and NK cells). This results in an increase in circulating monocytes, as we and others have observed[187–191]. The ratio of classical to intermediate and non-classical monocytes changes with age. $CD16^+$ monocytes (intermediate and classical) increase while classical monocytes decrease[187–191]. The degree to which changes in proportions of monocytes impact function is unclear due to major methodological differences in the way studies are performed. There are conflicting reports as to whether monocytes from older adults produce less[190,192] or more[193,194] pro-inflammatory cytokines at baseline and in response to toll-like receptor (TLR) stimulation when compared to those from younger adults. There is no compelling evidence that changes in cytokine production in response to TLR agonists is due to changes in TLR expression. Instead, it appears as though downstream signalling is compromised with age[195–198]. In addition to finding increased monocytes with age, we found that those from older adults produced more pro-inflammatory cytokines (e.g. IL-1β, TNF, IL-8)[187], which we believe may contribute to systemic inflammation. Increased circulating monocytes, particularly

inflammatory and Ly6C^{high} monocytes, and increased pro-inflammatory cytokines have been characterized in atherosclerosis[199–201], coronary artery disease[156,202], cancer[83,203], rheumatoid arthritis[204,205], inflammatory bowel disease[206–208], and dementia[209]. Thus we hypothesize that the increase in these monocytes with age contributes to the development of chronic diseases.

Age-associated changes to macrophage phenotype and function have also been studied with contradicting results. Macrophage functions including cytokine production[189,210], chemotaxis[211], antigen presentation[212], wound healing[213], and bacterial clearance (i.e. adherence, opsonization, phagocytosis, oxidative burst)[214–219] have all been shown to change with age in humans and rodents. We previously reported that monocyte-derived macrophages (MDMs) from the elderly are hyporesponsive when stimulated with heat killed *Streptococcus pneumoniae*[197]. Although there was no difference in binding and uptake of *S. pneumoniae*, intracellular killing of the bacteria was lower in old MDMs[197]. In bone marrow derived macrophages from old mice, we found increased production of pro-inflammatory cytokines following TLR stimulation[39]. We have also found lower phagocytic ability in bone marrow derived and alveolar macrophages[39]. Furthermore when we normalize for impaired bacterial uptake, intracellular bacterial killing by macrophages remains impaired with age[39]. These age-associated changes in macrophage function not only result in altered innate immune responses, but impaired orchestration of adaptive immunity[220]. Many of these changes in monocyte and macrophage function also occur in other chronic inflammatory

diseases, where they act as initiators of the disease or propagators of chronic inflammation[221].

1.5 - Myelopoiesis and the aging microenvironment

Monocytes and other innate immune cells are the first responders to infection or injury. As such, hematopoietic progenitors must be exquisitely sensitive to even slight elevations in systemic levels of inflammation in order to promptly mobilize a sufficient number of innate immune cells[222]. In adults, monocytes arise from myeloid progenitors derived from HSCs. Under homeostatic conditions the majority of HSCs remain in a quiescent state. During times of stress (e.g. infection or injury), levels of TNF, IFN-γ and toll-like receptor (TLR) ligands increase in circulation and stimulate HSCs to preferentially produce myeloid cells (e.g. monocytes, neutrophils)[210,223–225]. This process is called emergency myelopoiesis and it occurs at the expense of lymphopoiesis which gives rise to B, T and NK cells. The nature of the inflammatory stimulus[226] can determine whether myelopoiesis is more heavily weighted towards monopoiesis (e.g. during *L. monocytogenes* infection[227]) or granulopoiesis, (e.g. during *C. albicans* infection[228,229]). Once the inflammation is resolved, the balance in the rates of myelopoiesis and lymphopoiesis should be restored. Even acute inflammatory events like vaccination can impact myeloid development and innate immune function for weeks[230]. We hypothesize that age-related increases in inflammatory cytokines and circulating bacterial products results in elevated

myelopoiesis that resembles emergency myelopoiesis. This contributes to the myeloid bias in hematopoiesis that occurs with age. Consistent with this theory, repeated low-dose LPS[231] or IL-6[232] stimulations result in an increase in myelopoiesis and a decrease in lymphopoiesis, which resembles what we see with age.

Gene ontology analysis of transcriptomes from young and old HSCs reveal an increase in nitric oxide (NO)-mediated signalling, unfolded protein response and inflammation, while chromatin remodelling and DNA repair are decreased[233]. These changes are not only seen in hematopoietic cells with age. There is a vast body of literature investigating the biological mechanisms underlying the process of aging. Lopez-Otin et al., have described 9 hallmarks of aging, which are: genomic instability, telomere shortening, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication[234]. These changes can be sensed by hematopoietic progenitors and especially by monocytes and macrophages and trigger inflammatory signalling as well as directly influence their function. In fact, HSCs from old mice have higher nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) activation and increased inflammatory gene signatures compared to HSCs from young mice[235]. The progeny of activated HSCs in aged hosts are also capable of producing elevated levels of pro-inflammatory cytokines, establishing a feed forward mechanism that amplifies inflammation[225].

The ability of HSCs to repopulate decreases and they are biased towards the myeloid lineage[233,235]. A greater proportion of HSCs from the elderly are in a nonquiescent state[233] and the polyclonality of myeloid cells declines with age[236]. This loss in clonality is driven by the aged microenvironment and is associated with poor health outcomes[237]. Although certain cell intrinsic changes in hematopoiesis have been found[225], the microenvironment significantly influences hematopoiesis. In heterochronic bone marrow chimeras, we transplanted bone marrow from old mice into both young and old irradiated hosts[39]. The myeloid bias was conserved in an old host, however in a young host the number of myeloid cells was similar to a young mouse. Furthermore, bone marrow from a young mouse transplanted into an old host phenocopied the age-associated increase in myeloid cells. These data suggest that it is the aging microenvironment that drives the changes to hematopoiesis with age.

The interaction of HSCs with their niche changes significantly with age. Mesenchymal cells including osteocytes, adipocytes, chondrocytes, fibroblasts and epithelial cells are all involved in regulating hematopoiesis[238]. They undergo senescence as well, which alters the hematopoietic niche by decreasing bone formation, increasing adipogenesis and altering the extracellular matrix[225]. Increased adipogenesis and decreased osteogenesis skews hematopoiesis towards the myeloid lineage[225]. In the bone marrow, HSCs are further from the endosteum in old mice[239] and have increased mobilization potential following G-CSF stimulation[240], but are likely to have impaired homing back to the bone marrow[241].

Other soluble factors from the microenvironment like ROS[242] and RANTES[243] have been shown to increase myelopoiesis with age. Similar changes to the hematopoietic niche have been documented in myeloproliferative disorders where there is an increase in pro-inflammatory cytokines that lead to bone marrow failure[244,245]. The changes to the hematopoietic niche and increased inflammation with age can lead to epigenetic changes in HSCs and their progeny that can predetermine functional capacity of mature monocytes and macrophages[134,246,247].

1.6 – Myeloid derived suppressor cells as a model of impaired myeloid development during acute and chronic inflammation

MDSCs: products of impaired myelopoiesis

If myelopoiesis is sustained there is an accumulation of immature myeloid progenitors, which are phenotypically and functionally distinct from their terminally differentiated progeny (e.g. neutrophil, macrophage, dendritic cell). Myeloid derived suppressor cells (MDSCs) are heterogeneous, immunosuppressive cells that result from the incomplete differentiation of myeloid progenitors[248]. They are elevated in mice and humans with conditions of chronic or excessive inflammation (i.e. cancer, trauma, sepsis, infection, autoimmune diseases)[248–250]. MDSCs can either be monocytic or granulocytic and are identified by different markers in mice (mMDSCs: $CD11b^+Ly6G^-Ly6C^{high}$; gMDSCs: $CD11b^+Ly6G^+Ly6C^{low}$) and in humans (MDSCs:

$CD33^{+}CD11b^{+}HLA\text{-}DR^{low/-}Lin^{-}$(CD3/19/56); mMDSCs: $CD14^{+}$; gMDSCs: $CD14^{-}CD15^{+}CD66b^{+}$)[248].

MDSCs present antigen in the absence of co-stimulation while secreting immunosuppressive factors (e.g. prostaglandins, arginase, IL-10), leading to the suppression of cytotoxic T cells[250]. MDSCs additionally suppress T cells via inducible nitric oxide synthase (iNOS) and arginase-1 (ARG-1)[250]. gMDSCs are thought to increase production of reactive oxygen species (ROS), but not nitric oxide(NO). Peroxynitrite, the product of a ROS-triggered reaction, leads to post-translational modifications in the T cell receptor further contributing to decreased T cell responses to antigen[251]. Conversely, mMDSCs have increased production of NO but not ROS. The increased NO levels suppress T cells by inducing T cell apoptosis, as well as inhibiting MHC II expression[249]. mMDSCs increase the recruitment of T_{reg} cells via CCR5[252]. MDSCs suppress innate immune cells by downregulating macrophage IL-12 production and by increasing their own production of anti-inflammatory mediators (IL-10, TGFβ, PGE2)[250]. MDSCs are produced at the expense of dendritic cells, thus reducing efficient antigen presentation. The effect of MDSCs on NK cells remains controversial, with some studies showing NK cell activity is increased by MDSCs, while others show that it is inhibited. This could be due to the fact that IFN-γ, a cytokine produced by NK cells, is instrumental in the production and regulation of MDSCs.

MDSCs expand in response to many different factors including: prostaglandins, cyclooxygenase 2, growth factors (e.g. SCF, M-CSF, GM-CSF, VEGF), and cytokines

(IL-6). These factors primarily signal through the JAK-STAT pathway, upregulating the transcription factor STAT3, which is central to MDSC expansion. STAT3 increases survival and proliferation of myeloid progenitor cells, and likely maintains MDSCs in their immature state. Downstream of STAT3, the pro-inflammatory proteins S100A8 and S100A9 bind the receptor for advanced glycation end products (RAGE) and block the differentiation of MDSCs[253,254]. As mentioned earlier, the production of immature myeloid cells is a natural response to infection and vaccination, which occurs as part of emergency myelopoiesis. Following the expansion of MDSCs, their differentiation is blocked by a second group of factors including cytokines (e.g. IFN-γ, IL-1β, IL-4, IL-13) and TLR agonists that activate STAT1 and STAT6 and further activate MDSCs, enhancing their suppressive capacity[255]. *In vitro* culture experiments have shown that the timing and dosage of these pro-inflammatory factors is critical in the differentiation process of immature myeloid cells. Cultures with low concentrations of GM-CSF result in MDSCs and immature DCs by 8-10 days. Those with high concentrations of GM-CSF result in rapid production of MDSCs; however, without prompt activation by 3 to 4 days, they fully mature into DCs[256]. The increase in inflammation in the environment with age may be more conducive to the production of MDSCs.

MDSCs in chronic inflammatory conditions

Many of the soluble factors that expand and activate MDSCs are found during states of chronic inflammation. Pharmacological reduction of inflammatory

mediators (e.g. S100A9, IL-1β, IL-6, VEGF) lead to decreased MDSCs in mouse models of cancer[257]. In mice, tumours secreting higher levels of IL-1β lead to an increased number of splenic myeloid suppressor cells. This phenomenon is indirectly mediated by IL-1β and is independent of the lymphoid compartment[258]. These data suggest that inflammatory factors derived from structural and myeloid cells are sufficient for the production and activation of MDSCs, although the lymphoid production of cytokines likely contributes to their activation as well. MDSCs are also increased in mouse models of chronic inflammation of the gut, eye and skin[259]. MDSCs are found to be elevated in many chronic inflammatory conditions including inflammatory bowel disease, obesity, and chronic infections (i.e. HIV, hepatitis C)[109,155,260]. Increases in inflammatory Ly6C^{high}CCR2^{+} monocytes, which have similar phenotypes to mMDSCs[155,261], are found in mouse models of these conditions.

Evidence of impaired myelopoiesis with age & implications for age-associated immune dysfunction

As we age, the microenvironment of the tissues and circulation changes and we hypothesize that this influences the developmental programing of HSCs, particularly towards the myeloid lineage. Age-associated inflammation is characterized by elevated concentrations of the same inflammatory mediators that lead to the production and activation of MDSCs: PGE2, TNF, IL-1β, bacterial products[262–264]. In our aged mice, we have found elevated monocytes with impaired maturation and

increased pro-inflammatory cytokine production, and decreased bacterial killing by macrophages. This is driven by TNF, as TNF deficient animals are protected from these phenomena. In humans, we have shown that MDSCs[265] increase with age and neutrophils appear less mature , which correlates with circulating levels of TNF and mitochondrial DNA[263]. We hypothesize that increases in inflammation rather than age correlate with increases in circulating numbers of MDSC and markers of myeloid maturity with age.

1.7 - Central Aims and Hypotheses:

Individuals with higher levels of pro-inflammatory mediators are at higher risk of developing chronic disease[266]. The concomitant increase in pro-inflammatory cytokines and prevalence of monocyte and macrophage driven diseases with age lead us to hypothesize that chronic exposure to pro-inflammatory cytokines (i.e. tumour necrosis factor, TNF) impairs monocyte and macrophage phenotype and function and predisposes individuals to chronic inflammatory and infectious diseases. Much of the research on the aging immune system has revolved around cell-intrinsic changes, however the central aim of this thesis is to provide compelling evidence that age-associated inflammation drives the aging of myeloid cells. We hypothesize age-associated inflammation impairs myeloid development and monocyte and macrophage function.

AIMS:

1. Disentangle age-, inflammation- and health condition-induced changes to human monocyte maturation, activation and migratory potential
2. Determine if modulating systemic inflammation influences human monocyte development and activation
3. Determine how TNF influences monocyte development and monocyte and macrophage function in mice

CHAPTER 2

Age, Inflammation, and the Myeloid Compartment

Introduction:

During acute and chronic inflammation, the rate of myelopoiesis increases leading to the production of immature myeloid cells[246]. We have found increased levels of cytokines (i.e. TNF, IFNγ, IL-1β) and circulating bacterial products (e.g. MDP, LPS) with age, which are known to stimulate myelopoiesis[264,267]. Myeloid progenitors in the bone marrow or extramedullary sites, like the spleen, progress through defined stages of maturation. This process continues in the circulation as monocytes transition from classical to intermediate to non-classical monocytes. Surface markers can be used to identify the stage of myeloid development and monocyte maturation[268]. In humans, CD33 and CD13 are widely expressed by myeloid cells early in their development. Differentiation from GMPs to monoblasts involves the gain of CD4, which can be used to discriminate monocytic precursors from myelocytes that give rise to neutrophils[268]. Progression to promonocytes involves the loss of CD34 and the gain in CD11b, CD14, CD64. Maturation into monocytes involves the progressive gain in CD16 and HLA-DR among other surface antigens described in the appendix [268–270]. In certain diseases with altered myeloid development and maturation, monocyte numbers and phenotypes change. For

example, in acute myeloid leukemia (AML) and myelodysplastic syndromes monoblast and promonocyte populations expand[270] and there is a concomitant change in the expression of surface markers of maturity. One of the most common patterns includes a loss of myeloid markers like CD33 and CD13, while expressing higher levels of maturity markers like CD16 or CD64[270]. With age, we believe that the increased numbers of circulating monocytes are a result of altered myeloid development and that markers of maturity (e.g. CD64, $CD16^+$ monocytes), trafficking and activation (e.g. CCR2, CX_3CR_1) will change.

The relative contributions of intrinsic and extrinsic factors in myeloid aging remain unknown. Our data from mice and humans suggest that age-associated inflammation may be driving increased myelopoiesis that results in improper or incomplete myeloid differentiation. We hypothesize that inflammation is more important in determining monocyte numbers and phenotype than age. To test this hypothesis, we recruited individuals from the community between the ages of 20-100 and measured serum cytokines and performed immunophenotyping on whole blood to quantify cell populations and surface markers of monocyte maturity and activation. In order to discriminate the affect of age versus the presence of diseases whose frequencies increase with age, we collected information on health conditions. We hypothesized that there would be a stronger correlation between serum cytokines and markers of monocyte maturity and activation than with age. Alterations in the numbers of circulating monocytes are biomarkers for inflammatory diseases like atherosclerosis, cardiovascular disease, and rheumatoid

arthritis[155]. MDSCs have also been described as potential biomarkers for clinical prognosis in cancer[271,272], severe infections[273] and even pulmonary fibrosis[274]. As such, we hypothesize that health of an individual will be reflected in the numbers, proportions and phenotypes of monocytes and MDSCs.

Materials and Methods:

Participants

Individuals over the age of 18 were recruited from the community and consented under the Hamilton Integrated Research Ethics Board Project #1949. Participants completed a questionnaire collecting the following information: date of birth, sex, weight, height, smoking status, medical conditions, medication use. The questionnaire is included in the appendix at the end of the chapter. A breakdown of age, sex and health conditions can be found in Tables 1& 2 and medication use can be found in Supplementary Table 4. Data from participants with an infection or surgery in the 2 weeks before sample collection were excluded from the data analyzed in this chapter.

Serum Cytokine Measurement

Venous blood was collected and centrifuged at 1.5 x g for 10 min at 25°C and serum was stored at -140°C until processed. Serum cytokines were measured using Milliplex HSTCMAG-28SK (GM-CSF, fractalkine, TNF, IL-1β, IL-6, IL-10, IFNγ) and

HCYTOMAG-60K (VEGFA, MCP-1, IP-10) as per manufacturer's protocol (EMD Millipore). See Supplementary Table 1 for descriptive statistics.

Whole Blood Immunophenotyping

Venous blood was collected in heparinized tubes and 100 μL was stained for 30 min with monoclonal antibodies of the following specificities: CD45-BV510, CCR2-PE, CD15-BV610, CD14-BV421, CD56-AF700, CD64-BV605, CD13-PE, CD33-PE (BioLegend); CD16-PE-Cy7, HLA-DR-PerCP-Cy5.5, CD19-AF700, CD115-APC (eBioscience); CD11b-APC, CD3-AF700 (BD Pharmingen) CX3CR1-FITC (MBL Life Sciences). Samples were then incubated with 1X Fix/Lyse Buffer (eBioscience) for 10 min with frequent inversion and centrifuged at room temperature, washed and resuspended in FACS Wash (5 mM EDTA, 0.5% BSA in PBS). Samples were then run on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo 10 software (Treestar). Total cell counts were determined with 123 count eBeads (eBioscience). For gating strategy, see Supplementary Figures 1-3. Expression of surface markers were quantified by measuring geometric mean fluorescence intensity (gMFI) of each marker and subtracting background gMFI of isotype controls. See Supplementary Tables 2 & 3 for descriptive statistics.

Statistical Analysis

Spearman's rank correlation was performed using the rcorr function in the Hmisc package[275] in R[276]. Partial correlation analyses were performed using the pcor.test function in the ppcor package[277] to test relationships between variables

while controlling for factors like sex, age, BMI and self reported health conditions. Confidence intervals for correlation coefficients were generated using the CIrho function in mada package[278]. The lmer function from the lme4[279] package in R was used to perform linear mixed effects modeling to determine if changes in age and serum inflammation lead to changes in cellular inflammation. Analysis of variance was performed on our linear models using the Type II Wald chi-squared tests, which is part of the Anova function in the car package[280]. This approach was also used to determine how the number of health conditions influenced soluble and cellular inflammation. We used SPSS (Version 21; IBM, Armonk, NY, USA) to perform ANCOVA analysis was used to test the differences between individuals without chronic conditions and those with one or more, while using sex, age and BMI as covariates. The stepwise Akaike information criterion (stepAIC) function from the MASS package[281] was used to test which of the highly correlated variables to select as the best predictor variables for monocyte CD115 expression in a linear regression. Correlation plots were generated using the corrplot function in the corrplot package[282]. Corrections for multiple comparisons were made using the Benjamini-Hochberg false discovery rate in the p.adjust function of the stats package[283].

Results & Discussion:

Age-associated changes:

Consistent with previous reports[189], there was a bias towards the production of myeloid cells over lymphoid cells as the ratio of myeloid to lymphoid cells is higher in older individuals when controlling for sex (Table 3). With age there was a shift in the composition of the PBMC compartment. As previously reported[179,181,189,284–286], we found decreased B and T cells with age while the numbers of NK cells and monocytes increased (Table 3 & 4, Figures 2-4). Non-classical monocytes increased with age, but no significant differences were seen in classical and intermediate subsets (Table 3, Figures 2&4), consistent with findings that $CD16^+$ monocytes increase in circulation with age[188–190,193,267]. Although age correlated with number of non-classical monocytes, it was not significantly related to markers of monocyte maturity and activation. Age was weakly associated with intermediate monocyte CD16 ($r=-0.377$, $p=0.0035$, Table 4, Figure 5) and monocyte CX_3CR_1 ($r=0.301$, $p=0.021$, Table 4, Figure 5), but these associations were lost after controlling for sex and BMI.

In our cohort, higher body mass index (BMI) correlated with age ($r=0.318$, $p=0.020$, Table 4, Figure 1). Individuals with higher BMI had a higher proportion of CD4 T cells when controlling for sex, age and health conditions ($r=0.327$, $p=0.0247$, Table 5). Although the proportion of T cells is shown to correlate with age (Table 4),

BMI is a stronger driver of this relationship as it is the most significant predictor of CD4 T cells (% T cells) in a mixed model (Table 5, Figure 2). This is consistent with findings in obesity, where there is an expansion of CD4 T cells that are skewed towards T_{reg} and Th_2 phenotypes[287], due to fatty acid-induced metabolic stress driving differentiation of CD4 T cells via phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)[288]. Changes to glucose and fat metabolism have been well documented with age[289], and these findings underscore the importance of considering metabolism in studies of immunity.

An increase in circulating cytokines with age has previously been reported[23]. We found IP-10 and MCP-1 were positively correlated with age when controlling for sex and BMI (IP-10: r=0.502, p=0.0001; MCP-1: r: 0.311, p=0.0247, Table 4, Figure 1). Both IP-10 and MCP-1 have previously been found to increase with age[290]. Based on our sample, we predict that IP-10 would increase by 9.12 ± 3.86 pg/mL per year (p=0.0003274, Table 6), and MCP-1 would increase by 12.31 ± 3.86 pg/mL per year (p=0.00157, Table 6). There was no statistically significant increase in the other cytokines measured with age. This could be due to the small size of our cohort, as we previously found significant increases in populations that were older, or frail and had more chronic conditions[265]. We are able to detect IP-10 and MCP-1 more easily as they are present at higher concentrations in the serum and have a better range than the other cytokines (e.g. TNF, IL-1β and IFNγ) that are close to the limit of detection.

MCP-1 has been found to increase with age[189,291,292] and is higher in frail elderly[292,293]. One group found MCP-1 correlated with BMI, however this could be due to the fact that BMI is a covariate of age. When we performed partial correlation between MCP-1 and BMI while controlling for age, there was no statistically significant relationship (data not shown). BMI is not the most accurate measure of adiposity, which itself has been shown to elevate MCP-1 levels in circulation in mice[294]. Serum triglyceride levels were also predictive of MCP-1 in humans[291]. MCP-1 correlates with disease severity in Alzheimer's and in mild cognitive impairment[295] and mediates monocyte recruitment in models of fibrosis[296,297], obesity[298] and atherosclerosis[299]. As such it could be contributing to the development and progression of many age-associated diseases driven by monocytes.

Increased serum concentrations of IP-10 have previously been documented with age[194,290,300]. IP-10 is an IFNγ-inducible protein produced by monocytes, macrophages, neutrophils, and stromal cells (e.g. fibroblasts, mesenchymal and endothelial cells)[301–303]. Plasma MCP-1 and IP-10 were found to increase with age in men aged 18-84, and were associated with cardiovascular risk factors[304]. IP-10 was increased with age in leukocytes as well as in brain tissue and correlated with cognitive decline[300]. Although the mechanism driving increased IP-10 with age remains unknown, many cells can produce it. Unstimulated CD4 and CD8 T cells from old mice produce more of it when compared to young mice[305]. IP-10 is downregulated in T cells following maximal TCR/co-receptor stimulation [305]. With age the strength of TCR signalling decreases[170], which may result in unhindered

production of IP-10. Although we did not find an increase in IFNγ with age, others have[306,307], which could stimulate the production of IP-10 by stromal cells.

Cytokines and their relationship with circulating leukocytes:

There were no age-associated increases in cytokines except for MCP-1 and IP-10; however, there was variability in serum cytokines across our population, so we investigated the associations of each cytokine with myeloid development and monocyte maturity. Each cytokine had unique associations with markers of monocyte activation (e.g. CD64, $CD16^+$ monocytes), monocyte trafficking (e.g. CCR2, CX_3CR_1), and monocyte maturity and myeloid development (e.g. CD13, MDSCs).

IL-6, GM-CSF and IL-10 are positively associated with mature, activated monocytes. IL-6 and GM-CSF correlated with an increase in $CD16^+$ monocytes (Table 7, Figure 4). Both IL-6 and GM-CSF have been shown to drive monocyte differentiation, increasing the number of $CD16^+$ cells[155,308]. Furthermore, IL-6 and IL-10 were positively correlated to CD64 expression on non-classical monocytes (Table 7, Figure 2&5). During infections and G-CSF therapy, when levels of IL-6 are high, CD64 expression is upregulated on monocytes[309]. Expression of CD64 is also induced by IL-10, type I IFN, and TLR-7 and -9 agonists[310,311]. Elevated CD64 increases antibody-dependent cellular cytotoxicity and superoxide production by monocytes and has been implicated in diseases like SLE[312,313]. IP-10 and age individually correlated with CX_3CR_1 expression on monocytes (Figure 5), however

these associations were not statistically significant after controlling for sex, BMI and health conditions (data not shown).

Although we did not find TNF to be associated with age, it was positively correlated with CCR2 on intermediate monocytes (Table 7, Figure 5). This is consistent with our published findings in mice and humans, where TNF was associated with increased expression of CCR2 on $Ly6C^{high}$ and intermediate monocytes[39]. This suggests that TNF increases the migratory potential of circulating monocytes. CCR2 expressing monocytes contribute to cardiovascular disease[314,315], diabetes-associated renal disease[316], and arthritis[317], all of which increase in prevalence with age. We found that IL-6 and MCP-1 were inversely related to CCR2 expression on monocytes (Table 7, Figure 5). This is due to the fact that individuals with high levels of IL-6 and MCP-1 had a higher proportion of $CD16^{+}$ monocytes compared to classical monocytes, which express the highest levels of CCR2. It could be that classical monocytes are lower in these individuals because they have left the circulation and migrated to the tissues. CCR2 is rapidly downregulated during the differentiation of monocytes. When cultured in the presence of MCP-1, monocyte calcium signalling downstream of CCR2 is desensitized in the first 6 hours and the surface expression of CCR2 decreases[318]. CCR2 signalling is recovered after 24 hours, suggesting the receptor is transcriptionally regulated in response to MCP-1[318]. How chronic exposure to elevated levels of MCP-1 influences CCR2 expression and monocyte function remains unknown but is a relevant area of future research in aging monocytes as we found it to be increased with age.

VEGFA is released during angiogenesis and has been shown to increase myelopoiesis[319], which explains why we found it was positively associated with total monocytes (%CD45) (Table 7, Figure 4&5). VEGFA is also produced by Ly6C^{high} monocytes, the functional equivalents of classical and intermediate monocytes in humans, in mouse models of lung injury[320]. VEGFA was negatively related to CD13 on non-classical monocytes (Table 7, Figure 4&5). CD13 is a myeloid maturity marker that is expressed on early monoblasts and increases during differentiation[269,270]. It is most highly expressed on non-classical monocytes. The negative relationship between VEGFA and CD13 on non-classical monocytes suggests that VEGFA is related to incomplete maturation. In models of acute and chronic inflammation, VEGFA drives the production of myeloid derived suppressor cells (MDSCs)[257,321] and has been shown to inhibit the differentiation of other myeloid cells[322]. In addition to VEGFA, IL-1β and IFNγ block the differentiation of MDSCs[255] and we found them to be positively related to the proportion of mMDSCs (Table 8A, Figure 4). IL-1β, IFNγ and fractalkine were also directly related to monocyte expression of CD115 (Table 8A, Figure 5). IL-1β and IFNγ are upregulated in response to inflammation and induce emergency hematopoiesis[246], which requires signalling through CD115 to produce myeloid cells[246].

Immune correlates of self-reported health conditions:

Age is a risk factor for many diseases and health conditions. Consequently, it is a challenge to determine which immune changes are due to age and which are due

to health conditions that occur with age. To address this we looked at associations of self-reported health conditions with serum cytokines, markers of monocyte activation, trafficking, maturity and myeloid development. In participants over the age of 50, 34.3% reported no health conditions, 37.1% reported one health condition, 20% reported 2 health conditions, 2.9% reported 3 health conditions and 5.7% reported 4 health conditions (Table 2). This is consistent with findings from the 2008 Canadian Community Health Survey where in the population of individuals over the age of 50, 31-32% had one chronic condition, 13-29% had two chronic conditions, and 6-21% had three or more chronic conditions[10]. The majority of the conditions reported in our sample were joint disorders (e.g. osteoarthritis, osteoporosis) and past treatment for cancer (Table 2). Chronic respiratory conditions (e.g. asthma and COPD) and cardiovascular and metabolic conditions (e.g. hypertension, heart disease, type 2 diabetes) were also reported, making our sample an accurate representation of the Canadian population over the age of 50[10]. As expected, the number of health conditions positively correlated with age when controlling for sex and BMI (r=0.439, p=0.001, Table 9, Figure 1). Although MCP-1 and IP-10 were shown to correlate with health conditions, this was lost after controlling for age, which as mentioned earlier has a strong association with both cytokines. Serum cytokines and lymphocyte populations did not correlate with health status and we found the strongest associations were with the myeloid compartment.

The ratio of $CD16^+$ monocytes to $CD14^+$ monocytes was slightly elevated in individuals with health conditions compared to those without (mean difference [95%CI]: 0.028[-0.10-0.066], p=0.149, Figure 6), suggesting monocytes may be related to health status. As a result, monocyte CD16, was higher in individuals with more self-reported health conditions when controlling for age, sex and BMI(r=0.438, p=0.00129, Table 10, Figure 5). We found no associations with markers of monocyte activation or trafficking. We quantified MDSCs in this cohort, which are immature myeloid cells that are elevated following inflammation-induced myelopoiesis as well as in many chronic conditions[248]. We were able to distinguish between granulocytic and monocytic MDSCs (gMDSCs; mMDSCs) and found that the latter most strongly associated with health status. BMI and health conditions were both correlated with mMDSCs (cells/mL) when controlling for sex and age (BMI r=0.274, p=0.049; health conditions r= 0.353, p=0.010, Table 9, Figure 2). When we build a mixed model to predict circulating mMDSCs with the fixed effects of health conditions, BMI and age and the random effect of sex, the only significant predictor is the number of health conditions ($p=2.13 \times 10^{-5}$, Table 10, Figure 2). After setting age and BMI as random effects in addition to sex we found that each health condition resulted in an increase in 0.6817 ± 0.1324 MDSCs(%CD45) or 21,091 ± 5,385 cells/mL (Table 10). This is largely driven by mMDSCs, which have a strong correlation with self-reported health conditions (%MDSCs) (r=0.357, p=0.010).

Due to the strong association between MDSCs and health status in our cohort, we looked at their associations with the monocyte population. mMDSCs were

positively correlated with intermediate monocytes (r=0.293,p=0.035) while being inversely correlated with classical monocytes (r=-0.300, p=0.0343) when we controlled for age, sex, health conditions and BMI (Table 11, Figure 7). This could suggest a delay in the maturation process of myeloid/monocyte precursors to monocytes, with more immature myeloid cells remaining MDSCs rather than differentiating to monocytes. Although our immunophenotyping panels did not allow us to measure CD115 on mMDSCs, we saw a positive association between the proportion of mMDSCs and CD33 and CD115 expression on monocytes (r=0.281, p=.048; r=0.354, p=0.012, Table 11). CD33 is expressed throughout myeloid differentiation; however it decreases in expression as cells mature into monocytes[269]. These data suggest that a block in monocyte maturation occurs in individuals with a higher proportion of mMDSCs. IFNγ and IL-1β were the cytokines most strongly associated with mMDSCs and monocyte CD115 expression (Table 8A). Fractalkine was positively related to monocyte expression of CD115 but not mMDSCs (Table 8A). Only after controlling for fractalkine, IFNγ, IL-1β, and sex was age correlated with monocyte CD115 (r=0.289, p=0.0416, Table 8A). This suggests that age alone is not the most important factor in determining myeloid maturity. In fact, when we used stepwise Akaike information criterion(AIC) to select a model to predict CD115 expression on monocytes, we found IL-1β and age were the most important predictor variables (Table 8B).

Conclusions:

Based on our findings from this study, age and inflammation both have a hand in shaping circulating leukocytes. Age was significantly related to the composition of the leukocyte compartment, as it correlated with an increase in myeloid cells and a decrease in lymphoid cells. Interestingly, age was not correlated with markers of monocyte maturity or activation, except for CD115 after controlling for the cytokines with which it was highly correlated (i.e. IFNγ and IL-1β). The most significant observation from this study was the signature of impaired myeloid maturation that was associated with health status. Individuals with more health conditions had more mMDSCs, which were related to higher serum levels of IFNγ and IL-1β. These two cytokines were also significantly related to higher CD115 expression on monocytes. In mice, excess M-CSF signalling through CD115 has been shown to lead to the expansion of MDSCs[323]. IL-1β in acute settings stimulates normal emergency myelopoiesis via the transcription factor PU.1, which promptly upregulates CD115, however, in chronic settings IL-1β drives constant myelopoiesis and results in HSC exhaustion (i.e. decreased self-renewal capacity)[324]. This may result in adaptations where myelopoiesis relies on more differentiated myeloid progenitors downstream of the HSC[325,326]. In other models, overexpression of IL-1β leads to the activation of mobilization of MDSCs[327]. IFNγ similarly stimulates emergency myelopoiesis through the transcription factor C/EBPβ in both acute and chronic settings[328,329], and it is instrumental in the activation of MDSC cytokine production and suppressive activity[330].

In the future, our study would benefit from further examination of the transcriptional and epigenetic landscapes of monocyte subsets from humans to determine how markers of monocyte maturation (i.e. transcription factors PU.1, IRF-8, KLF4) are altered with age and inflammation[88,331]. As we have demonstrated, each cytokine plays a unique role in tuning the myeloid compartment. *In vivo,* cytokines are never present in isolation and their concentrations and gradients fluctuate. In order to disentangle which cytokines are most important in instructing myeloid development, we have to understand the effect of their changes in dose, time and co-administration with cocktails of other cytokines. In the following chapter, we demonstrate the importance of inflammation in shaping the monocyte compartment in an advanced model of myeloid aging (e.g. rheumatoid arthritis). We additionally follow patients along immunomodulatory treatment that reduces systemic inflammation to demonstrate the importance of inflammation on the monocyte compartment.

<u>Tables & Figures:</u>

Table 1. Study Cohort. Age, sex, BMI and the prevalence of health conditions.

	n	Mean	SD	Median	Minimum	Maximum
Age	58	55.62	20.33	59	20	93
BMI (kg/m^2)	54	25.23	4.30	24.93	15.22	34.33

		Age				
Sex	**n (%)**	**Mean**	**SD**	**Median**	**Minimum**	**Maximum**
Female	39 (67.2)	57.5	20.1	63	20	92
Male	19(32.8)	51.7	20.8	53	21	93

Age	Freq(%)	n	Sex (%)
<50 years	39.7	23	60.9% Female 39.1% Male
>50 years	60.3	35	71.4% Female 28.6% Male

Number of Health Conditions	n	Freq(%)
0	30	51.7
1	18	31.0
2	7	12.1
3	1	1.7
4	2	3.4

Table 2. Self-reported Health Conditions. The cohort was divided into those under and those over the age of 50. The number of health conditions per person and the number of individuals who reported certain health conditions were quantified.

Number of Health Conditions	Participants under age 50	
	n	Freq(%)
0	18	78.3
1	5	21.7
2	0	0.0
3	0	0.0
4	0	0.0
Total	23	100.0

Number of Health Conditions	Participants over age 50	
	n	Freq(%)
0	12	34.3
1	13	37.1
2	7	20.0
3	1	2.9
4	2	5.7
Total	35	100.0

Health Conditions reported by Participants under age 50	
n	Condition
2	Asthma
1	Chronic shoulder dislocations
1	Ulcerative Colitis
1	Hypothyroidism

Health Conditions reported by Participants over age 50	
n	Condition
1	Allergic rhinitis
1	Arrhythmia
1	Autoimmune acrodermatitis
1	Depression
1	Goiter
1	Migraine
1	Recent *C.difficile* infection (within a year)
1	Recent heart attack (within a year)
2	COPD
3	Asthma
3	Heart disease
3	Hypertension
4	Type 2 Diabetes
6	Past treatment of cancer
9	Joint disorder (osteoarthritis, osteoporosis)

Table 3. Age-associated changes in leukocyte numbers and proportions with age. To determine if a change in age results in changes in leukocyte populations we performed linear mixed models controlling for sex, BMI and self-reported health conditions. Statistical significance was tested by Analysis of deviance (Type II Wald chi square test).

Ratio of Myeloid to Lymphoid Cells ~ Age + (1|Sex)						
n	55					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	1.577	0.33108	4.762	4.04	1	**0.04443**
Age	0.011	0.0056	2.01			
Random Effects	**Variance**	**SD**				
Sex	0.00	0.00				
Residual	0.71	0.84				

B cells (%CD45) ~ Age + (1|Sex) + (1|BMI) +(1|Health Conditions)						
n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	4.457	0.884	5.040	5.4832	1	**0.0192**
Age	-0.0284	0.0123	-2.342			
Random Effects	**Variance**	**SD**				
Sex	0.523	1.440				
BMI	1.072	1.439				
Health Conditions	0.046	0.214				
Residual	0.766	0.857				

Non-classical monocytes (cells/mL) ~ Age + (1|Sex) + (1|BMI) +(1|Health Conditions)						
n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	679.39	1201.17	0.566	4.3674	1	**0.03663**
Age	43.27	20.71	2.090			
Random Effects	**Variance**	**SD**				
Sex	0	0				
BMI	0	0				
Health Conditions	0	0				
Residual	$9x10^6$	3000				

Table 4. Correlations of immune parameters with age. Spearman correlation was performed to determine which variables of immunity correlated with age. To control for the effects of sex and body mass index, partial correlation analysis was applied.

Correlation with Age	Spearman Correlation				Partial Correlation controlling for sex and BMI			
	n	r	95% CI	p	n	r	95% CI	p
BMI (kg/m^2)	54	0.300	0.035-0.526	**0.028**	54	0.318	0.055-0.540	**0.020**[$]
Monocytes (cells/mL)	58	0.092	-0.170-0.342	0.492	54	0.137	-0.136-0.391	0.3322
NK cells (cells/mL)	56	0.044	-0.221-0.305	0.747	52	0.151	-0.128-0.407	0.2965
B cells (cells/mL)	55	-0.305	-0.527- -0.043	**0.024**	51	-0.313	-0.542- -0.042	**0.0283**
T cells (cells/mL)	55	-0.174	-0.420-0.096	0.205	51	-0.114	-0.377-0.167	0.4367
Monocytes (%CD45)	58	0.108	-0.154-0.357	0.418	54	0.137	-0.136-0.390	0.3342
NK cells (%CD45)	56	0.118	-0.149-0.370	0.386	52	0.209	-0.06770- 0.456	0.1451
B cells (%CD45)	55	-0.345	-0.559-0.088	**0.010**	51	-0.345	-0.567- -0.077	**0.0151**
T cells (%CD45)	55	-0.286	-0.513- -0.023	**0.032**	52	-0.180	-0.432-0.098	0.2112
CD4 T cell (%T cells)	54	0.259	-0.021-0.501	*0.058*	50	0.096	-0.188-0.364	0.518
IP-10 (pg/mL)	58	0.512	0.292-0.680	**3.98×10^{-5}**	54	0.502	0.270-0.678	**0.0001**
MCP-1 (pg/mL)	58	0.239	-0.021-0.468	*0.071*	54	0.311	0.047-0.534	**0.0247**
Intermediate Monocyte CD16 (MFI)	58	-0.377	-0.578- -0.153	**0.0035**	54	-0.262	-0.495-0.006	0.0603
Monocyte CX_3CR_1 (MFI)	58	0.301	0.051-0.523	**0.021**	54	0.232	-0.039-0.470	0.0985

[$]Controlling for sex only

Table 5. BMI is a stronger correlate of the CD4 T cell compartment.

Partial Correlation with BMI	Controlling for sex, age, and health conditions			
	n	r	95% CI	p
CD4 T cell (%T cells)	50	0.327	0.054-0.555	**0.0247**

CD4 T cells (% T cells) ~ Age + BMI + (1|Sex)						
n	50					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	42.39712	8.38299	5.058			
Age	0.00628	0.07268	0.086	0.0075	1	0.93114
BMI	0.8222	0.34055	2.414	5.8289	1	**0.01576**
Random Effects	**Variance**	**SD**				
Sex	0	0				
Residual	94.97	9.75				

Table 6. Age-associated changes in serum inflammation.

MCP-1 (pg/mL) ~ Age + (1|Sex) + (1|BMI) +(1|Health Conditions)						
n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	1094.756	277.163	3.95	9.9838	1	**0.001579**
Age	12.307	3.895	3.16			
Random Effects	**Variance**	**SD**				
Sex	0.00	0.00				
BMI	0.00	0.00				
Health Conditions	54133	232.7				
Residual	309609	556.4				

IP-10 (pg/mL) ~ Age + (1|Sex) + (1|BMI) +(1|Health Conditions)						
n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	411.162	186.696	2.202	10.166	1	**0.00143**
Age	8.63	2.707	3.188			
Random Effects	**Variance**	**SD**				
Sex	4001.00	63.25				
BMI	0.00	0.00				
Health Conditions	17060.00	130.60				
Residual	128500.00	358.50				

Table 7. Cytokines and their relationships with circulating leukocytes.

Partial Correlations controlling for age, sex, BMI and health conditions					
Variable 1	**Variable 2**	**n**	**r**	**95% CI**	**p**
Ratio CD14+ to CD16+ Monocytes	**IL-6**	54	-0.249	-0.485-0.020	0.0811
	GM-CSF	54	-0.277	-0.507- -0.101	0.051
Non-classical Monocyte CD64 (MFI)	**IL-6**	54	0.299	0.0340-0.525	**0.0349**
	IL-10	54	0.354	0.095-0.568	**0.0117**
Monocyte CCR2 (MFI)	**IL-6**	54	-0.273	-0.504- -0.006	**0.0551**
	MCP-1	54	-0.317	-0.539- -0.053	**0.0222**
VEGFA (pg/mL)	**Non-classical Monocyte CD13 (MFI)**	54	-0.400	-0.608- -0.140	**0.00537**
	Monocytes (%CD45)	51	0.339	0.070-0.562	**0.0196**
TNF (pg/mL)	**Neutrophils (%CD45)**	54	0.329	0.076-0.548	**0.019**
	Intermediate Monocyte CCR2 (MFI)	54	0.303	0.038-0.528	**0.0326**

Table 8. Correlates of mMDSCs and monocyte CD115 expression.

A)

Partial Correlation controlling for age, sex, BMI and health conditions					
Variable 1	**Variable 2**	**n**	**r**	**95% CI**	**p**
mMDSC(%MDSC)	**IFNγ**	54	0.35	0.091 - 0.565	**0.013**
	IL-1β	54	0.356	0.099 - 0.570	**0.011**
Monocyte CD115 (MFI)	**Fractalkine**	54	0.336	0.0745 - 0.554	**0.0172**
	IFNγ	54	0.297	0.0320 - 0.523	**0.0361**
	IL-1β	54	0.335	0.0733 - 0.553	**0.0176**
Partial Correlation controlling for Fractalkine, IFNγ, IL-1β, sex					
Variable 1	**Variable 2**	**n**	**r**	**95% CI**	**p**
Age	**Monocyte CD115 (MFI)**	54	0.289	0.023-0.517	**0.0416**

B)

Initial Model: lm(ln(Monocyte CD115) ~ zscore(ln(IL-1β)) + zscore(ln(IFNγ)) + zscore(ln(Fractalkine)) + Age+ Sex +BMI + Health Conditions)

Final Model: lm(ln(Monocyte CD115) ~ zscore(ln(IL-1B)) + Age)

	Step	**Df**	**Deviance**	**Residual Df**	**Residual Deviance**	**AIC**
1	Full Model			44	58.012	21.690
2	– Health Conditions	1	0.126	45	58.138	19.802
3	– Fractalkine	1	0.326	46	58.464	18.093
4	– Sex	1	1.560	47	60.024	17.462
5	– BMI	1	1.606	48	61.630	16.835
6	– IFNg	1	1.984	49	63.614	16.483

Model: lm(ln(Monocyte CD115) ~ zscore(ln(IL-1β) + Age)				
Coefficients:				
Estimate	**Std.**	**Error**	**t**	**p**
(Intercept)	4.293491	0.472733	9.082	
zscore(ln(IL-1β))	0.476718	0.159225	2.994	0.00431
Age	0.017453	0.008063	2.165	0.03532
Residuals:				
Min	**1Q**	**Median**	**3Q**	**Max**
-3.1697	-0.7342	-0.1202	0.6634	2.2059
Adjusted R^2: 0.1746	**$F_{(2,49)}$**: 6.396	**p**=0.003405	**Residual, SE, df**	1.139, 49

Table 9. Immune correlates of health.

Partial Correlation controlling for sex and BMI					
Variable 1	**Variable 2**	**n**	**r**	**95% CI**	**p**
Age	**Health Conditions**	54	0.439	0.195-0.633	**0.001**
Partial Correlation controlling for age and sex					
Variable 1	**Variable 2**	**n**	**r**	**95% CI**	**p**
mMDSC (cells/mL)	**BMI**	54	0.274	0.007-0.505	**0.049**
	Health Conditions	54	0.353	0.094-0.567	**0.010**
mMDSC (%MDSCs)	**Health Conditions**	54	0.357	0.098-0.570	**0.010**
Partial Correlation controlling for age and sex and BMI					
Variable 1	**Variable 2**	**n**	**r**	**95% CI**	**p**
Monocyte CD16 (MFI)	**Health Conditions**	54	0.438	0.193-0.632	**0.001**

Table 10. MDSCs correlate with self-reported health conditions.

mMDSCs (cells/mL) ~ Age + BMI + Health Conditions + (1|Sex)						
n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	-12501.18	29236.17	-0.428			
Age	-42.84	273.32	-0.157	0.0246	1	0.8754
BMI	1000.96	1164.82	0.859	0.7384	1	0.3902
Health Conditions	23096.97	5433.24	4.251	18.0714	1	**$2.13x10^{-5}$**
Random Effects	**Variance**	**SD**				
Sex	0	0				
Residual	$1.15x10^{9}$	33928				

MDSCs (%CD45) ~ Health Conditions +(1|Age) + (1|BMI) + (1|Sex)						
n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	0.9346	0.1827	5.115			
Health Conditions	0.6817	0.1324	5.151	26.532	1	**$2.59x10^{-7}$**
Random Effects	**Variance**	**SD**				
Age	0	0				
BMI	0	0				
Sex	0	0				
Residual	0.39	0.63				

MDSCs (cells/mL) ~ Health Conditions +(1|Age) + (1|BMI) + (1|Sex)						
n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	23741	6796	3.493			
Health Conditions	21091	5385	3.917	15.34	1	**$8.98x10^{-5}$**
Random Effects	**Variance**	**SD**				
Age	0	0				
BMI	0	0				
Sex	0	0				
Residual	$1.55x10^{9}$	39326				

Table 11. Associations between MDSCs and Monocytes.

Partial Correlation controlling for age, sex, BMI and health conditions					
Variable 1	**Variable 2**	**n**	**r**	**95% CI**	**p**
mMDSC(%MDSC)	**Intermediate Monocytes (% Monocytes)**	54	0.298	0.033-0.524	**0.0354**
	Classical Monocytes (% Monocytes)	54	-0.300	-0.526—0.035	**0.0342**

Partial Correlation controlling for age, sex, BMI and health conditions					
Variable 1	**Variable 2**	**n**	**r**	**95% CI**	**p**
mMDSC(%MDSC)	**Monocyte CD33 (MFI)**	54	0.281	0.013-0.510	**0.048**
	Monocyte CD115 (MFI)	54	0.354	0.095—0.568	**0.012**

Figure 1. Cytokine correlations with age, sex and health conditions. Spearman correlation analyses were performed. A black asterisk indicates correlations where p<0.05, a red asterisk indicates correlations where p<0.05 after FDR adjustment.

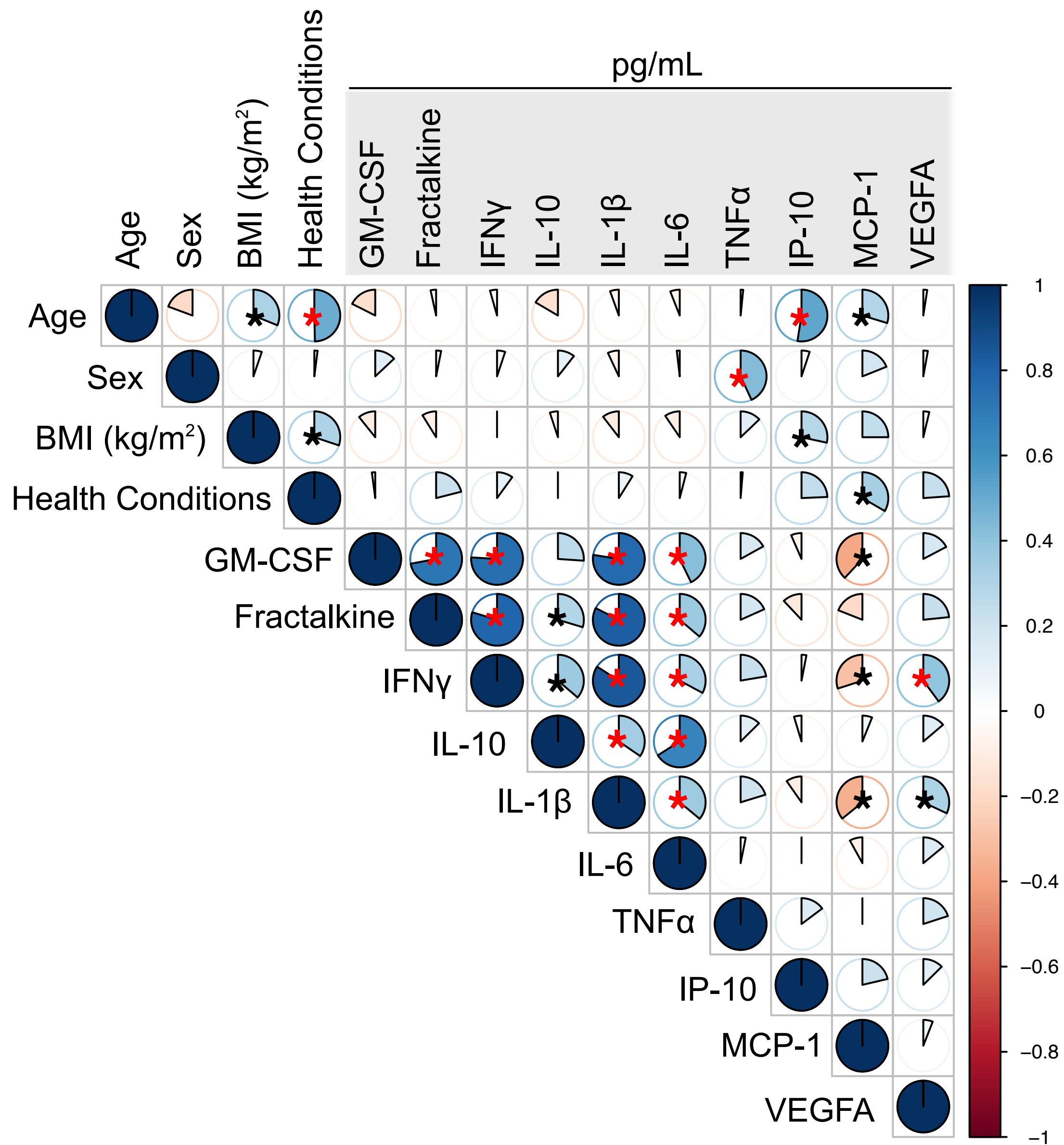
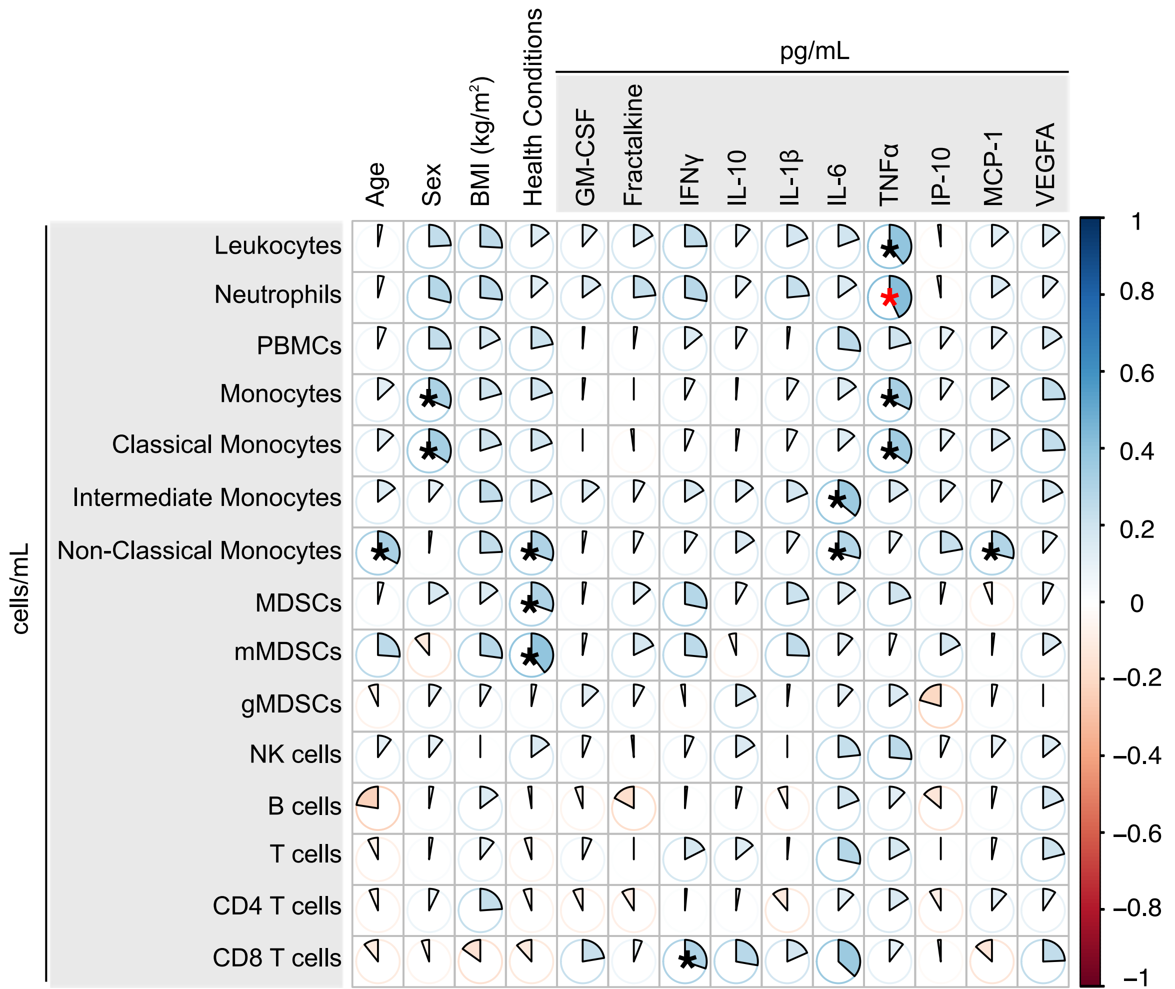


Figure 2. Cytokine correlations with cell numbers. Spearman correlation analyses were performed. A black asterisk indicates correlations where p<0.05, a red asterisk indicates correlations where p<0.05 after FDR adjustment.



Non-classical Monocytes (%CD45)
Non-classical Monocytes (%Monocytes)
MDSCs (%CD45)
mMDSC (%MDSC)
gMDSCs (%MDSC)

Figure 3. Cytokine correlations with lymphoid cells. Spearman correlation analyses were performed. A black asterisk indicates correlations where $p<0.05$, a red asterisk indicates correlations where $p<0.05$ after FDR adjustment.

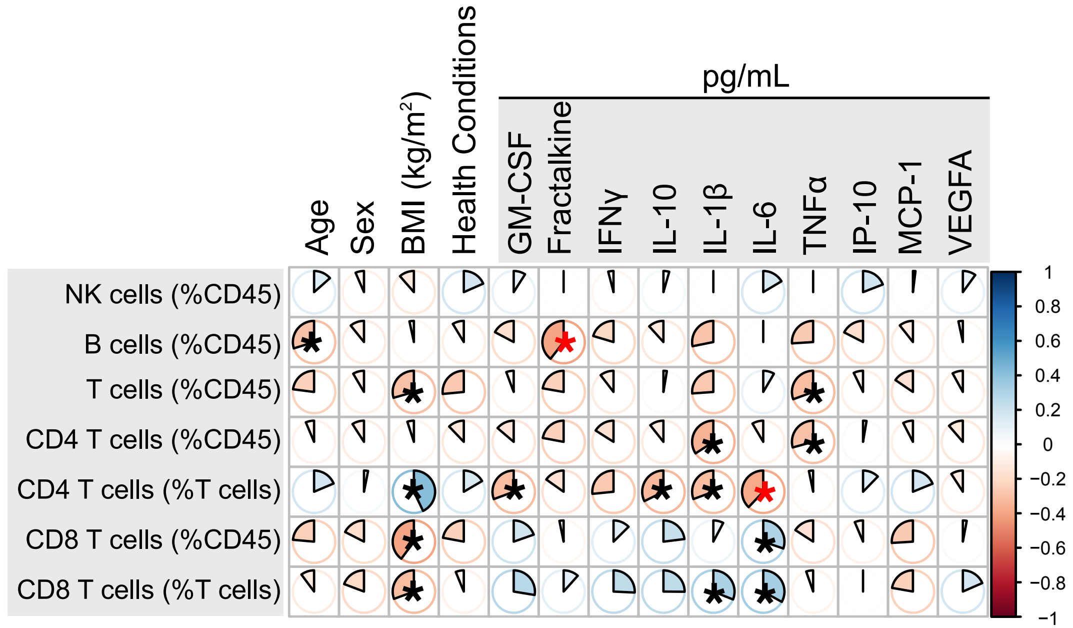


Figure 4. Cytokine correlations with myeloid cells. Spearman correlation analyses were performed. A black asterisk indicates correlations where $p<0.05$, a red asterisk indicates correlations where $p<0.05$ after FDR adjustment.

Figure 5. Cytokine correlations with markers of monocyte activation and maturity. Spearman correlation analyses were performed. A black asterisk indicates correlations where p<0.05, a red asterisk indicates correlations where p<0.05 after FDR adjustment.

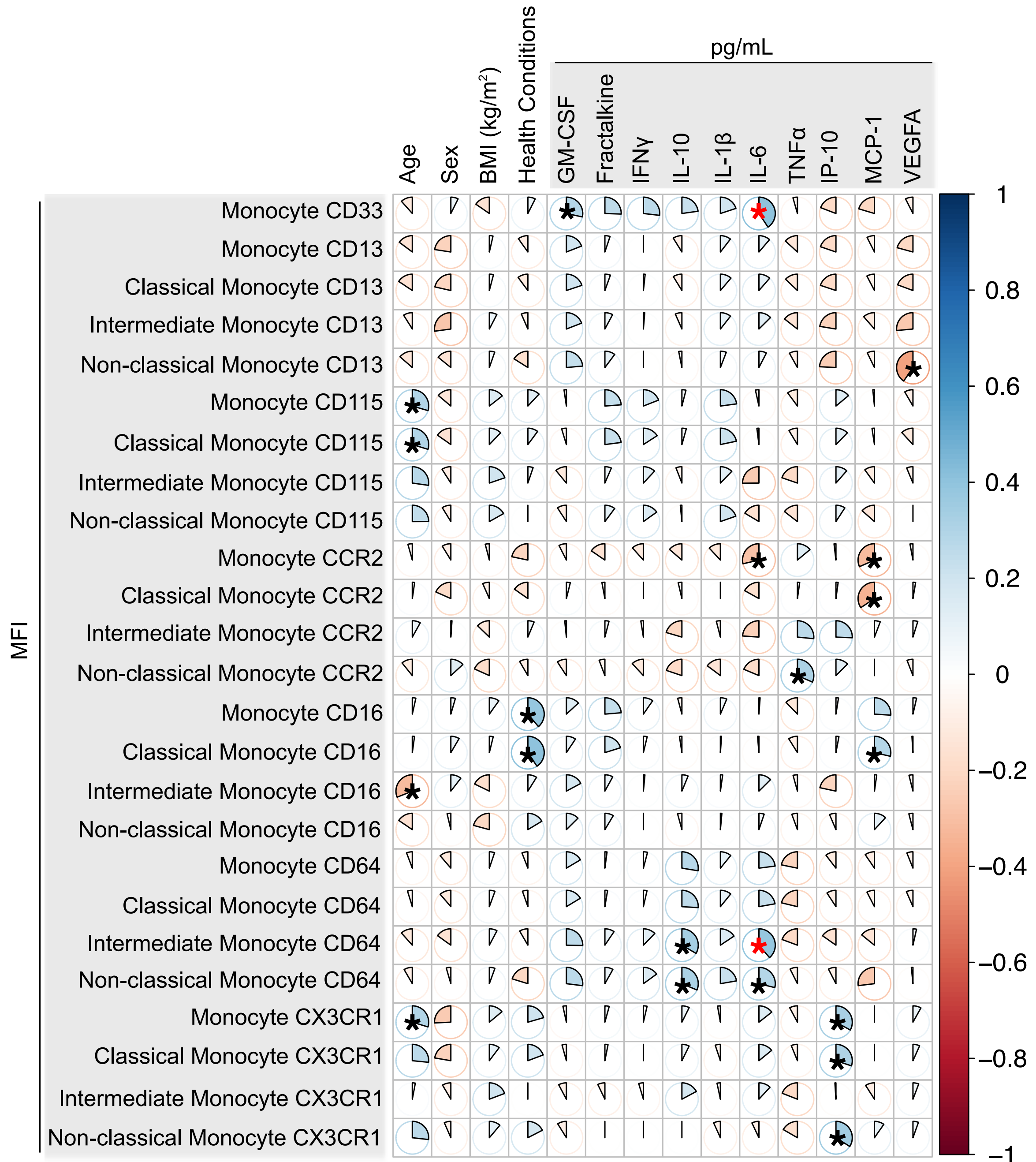
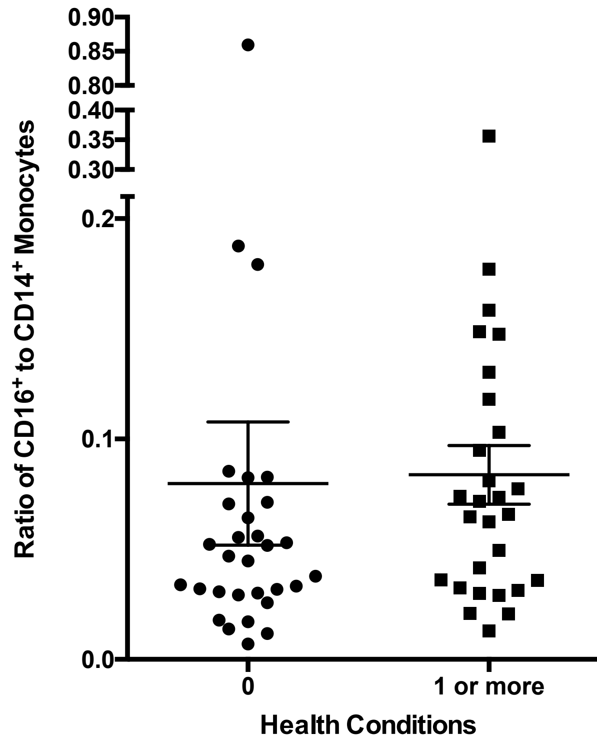
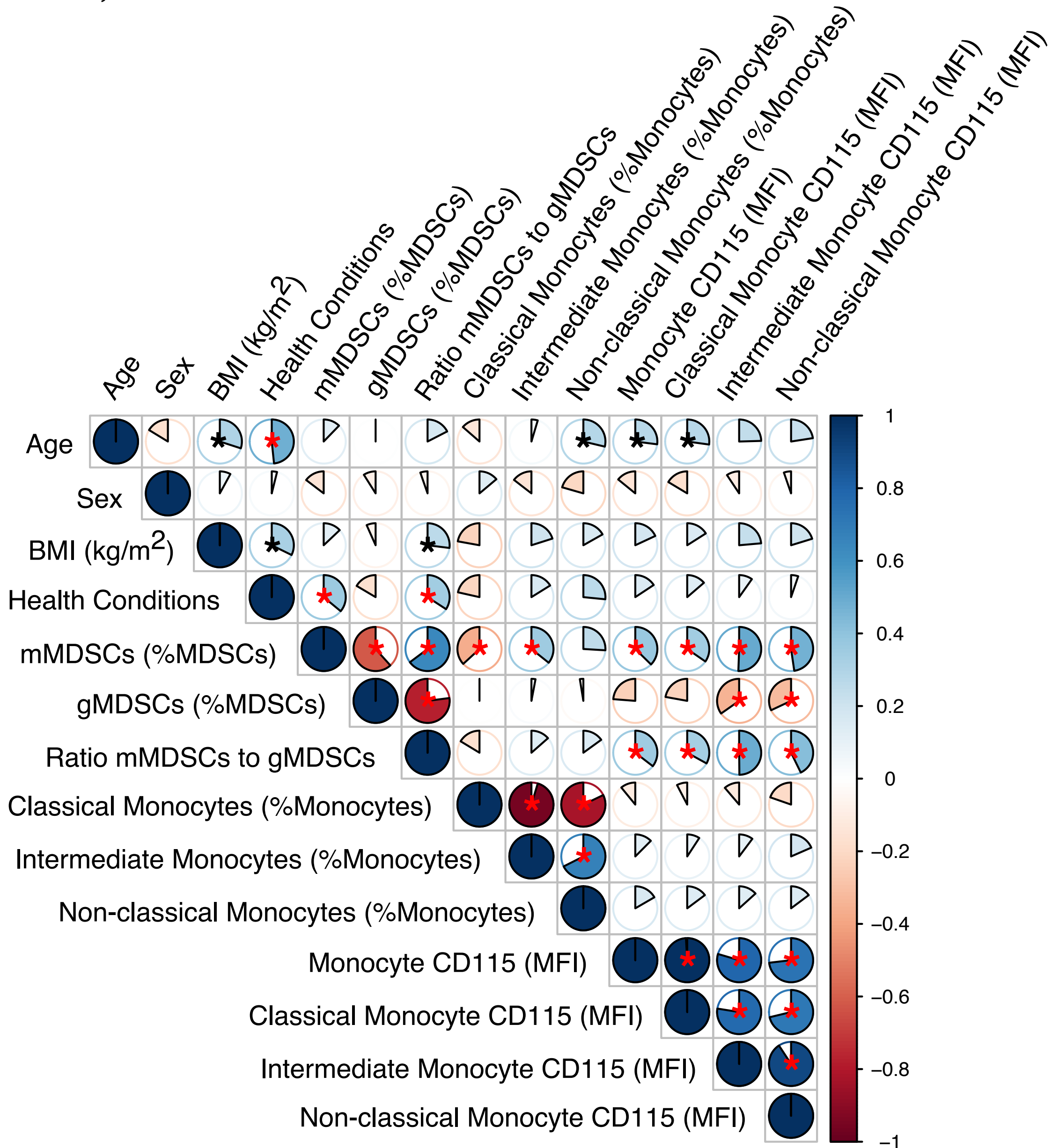


Figure 6. Individuals with health conditions have an expansion in CD16⁺ monocytes compared to individuals with no health conditions. The ratio of the sum of the number of intermediate and non-classical monocytes to the number of classical monocytes was calculated for every individual and the cohort was stratified into those without self-reported health conditions and those with 1 or more. The comparison between these two groups was made while controlling for sex, age and BMI using ANCOVA analysis.



ANCOVA comparing Ratio of CD16⁺ to CD14⁺	F	P	Mean [95%CI]
No Health Conditions vs 1 or more Health Conditions	2.147	.149	No Health Conditions: 0.055[0.030-0.080] 1 or more Health Conditions: 0.083[0.058-0.108]
Sex	1.073	0.305	
Age	0.002	.968	
BMI	0.106	.747	
$df, df_{error}(1,49)$			

Figure 7. Correlates of mMDSCs and monocyte CD115 expression.
Spearman correlation analyses were performed. A black asterisk indicates correlations where p<0.05, a red asterisk indicates correlations where p<0.05 after FDR adjustment.



Supplementary Table 1. Distribution of serum cytokines.

Serum Cytokine (pg/mL)	n	Mean	SD	Median	Minimum	Maximum
GM-CSF	58	80.61	285.85	35.74	5.21	2207.00
Fractalkine	58	113.78	56.67	102.08	44.67	373.03
IFNγ	58	28.06	17.10	24.76	6.31	73.85
IL-10	58	18.47	20.97	10.80	0.19	111.98
IL-1β	58	2.42	1.25	2.04	0.81	6.07
IL-6	58	13.85	25.89	4.89	0.17	132.26
TNF	58	10.88	2.87	10.29	5.46	19.18
IP-10	58	900.95	532.79	783.17	250.53	3492.00
MCP-1	58	1668.83	625.41	1568.00	544.98	3373.00
VEGFA	55	668.30	790.28	447.39	3.12	5104.00

Supplementary Table 2. Distribution of monocyte markers of maturity and activation.

MFI		n	Mean	SD	Median	Minimum	Maximum
CD33	Monocytes	58	7457.7	3121.6	7008.0	1221.0	15856.0
CD115	Monocytes	58	344.7	358.6	212.0	0.0	1660.5
	Classical	58	330.5	347.4	192.6	0.0	1635.5
	Intermediate	58	563.4	696.2	289.4	0.0	3828.5
	Non-classical	58	1060.8	856.8	784.0	13.8	3122.5
CD13	Monocytes	58	4950.5	4199.8	4213.2	220.0	17557.7
	Classical	58	4773.8	4044.7	3969.1	220.0	17213.7
	Intermediate	58	8722.9	7085.5	7690.3	314.0	27448.7
	Non-classical	58	7091.5	5345.3	6313.2	187.3	20827.0
CD64	Monocytes	58	18688.9	25024.9	11533.4	5375.7	149130.8
	Classical	58	18621.7	23989.5	11725.8	5442.7	143167.8
	Intermediate	58	26184.0	44055.2	12038.9	4896.7	226345.8
	Non-classical	58	12402.8	20462.2	6942.4	228.8	125308.8
CX3CR1	Monocytes	58	1959.7	1191.5	1712.1	1008.0	7804.0
	Classical	58	1816.8	1180.0	1639.8	831.0	7697.0
	Intermediate	58	4675.1	1987.3	4216.5	1660.0	12396.0
	Non-classical	58	6378.9	1839.1	6280.5	2485.0	12597.0
CD16	Monocytes	58	91.8	97.9	74.9	0.0	636.7
	Classical	58	60.0	41.8	56.7	0.0	220.0
	Intermediate	58	2837.4	1284.4	2691.0	495.2	6680.7
	Non-classical	58	3937.0	2337.4	3465.0	407.2	10179.7
CCR2	Monocytes	58	21281.2	6416.5	21609.6	2932.0	36745.8
	Classical	58	25110.3	6006.3	24208.8	13982.0	41211.8
	Intermediate	58	5973.6	4625.7	5381.4	384.7	23425.3
	Non-classical	58	1442.2	3051.8	604.1	1.7	21575.0

Supplementary Table 3. Distribution of leukocyte numbers and proportions.

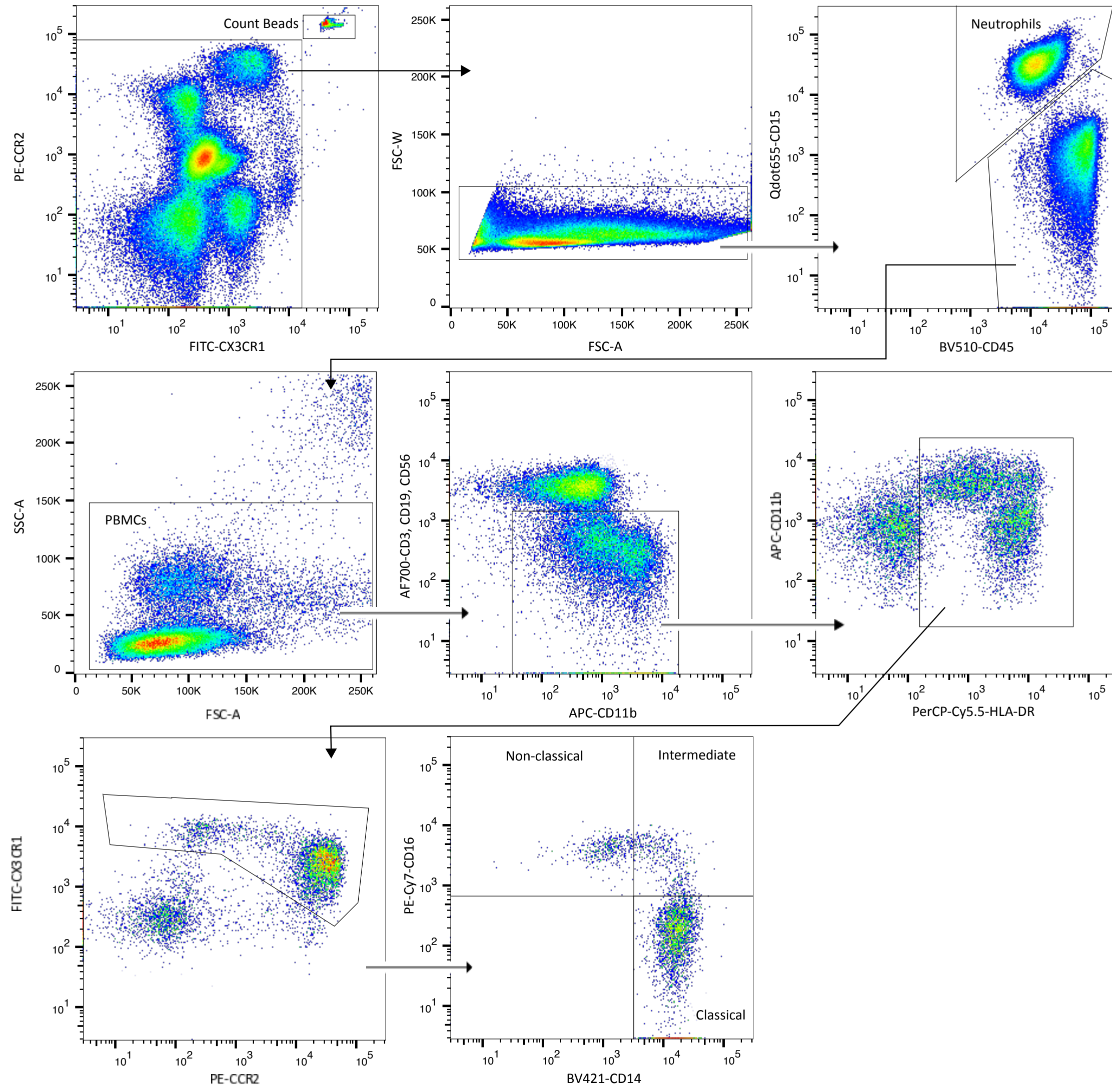
Cells/mL blood		**n**	**Mean**	**SD**	**Median**	**Minimum**	**Maximum**
Leukocytes		58	3.14×10^6	2.92×10^6	2.38×10^6	5.19×10^5	1.72×10^7
Neutrophils		58	1.85×10^6	1.75×10^6	1.42×10^6	2.52×10^5	1.02×10^7
PBMCs		58	1.28×10^6	1.21×10^6	9.45×10^5	2.67×10^5	6.96×10^6
Monocytes		58	1.39×10^5	1.38×10^5	1.01×10^5	1.43×10^4	9.02×10^5
	Non-classical	58	3.12×10^3	3.30×10^3	1.70×10^3	1.92×10^2	1.47×10^4
	Intermediate	58	4.87×10^3	5.64×10^3	2.35×10^3	2.37×10^2	2.74×10^4
	Classical	58	1.31×10^5	1.34×10^5	9.56×10^4	1.33×10^4	8.86×10^5
NK cells		56	1.22×10^5	9.64×10^4	8.62×10^4	1.46×10^4	4.39×10^5
B cells		55	1.12×10^5	1.98×10^5	7.07×10^4	8.06×10^2	1.19×10^6
T cells		55	7.64×10^5	8.56×10^5	5.92×10^5	1.60×10^5	6.49×10^6
CD4 T cells		55	4.79×10^5	5.03×10^5	3.62×10^5	1.36×10^5	3.68×10^6
CD8 T cells		56	2.21×10^5	2.00×10^5	1.81×10^5	2.18×10^4	1.42×10^6
Ratio of Myeloid to Lymphoid cells		55.00	2.20	0.87	2.14	0.65	4.64
% of Leukocytes		**n**	**Mean**	**SD**	**Median**	**Minimum**	**Maximum**
Neutrophils		58	57.75	9.52	59.45	28.40	75.90
PBMCs		58	42.05	9.46	40.05	24.10	71.20
Monocytes		58	4.55	1.62	4.26	1.69	9.60
	Non-classical	58	2.84	3.40	1.74	0.25	21.30
	Intermediate	58	3.97	3.94	2.48	0.38	24.50
	Classical	58	93.00	7.10	94.55	53.30	99.30
MDSCs		58	1.41	1.19	1.03	0.24	7.06
NK cells		56	4.20	2.45	3.49	1.02	11.70
B cells		55	3.01	1.79	2.71	0.04	7.94
T cells		56	24.64	7.71	23.35	12.00	44.30
CD4 T cells		54	15.67	4.88	15.05	5.90	31.50
CD8 T cells		56	7.54	4.04	6.96	1.08	21.50
% of PBMCs		**n**	**Mean**	**SD**	**Median**	**Minimum**	**Maximum**
Monocytes		58	11.14	4.28	10.70	4.75	24.00
	Non-classical	58	0.28	0.28	0.19	0.02	1.56
	Intermediate	58	0.41	0.37	0.28	0.03	1.79
	Classical	58	10.17	4.23	10.20	3.70	22.15
% of MDSCs		**n**	**Mean**	**SD**	**Median**	**Minimum**	**Maximum**
	mMDSCs	58	10.70	12.15	8.48	0.24	56.10
	gMDSCs	58	79.48	17.19	86.15	17.70	96.50
% of Monocytes		**n**	**Mean**	**SD**	**Median**	**Minimum**	**Maximum**
	Non-classical	58	2.84	3.40	1.74	0.25	21.30
	Intermediate	58	3.97	3.94	2.48	0.38	24.50
	Classical	58	93.00	7.10	94.55	53.30	99.30
Ratio of CD14+ to CD16+ Monocytes		58	27.41	28.33	18.52	1.16	143.91
% of T cells		**n**	**Mean**	**SD**	**Median**	**Minimum**	**Maximum**
CD4 T cells		54	64.18	11.12	64.35	37.50	88.80
CD8 T cells		55	29.74	10.74	29.00	8.25	57.90
Ratio of CD4 to CD8 T cells		55	2.66	1.69	2.28	0.65	10.77

Supplementary Table 4. Medication Use and Associated Conditions.

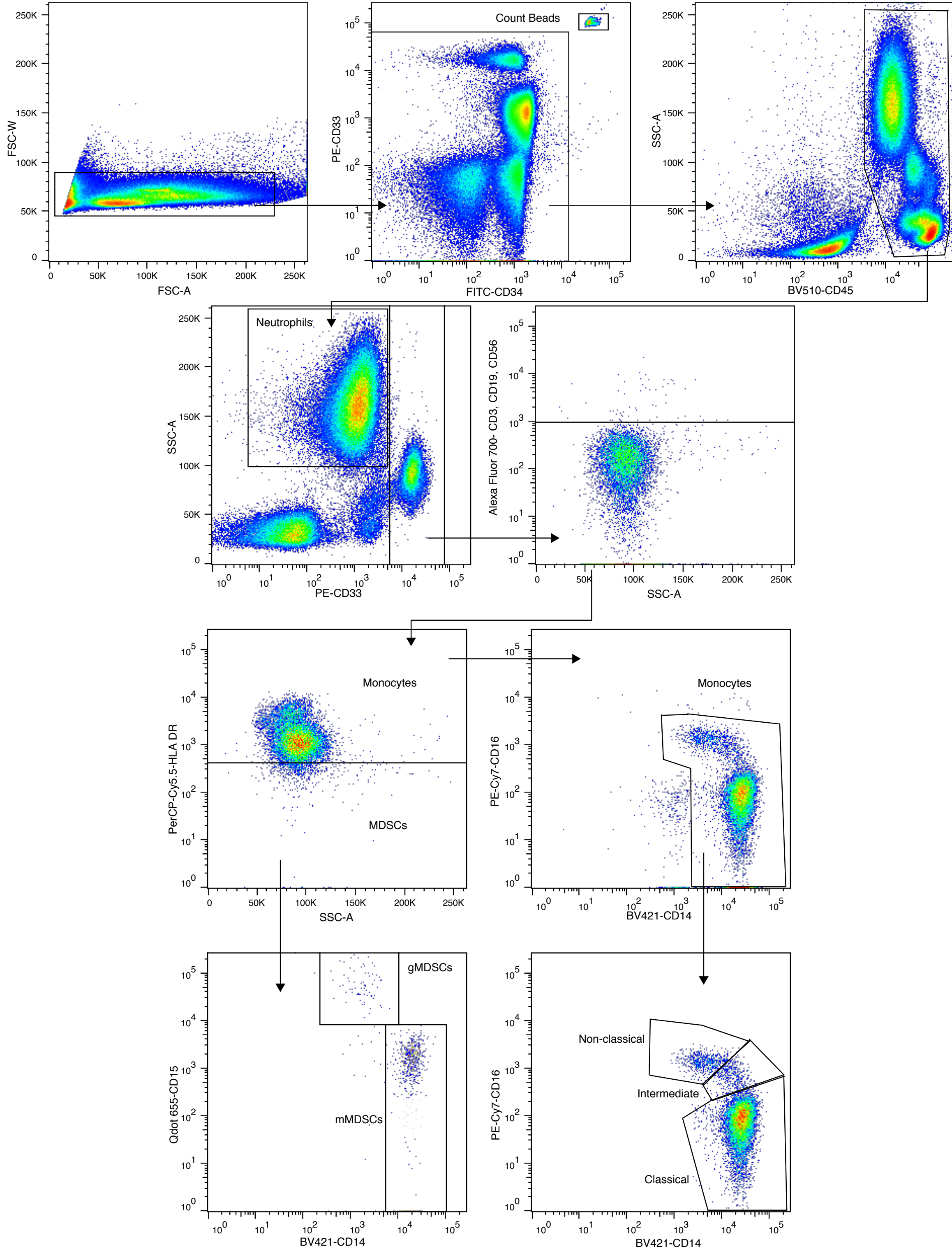
Medications		n
Anxiety	Effexor	1
	Lipralex	1
	Pristiq	1
ADHD	Adderall	1
Allergy	Nasonex	1
Asthma	Astrovent	1
	Avamys	1
	Fluticasonefuroate	1
	Qvar	1
COPD	Spiriva	1
Depression	Citalopram	1
	Paxil	1
	Sertraline	1
Diabetes	Glumetza	1
	Metfomin	2
GERD	Ranitidine	1
	Pantoprazole	1
Gout	Allopurinol	1
	Feboxostat	1
Hepatic Encephalopathy	Zaxine	1
Hormone Replacement & Contraceptions	Premarin	1
	Nuvaring	1
Hyperlipidemia, Hypertension, Thrombosis	Accupril	1
	Amlodipine	2
	Atenolol	1
	Bisoprolol	2
	Diltiazem	1
	Ezetimibe	1
	Furosemide	2
	Hydrochlorothiazide	2
	Lipitor/Atorvastatin	3
	Metoprolol	1
	Nitroglycerine Patch	1
	Olmetec Plus	1
	Plavix/Clopidogrel	1
	Ramipril	4
	Rivaroxaban	1
	Rosuvastatin	4
	Spironolactone	1

Medications		n
Hypothyroidism	Synthroid/Levothyroxine	3
Insomnia	Temazepam	1
	Zopiclone	1
Migraine	Amitriptyline	1
	Candesartan	1
	Zolmitriptan	1
Non-steroidal anti-inflammatory	Aspirin	6
	Celebrex	1
	Celecoxib	1
	Ibuprofen	2
	Tylenol/Acetaminophen	1
Osteoporosis	Actonel	1
	Prolia/Denosumab	3
Vitamins and Supplements	Calcium	1
	Folic Acid	1
	Iron	2
	Multivitamin	1
	Omega 3	1
	Vitamin B6	1
	Vitamin B12	1
	Vitamin D	16

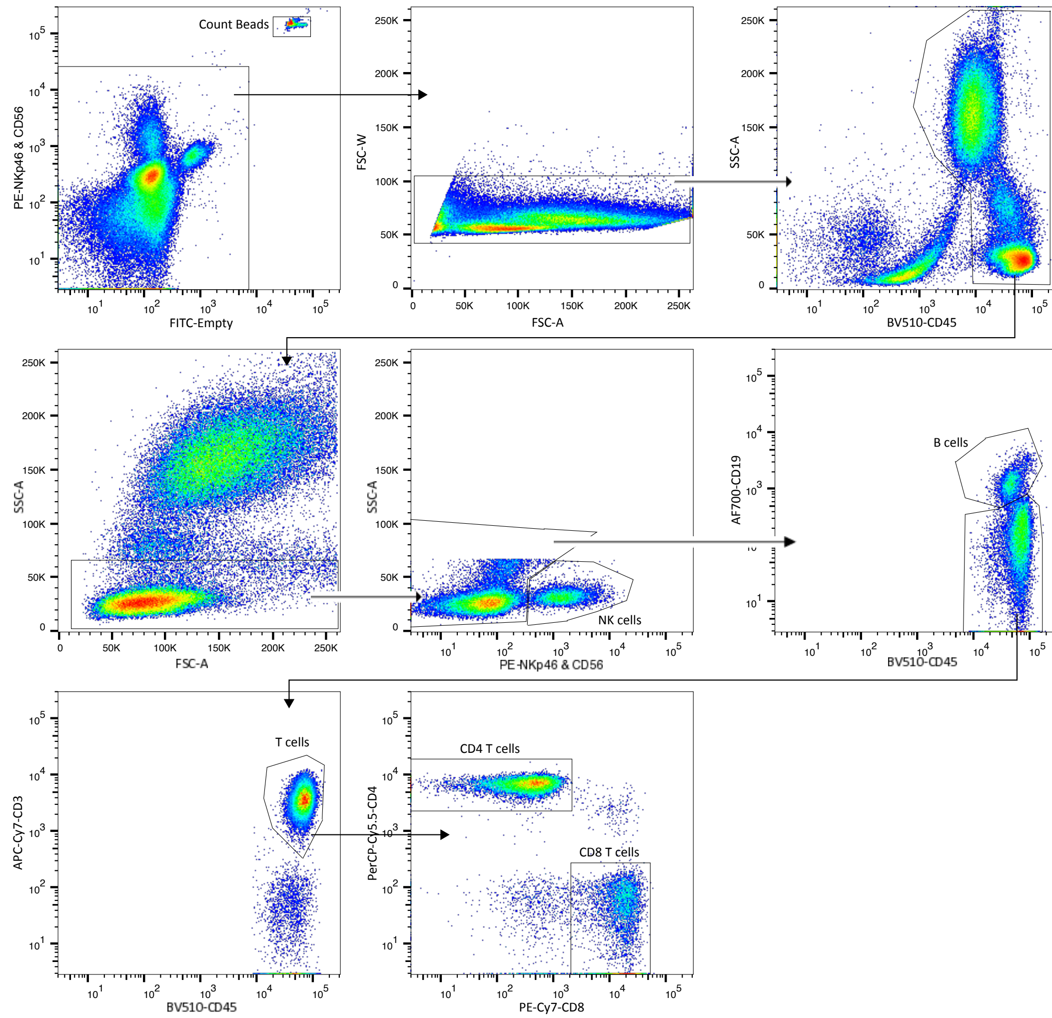
Supplementary Figure 1. Monocyte Gating Strategy.



Supplementary Figure 2. MDSC Gating Strategy

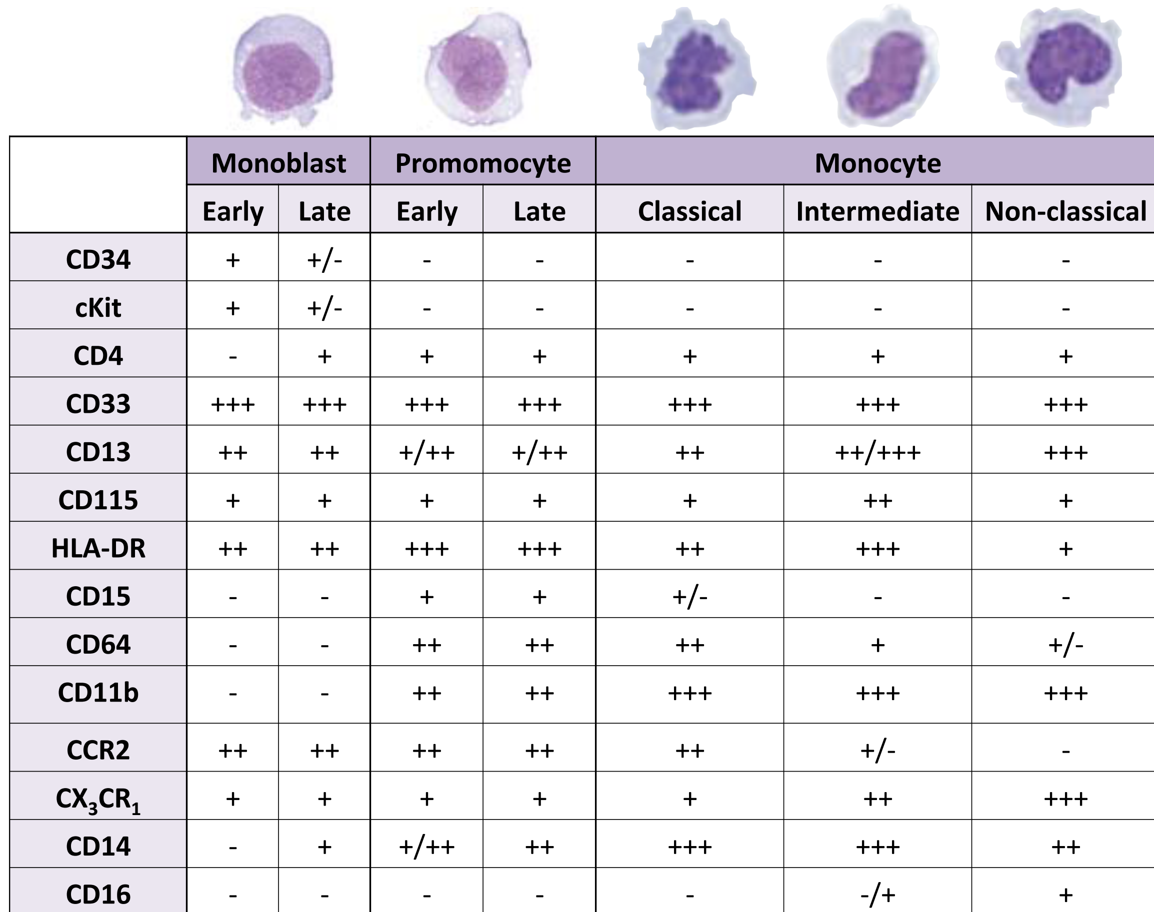


Supplementary Figure 3. Lymphocyte Gating Strategy



Appendix:

Figure 1. Human Monocyte Maturity. A summary of how the expression of surface markers change during monocyte differentiation, previously described by many groups[268,269,309,332].



	Monoblast		Promomocyte		Monocyte		
	Early	Late	Early	Late	Classical	Intermediate	Non-classical
CD34	+	+/-	-	-	-	-	-
cKit	+	+/-	-	-	-	-	-
CD4	-	+	+	+	+	+	+
CD33	+++	+++	+++	+++	+++	+++	+++
CD13	++	++	+/++	+/++	++	++/+++	+++
CD115	+	+	+	+	+	++	+
HLA-DR	++	++	+++	+++	++	+++	+
CD15	-	-	+	+	+/-	-	-
CD64	-	-	++	++	++	+	+/-
CD11b	-	-	++	++	+++	+++	+++
CCR2	++	++	++	++	++	+/-	-
CX_3CR_1	+	+	+	+	+	++	+++
CD14	-	+	+/++	++	+++	+++	++
CD16	-	-	-	-	-	-/+	+

Surface markers of monocyte maturity and activation:

CD33, also known as sialic acid-binding Ig superfamily lectin (Siglec)-3[333], is a pan myeloid marker[334], which can be expressed on some normal B cells and activated T and NK cells, but not on pluripotent HSCs or non-hematopoietic cells[335,336]. It is most highly expressed on promonocytes and classical monocytes and decreases following differentiation into more mature monocytes and macrophages. There is some evidence that it is required for the maturation from $CD34^+$ myeloid precursors and monocytes to macrophages and dendritic cells[337]. CD33 conjugated to a toxic payload has been successful in a subset of AML patients[338].

CD13 is a pan-myeloid marker a metalloproteinase that cleaves N-terminal peptides. It can also be expressed on the surface of fibroblast like synoviocytes, intestinal and renal epithelia, on angiogenic or activated endothelial cells and at the synaptic membranes of pericytes[339]. CD13 is upregulated by TGFβ and downregulated by IL-10. Myeloid cells positive for CD13 have been found to contribute to angiogenesis, tumour growth and metastasis[339,340]. CD13 mediates cell adhesion and motility and has shown to be important in endocytosis and uptake of cholesterol. It can cleave chemokines and cytokines to further influence cell recruitment. CD13 enzymatic activity also plays a role in extracellular processing of antigenic peptides loaded on MHC II[339]. Membrane bound CD13 is cleaved by an unknown enzyme, and its soluble form has been correlated with rheumatoid arthritis[341], angiogenesis and cancer[342].

CX_3CR_1 is a chemokine receptor that binds fractalkine (CX3CL1) and is expressed by NK cells, cytotoxic T cells, γδ cells and monocytes. The receptor and its ligand are upregulated under inflammatory conditions and their interaction promotes leukocyte recruitment by providing the initial tethering and adhesion which leads to transmigration[343]. See *Monocyte Trafficking* section of the introduction for more detail.

CCR2 is a chemokine receptor that binds MCP-1 (CCL2) and is expressed on mononuclear cells, activated NK cells and smooth muscle vascular cells. It mediates the recruitment of T cells and monocytes and has been implicated in inflammatory diseases mediated by these cell types (e.g. RA, MS, IBD, CVD, cancer)[344]. See *Monocyte Trafficking* section of the introduction for more detail.

CD115 also referred to as c-fms, colony stimulating factor 1 receptor (CSF-1R), and macrophage colony stimulating factor receptor (MCSFR). It belongs to the same family of receptor tyrosine kinases as cKit and Flt-3. It binds IL-34 and CSF-1 (MCSF) and leads to proliferation, survival and differentiation of macrophages[108]. Ligand binding results in dimerization, autophosphorylation and downstream activation of MAPK, PI3K and Akt[345,346]. CD115 is primarily expressed by myeloid and monocytic progenitors, monocytes and macrophages. The receptor is additionally expressed on trophoblasts during embryonic development and on osteoclasts, but the gene is under regulation from a separate promoter than in myeloid cells[347]. Cells of mesenchymal origin (i.e. blood vessel endothelial cells, fibroblasts, bone marrow stromal cells), macrophages, and tumour cells produce CD115's ligand, CSF-1[95]. CSF-1 is found at concentrations of 10ng/mL in circulation and in the pg/mL range in

tissues[96]; without the protein, macrophages and their precursors undergo cell death as it is needed to enter cell cycle[94].

CD64 FcγRI is a high-affinity receptor for monomeric IgG. It is a granulo-monocytic lineage marker on $CD34^+$ hematopoietic progenitor cells[348] and is expressed by monocytes, macrophages and activated neutrophils. It is capable of initiating and activating effector immune responses at low concentrations of IgG. Monocytes expressing CD64 have increased phagocytic ability and superoxide production[349] and elevated expression has been found in many chronic inflammatory diseases[350].

CD16 or FcγRIII is a low-affinity receptor for IgG expressed on intermediate and non-classical monocytes as well as NK cells and neutrophils. It can bind IgG in monomeric form or in immune complexes and mediates antibody dependent cell mediated cytotoxicity, phagocytosis and degranulation[113].

Health & Immune Status Questionnaire

Participant ID #:

Date: yyyy/mm/dd Participant Name:_______________

PART A: PATIENT INFORMATION

Date of Birth (YYYY/MM/DD):
Sex:
Age:
Weight (lbs):
Height:
Smoker? Yes No Prior (If so, how long?:____________________)

PART B: INCLUSION CRITERIA (PROCEED ONLY IF YES)

≥ 18 years? Yes No

PART C: CHRONIC CONDITIONS/COMORBDITIES

Check if it applies	Condition
	Cardiovascular disease (i.e. atherosclerosis/hyperlipidemia)
	Type 2 Diabetes
	Dementia
	Autoimmune condition (i.e. Rheumatoid arthritis, lupus, etc.) Please specify:____
	Joint disorder (i.e. osteoarthritis)
	Chronic Obstructive Pulmonary Disorder (COPD)

	Asthma, Emphysema, or Lung Fibrosis (please circle which one applies)
	Past/Current history of cancer Have you had past treatment of cancer: No Yes Are you under current treatment of cancer: No Yes If yes please specify:______________________________

Please list any other conditions that were not included in the list above:

____
____
____

PART D: CURRENT MEDICATION PROFILE (regular medications only)

CONDITION	ASSOCIATED MEDICATION
1.	
2.	
3.	
4.	
5.	
6.	
7.	
8.	
9.	
10.	
Are you currently taking Vitamin D supplements? Yes No	

PART E: RECENT INFECTION

Have you had an infection in the last two weeks? Yes No If **yes**, was it respiratory; or other If yes, please check which symptoms you have below and provide a nasal swab and saliva sample

PART F: RESPIRATORY SYMPTOMS

				Duration
In the past week, have you had a fever ≥ 38°C?	Yes	No	Temperature:	
In the past week, did you have any *new* symptoms?	Yes	No	If yes, check all that apply: Cough	
			Runny nose	
			Sore throat	
			Headache	
			Sinus problems	
			Muscle aches	
			Fatigue	
			Ear ache or infection	
			Chills	

BECAUSE OF YOUR ILLNESS…

			If yes, please provide additional information
Did you go to see a medical doctor?	Yes	No	
Did you go to the hospital?	Yes	No	

SAMPLES

			Specimen ID
Nasopharyngeal swab	Yes	No	
Saliva sample	Yes	No	

PART G: ANTIBIOTIC USE

Have you taken antibiotics in the last 2 weeks? Yes No	**Are you currently on any antibiotics?** Yes No

PART H: VACCINATION STATUS AND RISK FACTORS FOR *S. pneumoniae*

Did you receive the influenza vaccine **this season** (2017-2018)?	Yes No Unknown	If yes, specify date if known:
Did you receive the influenza vaccine **last season** (2016-2017)?	Yes No Unknown	If yes, specify date if known:
Have you **ever** received the influenza vaccine?	Yes No Unknown	If yes, specify date if known:
Have you received the pneumonia vaccine (23-valent polysaccharide vaccine) within the last 5 years?	Yes No Unknown	If yes, specify date if known:

PART I: CONTACT WITH CHILDREN

Does the participant have contact with children? No Yes If **yes,** list ages of children: ___________________________

PART J: FRIED FRAILTY SCALE

In the last year, have you lost more than 10 pounds unintentionally? □ No □ Yes if yes, why? ________________________________
How often in the last week did you feel this way? "I have felt that everything I did was an effort and I could not get going" □ 0 = Rarely or none of the time (<1 day) □ 1 = Some of little of the time (1-2 days) □ 2 = Moderate amount of the time (3-4 days) (Frail) □ 3 = Most of the time (Frail)

PART K: LAB SAMPLES

Specimen Type	Specimen ID#	Date of Collection	
Anterior nares swab (x2)			Baseline
			End of study
Saliva			Baseline
			End of study
Blood			Baseline
			End of study

CHAPTER 3

Modulation of inflammation to reshape the monocyte compartment

Introduction:

Rheumatoid arthritis (RA) is a chronic inflammatory disease which results in the destruction of joints leading to loss of function, disability and shortened life expectancy[351]. Although the initiating event remains unknown, an inflammatory environment drives RA pathogenesis. Synovial fibroblasts assume a pro-inflammatory role, and the elevated inflammation increases catabolism of chondrocytes and activation of osteoclasts[352]. These changes culminate in the inflammation of the synovium, degradation of cartilage, and bone erosion. Both the innate and adaptive immune responses contribute to inflammation, while the adaptive arm drives the auto-antigen specific response[353].

Autoantibodies to citrullinated proteins (ACPAs) and the Fc portion of IgG (rheumatoid factor; RF) are present in 50-70% of patients at diagnosis. Increased anti-citrullinated protein antibodies can be found long before disease and an abnormal response is thought to be important in driving disease, as they are found in 67% of patients and have >97% specificity in clinical diagnosis of RA[354]. Bacterial infections have been thought to initiate disease by increasing ACPAs. It has been

proposed that infections trigger increased protein citrullination, which leads to a break in immune tolerance to endogenous, citrullinated proteins (i.e. fibrin, vimentin, fibronectin, and type II collagen) all of which can be found in the joints. This hypothesis has yet to be confirmed as no rigorous epidemiological studies have demonstrated this association, likely due to the fact that the proposed infectious agents are found in the population at large at about the same incidence as in RA[355].

Patients positive for ACPAs have increased disease severity with enhanced progression and extra-articular manifestation and require more aggressive treatment than patients negative for ACPAs[356,357]. The production of autoantibodies by B cells requires T cell help[358] and has been supported by the fact that the strongest genetic link identified in RA has been the HLA DRB1*0404 allele[359], and its risk primarily applies to the subgroup of RA patients positive for ACPAs[360,361]. Autoantibody secreting B cells have been found in the circulation as well as in the joints of individuals with RA[362,363]. High levels of autoantibodies form immune complexes, which activate complement, macrophages and osteoclasts leading to vascularization of the joint, the release of chemotactic factors, as well as bone loss[353,364,365]. Vascularization leads to the recruitment of leukocytes and one of the most highly recruited cells to the inflamed synovium are monocytes. Almost every type of immune cell has been implicated in the destructive processes that occur in arthritic joints. There is some evidence that there may be different subtypes of synovial inflammation based on cellular composition of the joint that can be classified into either myeloid-, lymphoid- or fibroid-dominant[366].

The innate immune system is involved in antigen presentation to initiate the autoantigen specific adaptive response; however, it plays a significant role in propagating inflammation. Monocytes are initially recruited to the synovial membrane using chemokine receptors CCR2, CX_3CR_1, CXCR3, CCR5[367]. Once they have entered the synovium they differentiate into macrophages under the influence of the local microenvironment. In arthritic joints, immune complexes and the products resulting from cartilage breakdown and bone erosion activate macrophages. This leads to their production of TNF, IL-1β and IL-6, which further activate surrounding chondrocytes, osteoclasts, B cells and T cells[352]. Monocytes and macrophages are central to disease progression[368], as evidenced by the relationship between the number of synovial macrophages and articular destruction[369] as well as clinical response to therapy[370–372].

Treatment of RA

No cure exists for RA; instead disease-modifying anti-rheumatic drugs (DMARDs) are used to relieve swelling and pain and slow, but not stop, disease progression and joint deformity[354]. Methotrexate is the most commonly used DMARD, but others include sulfasalazine, leflunomide and hydroxychloroquine. Low-dose corticosteroids can additionally be used to rapidly relieve symptoms and offer more structural protection compared to DMARD monotherapy[373,374]. If patients do not respond to their first round of treatment, or they have autoantibodies or early joint damage confirmed by radiography, they are started on biologic

therapies[375]. Biologic DMARDs target cytokines (i.e. TNF or IL-6, IL-1, GM-CSF), block T cell co-stimulation or deplete B cells[353]. Biologics are used in concert with synthetic DMARDs like methotrexate, which has been shown to increase their efficacy[353]. A review comparing biologics has shown that although they may have different targets, they show similar response rates[376]; however each biologic comes with its own challenges in terms of dosing, routes of administration, half-lives and length of time until onset of action[377]. All RA therapies have differing mechanisms of action, sometimes affecting multiple cell populations[354]; however, a reduction in the number of synovial macrophages is consistently found in patients with improved clinical outcomes to a range of treatments, even in those primarily depleting B cells[378].

Rheumatoid Arthritis – A model of accelerated myeloid/monocytic aging

Individuals with RA develop comorbidities including cardiovascular disease, infection, and osteoporosis, which cause significant morbidity and mortality in the elderly as well[205,379–382]. RA patients have changes to the monocyte compartment that are similar to those that occur with age. RA patients have elevated circulating monocytes as well as an expansion of intermediate and non-classical monocytes[158,204,383–385], with the exception of one study that showed lower $CD16^+$ monocytes[386]. There is a body of data that demonstrates impaired hematopoiesis in patients with RA, particularly abnormal myeloid cell development in the bone marrow[387–389]. Hematopoietic progenitors cells from patients with RA show

decreased proliferative capacity and accelerated telomeric shortening[390], which is consistent with what is seen with advanced age[391]. Another hallmark of age that is recapitulated in RA is an enhanced myelopoiesis. More myeloid precursors are found adjacent to rheumatoid joints where the concentration of TNF and other myeloid growth factors (e.g. M-CSF, GM-CSF) are in high concentrations[392,393]. Bone marrow progenitors from RA patients produce more $CD14^+$ myelomonocytic cells faster than trauma patients or those with osteoarthritis[394]. These data are consistent with our observations that TNF drives myelopoiesis in humans[263,395]. Transcriptional analyses show that myeloid precursors in the bone marrow of RA patients are enriched for genes found in early hematopoiesis, and that circulating non-classical monocytes have lower expression of genes found in fully differentiated cells[396]. Thus advanced myelopoiesis does not necessarily result in more mature monocytes, as RA patients have a reduced frequency of non-classical monocytes compared to individuals with osteoarthritis[396]. These changes in maturation result in functional changes like slower complement mediated phagocytosis than healthy controls[397]. Monocytes in RA patients are also primed to be in an "activated" state prior to their arrival at the affected joints[398], producing increased levels of pro-inflammatory cytokines and ROS, which correlate with increased TNF[399]. This may be due to inflammation-driven epigenetic changes of hematopoietic progenitors, previously reported in models of trained innate immunity[134]. Furthermore, when monocytes reach the affected joint they remain in a microenvironment rich in cytokines and pattern recognition receptor ligands and retain the activated status

documented in circulation[385]. In the previous chapter we demonstrated that inflammation is a stronger influence on monocyte phenotype than age. We hypothesize the chronically elevated inflammatory status of RA patients will result in changes to myeloid cell development and function similar to what we see with age-associated inflammation. To test this we recruited 19 newly referred, treatment-naïve RA patients with age- and sex-matched healthy controls. We compared serum cytokines, and monocyte maturity, migratory potential and activation status based on expression of CCR2, CX_3CR_1 and CD16 and the numbers and proportions of monocyte subsets. We followed the RA patients over 6 months of DMARD treatment to determine if decreasing inflammation would reverse inflammation-induced changes to the monocyte compartment.

Materials and Methods:

Study Design

RA patients 18 years and older were recruited and consented by a clinical research nurse following their scheduled visit with Dr. Maggie Larché or Dr. Raja Bobba (Hamilton Integrated Research Ethics Board Project # 14-528T). RA patients eligible for the study were newly referred, diagnosed according to the 2010 American College of Rheumatology criteria[400], and had not received DMARDs, non-steroidal anti-inflammatory drugs (NSAIDs) or oral corticosteroids. Venous blood was collected from patients at diagnosis and 3 and 6 months post-diagnosis. Clinical

erythroid sedimentation rate (ESR) and C-reactive protein (CRP) measurements were performed on same day. Patient medications were prescribed as seen fit by physicians and were documented at each visit. Disease activity was quantified using the clinical disease activity index (CDAI), which is a composite index used to quantify clinical remission of RA (score≤2.8) without the need of laboratory tests like CRP[401]. It is one of the most stringent and includes the summation of the number of 28 joints that are swollen and tender, as well as a patient and physician global score (Disease activity: Low <2.8 to 10, Moderate 10-22, High >22) [401]. The study questionnaire was filled out by the patient and clinical research nurse and is included in the appendix of this chapter.

Each RA patient was matched with a healthy control of the same sex within 1-2 years of his or her age that did not have any chronic conditions or any form of joint disorder or arthritis (Table 1). Age, sex, smoking status, disease activity and other reported chronic conditions are listed in Tables 1 and 2. A detailed breakdown of medications used can be found in Table 3.

Serum Cytokine Measurement

Venous blood was collected and centrifuged at 1.5 x g for 10 min at 25°C and serum was stored at -140°C until processed. Serum cytokines were measured using Milliplex HSTCMAG-28SK (GM-CSF, fractalkine, TNF, IL-1β, IL-6, IL-10, IFNγ) and HCYTOMAG-60K (VEGFA, MCP-1, IP-10) as per manufacturer’s protocol (EMD Millipore).

Whole Blood Immunophenotyping

Venous blood was collected in heparinized tubes and 100 μL was stained for 30 min with monoclonal antibodies of the following specificities: CD45-BV510, CCR2-PE, CD15-BV610, CD14-BV421, CD56-AF700 (BioLegend); CD16-PE-Cy7, HLA-DR-PerCP-Cy5.5, CD19-AF700 (eBioscience); CD11b-APC, CD3-AF700 (BD Pharmingen) CX3CR1-FITC (MBL Life Sciences). Samples were then incubated with 1X Fix/Lyse Buffer (eBioscience) for 10 min with frequent inversion and centrifuged at room temperature, washed and resuspended in FACS Wash (5 mM EDTA, 0.5% BSA in PBS). Samples were then run on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo 10 software (Treestar). Total cell counts were determined with 123 count eBeads (eBioscience). For gating strategy, see Chapter 2 Supplementary Figures 2 & 5. Expression of surface markers were quantified by measuring geometric mean fluorescence intensity (gMFI) of each marker and subtracting background gMFI of isotype controls.

Inflammatory Index Calculation

ESR, CRP and all serum cytokines measured were transformed using a natural log (ln) function. T-scores were then generated for each by centering to the mean and scaling to the standard deviation of either healthy controls when comparing differences between RA and healthy control, or baseline values of the RA population when comparing longitudinal changes following DMARD therapy. The inflammatory

index was then created by the summation of t-scores for each cytokine measured in a patient

Statistical Analysis

Principal component analysis was performed using the prcomp function of the stats package in R[276]. A pairwise Adonis was performed and Bonferroni post hoc test was used to adjust for multiple comparisons using the pairwiseAdonis package[402]. ANCOVA analysis was used to test the differences between RA and healthy controls while using sex and age as covariates and was performed using SPSS (Version 21; IBM, Armonk, NY, USA). To determine if immune parameters measured in RA patients changed in response to DMARD therapy we used lmer function from the lme4[279] package in R to perform linear mixed effects modeling using time as a fixed effect and patient ID as a random effect. Analysis of Deviance Table (Type II Wald chi square tests) was performed to test significance of treatment time (visit) in the model using the Anova function in the car package[280]. The same approach was used to determine if changes in serum inflammation lead to changes in cellular inflammation. If variables did not follow a normal distribution, they were ln-transformed. Spearman's rank correlation was performed using the rcorr function in the Hmisc package[275]. Correlation plots were generated using the corrplot function in the corrplot package[282]. Partial correlation was performed using the pcor.test function in the ppcor package[277]. Confidence intervals for spearman's rho were generated using the CIrho function in mada package[278].

Results:

We measured serum cytokines (e.g. GM-CSF, fractalkine, IFNγ, IL-10, IL-6, IL-1β, TNF, IP-10, MCP-1, VEGFA) and quantitated circulating leukocyte populations in addition to surface markers of monocyte activation and trafficking (e.g. CCR2, CX_3CR_1, CD16) in RA patients at diagnosis and compared them to age- and sex-matched healthy controls. Based on all the immunological parameters measured, RA patients were significantly different from controls ($p=0.043$, $R^2=0.119$, Supplementary Figure 1). The difference was mainly driven by lower circulating leukocytes (Table 5). There was a lower proportion of peripheral blood mononuclear cells (PBMCs) (Cohen's d[95%CI]:-0.93[-1.60 - -0.26] p=0.007) and a higher proportion of neutrophils in RA patients compared to controls (p=0.081, Table 5, Supplemental Figure 3). The lower numbers of PBMCs were not specific to any one cell type as NK, T and B cells and monocytes were all lower in RA patients compared to controls (Table 5). Serum cytokines were another significant driver of differences between RA patients and controls. An elevated composite inflammatory index summating all markers of serum inflammation, captures the elevated inflammatory status in RA patients (Table 4). Cytokine correlation plots in controls and RA patients reveal that the positive associations between cytokines are stronger

in RA patients than in controls(Figure 1), suggesting increased synchronicity between cytokines in RA.

Contrary to what we expected, monocyte numbers were lower in RA patients compared to controls (Cohen's d[95%CI]: -0.69 [-1.36 - -0.29] p=0.043, Table 5), but there was no difference in the distribution of the monocyte population between classical, intermediate and non-classical monocytes. Although there were no statistically significant differences in markers of monocyte maturity and activation, RA patients trended towards higher expression of CCR2 on intermediate monocytes (p=0.198) and CX_3CR_1 on classical monocytes (p=0.147), and lower expression of CD16 on intermediate monocytes (p=0.160; Table 6).

To determine how soluble inflammation affects cellular inflammation, we tracked both in RA patients along the course of their standard care with DMARDs at 3 and 6 months post-diagnosis. Soluble inflammation decreased with treatment, demonstrated by a decrease in the inflammatory index (Table 7). ESR, CRP, GM-CSF, IL-10 and IL-1β (Table 7) were the most significantly decreased markers of soluble inflammation with treatment. Treatment also increased circulating PBMCs, particularly monocytes (Table 8). With treatment there was a decrease in the expression of CCR2 on intermediate monocytes and the expression of CD16 and CX_3CR_1 on classical monocytes, indicating a decrease in monocyte activation (Table 9). IL-1β correlated with CD16, while IL-10 and IL-6 correlated with CX_3CR_1 (Figure 4, Supplementary Table 4).

Lower scores in the inflammatory index indicated lower CD16 expression on monocytes as well as an increase in circulating PBMCs (Supplementary Table 5). Using our longitudinal data, we performed mixed linear models to determine how specific cytokines were related to cellular inflammation. Higher concentrations of VEGFA, IL-6, IL-1β and IL-10 were positively associated with neutrophils and negatively associated with PBMCs (Figure 3A, Supplementary Table 6). Higher serum TNF, IL-1β, and IP-10 were associated with higher total monocytes (Figure 3A, Supplementary Table 7). IP-10 positively associated with intermediate and non-classical monocytes(Figure 3A, Supplementary Table 7). Higher serum TNF was associated with increased classical and intermediate monocytes, while IL-1β was only associated with intermediate monocytes (Figure 3A, Supplementary Table 7). Higher MCP-1 correlated with higher proportions of non-classical monocytes and lower expression of CCR2 by monocytes (Figure 3A&4, Supplementary Table 7).

Discussion:

Serum cytokines were elevated in our RA patients compared to healthy controls; however, none were individually statistically significant. The median levels of ESR and CRP in our patients (i.e. 17mm/hr, 3.4mg/L) were lower than documented in other RA studies (i.e. ~40-80mm/hr, 12-94mg/L)[204,384]. This could be due to the disease activity or duration, which when increased are associated with higher levels of inflammation[403]. The expression of monocyte chemokines has been

shown to increase with disease progression[404]. Of the monocyte chemokines that we measured in serum (e.g. MCP-1, IP-10 and fractalkine), only IP-10 was elevated. Many other published studies with rheumatoid arthritis cohorts include patients with >10 years of disease duration, while patients in our study were recently referred to rheumatologists. We had CDAI scores for 10 of 19 patients, and half were classified as high disease activity, while the others were low-moderate. Cytokines remain at higher concentrations in affected joints, only travelling a few microns away from their site of origin[405]. This suggests that increased disease activity over a longer period of time may be needed before detecting significant inflammation in circulation. Even though the magnitude of inflammation in our cohort was not as high as others reported, we still found elevations in cytokines characteristic of RA[404]. The serum cytokines that were most elevated in our RA patients were GM-CSF, IL-1β, TNF and IP-10. Intermediate monocytes are essential producers of IL-1β and TNF[406,407], while IP-10 is a chemotactic ligand used to recruit monocytes and T cells to sites of inflammation[408]. GM-CSF and TNF are prominent cytokines in RA that drive increased myelopoiesis[409], and in concert with IL-1β, they increase monocyte survival in synovial joints[410].

Contrary to what we expected, RA patients had lower monocytes as a proportion of circulating leukocytes than healthy controls. Many other studies have reported increases in circulating monocytes and an expansion in intermediate and non-classical monocytes as a proportion of monocytes[158,383–385], with the exception of one[386]. It is difficult to make direct comparisons to these studies as many of the

RA patients included were on DMARD therapy and did not follow a 1-1 case control study design. This presents a challenge in determining which immunological differences are due to disease or treatment. Additionally, we controlled for age and sex in our analyses, which are two factors that influence both inflammation and the numbers of circulating monocytes[411–413]. Furthermore, the identification of monocytes based on CD14 and CD16 expression was variable between studies. Only one other study followed a stringent identification of monocytes, similar to ours, where they eliminated lymphocytes and neutrophils and ensured monocytes were HLA-DR^{+} [385]. Although this group did not quantify absolute numbers, they found an expansion in intermediate monocytes in RA; however, they did not report disease activity or treatment. These differences in monocyte phenotyping and patient populations across studies, makes it challenging to compare our findings to those that have been reported to date.

We may be capturing the initial phases of the disease where there is a mass infiltration of monocytes to inflamed joints, explaining the lower proportion of circulating monocytes. RA patients had slightly elevated expression of CCR2 on intermediate monocytes and CX_3CR_1 on classical monocytes when compared to controls. This suggests that monocytes in RA may have enhanced migratory capacity, as CCR2 and CX_3CR_1 have been implicated in monocyte trafficking to arthritic joints[414–417]. Blockade of these receptors has led to decreased disease activity and monocyte infiltration in animal models[418], but have had less success in clinical trials, due to the redundancy in chemokine receptors[419]. The elevated CCR2

expression on intermediate monocytes also implies maturation of classical monocytes to intermediate monocytes may be incomplete[420,421].

With 6 months of DMARD treatment there was a significant decrease in systemic inflammation, primarily due to decreases in ESR, CRP, GM-CSF, IL-10 and IL-1β and IL-6. Although there was a global decrease in soluble inflammation with treatment, some cytokines showed little change (e.g. TNF, fractalkine, IP-10, VEGFA). There is precedent for this since TNF-neutralizing therapies show great efficacy in RA, but serum TNF does not correlate with improved clinical outcomes[405]. This could be due to the fact that systemic levels do not reflect local concentrations at affected joints and fail to capture the regulation of the cytokine, which is naturally inhibited by soluble TNF receptors[405]. Rather, serum IL-6 has proven to be the best correlate of clinical efficacy as it is bioactive in serum, unlike most other cytokines[422].

DMARD treatment also lead to a decrease in the expression of monocyte activation markers CCR2, CD16 and CX_3CR_1 on classical monocytes, consistent with previous findings[383,423]. The expression of intermediate monocyte CCR2 returned to levels of healthy controls following DMARD treatment, as did the number of circulating monocytes. The decreased expression of CCR2 and CX_3CR_1, could reduce the invasive potential of monocytes to get into the joint, resulting in more monocytes remaining in circulation. Although MCP-1 did not show a statistically significant change with DMARD treatment, it increased, in contrast to all other cytokines. We found MCP-1 could significantly predict the expression of CCR2 on

monocytes. During monocyte differentiation, levels of MCP-1 increase as CCR2 expression decreases [420], thus we believe DMARD treatment is normalizing the maturation of monocytes in RA patients. This is further supported by the positive association between MCP-1 and non-classical monocytes. IL-10 and IL-6 both decreased with DMARD treatment and correlated with CX_3CR_1 on classical monocytes. These relationships are consistent with findings that IL-10 upregulates the expression of CX_3CR_1 on monocytes[424], while IL-6 stimulates the release of the ligand for CX_3CR_1 , fractalkine[425].

The inflammatory index could predict CD16 expression on monocytes, and IL-1β was strongly correlated to CD16 expression on classical monocytes. This is consistent with findings that IL-1β drives expression of CD16 during macrophage differentiation[426]. These data suggest IL-1β contributes to monocyte activation, seeing as IL-1β and CD16 are both elevated in RA compared to healthy controls, and both decrease with DMARD therapy. Anti-IL-1β treatments have shown some promise in mitigating disease progression[427]; however, the efficacy is not as robust as anti-TNF treatment, suggesting IL-1β is a contributor but not a key mediator of disease.

Although we did not find the expansion of intermediate and non-classical monocytes at baseline in RA patients, we were able to assess which cytokines most influence the composition of the monocyte compartment. Higher serum TNF, IL-1β and IP-10 were associated with higher total monocytes. IP-10 positively associated with intermediate and non-classical monocytes, TNF associated with increased

classical and intermediate monocytes, while IL-1β was only associated with intermediate monocytes. TNF was associated with newly-formed monocytes, in line with data that TNF drives monopoiesis and monocyte survival[413,428,429]. IL-1β and IP-10 are most associated with $CD16^+$ monocytes, which is consistent with their increased production of IL-1β and IP-10[430].

RA-specific gene signatures are different from other autoimmune conditions like SLE due to the dominance of different cytokines[431]. Our findings demonstrate that the unique cytokine environment in RA shapes monocyte phenotype. Other individuals have shown that culturing monocytes from healthy individuals in RA synovial fluid is sufficient to induce the activated monocyte phenotype documented in RA[385,432]. The cytokine environment additionally primes synovial fibroblasts to secrete factors driving monocyte survival in RA[410]. All these data emphasize the importance of understanding how each cytokine differentially regulates monocyte maturation, phenotype, and function. This advance in knowledge will allow us to more precisely modulate inflammation to target desired monocyte subsets and functions.

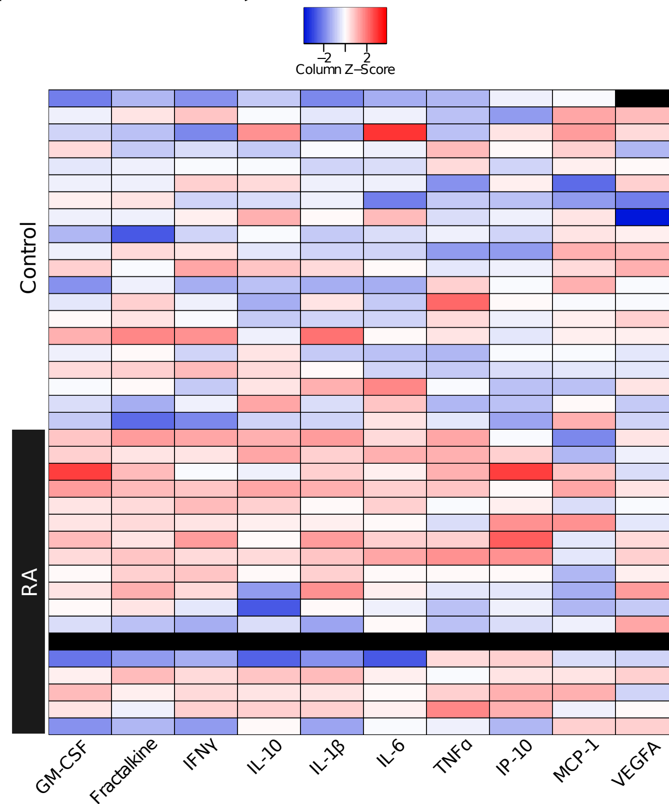
Future directions:

Although DMARDs lower inflammation, their mechanisms of action are non-specific[354] making it difficult to interpret which changes are attributable to TNF specifically. Thus, a continuation of this study is in progress, following RA patients over the course of anti-TNF biologic therapy. This will allow us to determine which

of the changes to the monocyte compartment in RA are specific to TNF, since we have found it to be a driving force of myeloid aging in our mouse models. Furthermore samples collected in these studies can be used to address the question of how chronic inflammation affects monocyte and monocyte-derived macrophage function, particularly their capacity to kill bacterial. Patients with RA are at increased risk of bacterial infections. Pneumonia is one of the leading causes of mortality in RA[433]. Some literature suggests that treatment with anti-TNF increases the risk for bacterial infections; however, two studies have shown that these biologics have risks of infection no greater than other conventional DMARD therapy, such as methotrexate[434]. In RA patients, glucocorticoid treatment is associated with the highest risk for developing an infection, including low dose prednisone[380]. Anti-TNF biologics are commonly used in concert with other DMARDs, which are known to influence cellular immunity in ways that may impact responses to infectious disease[354]; however, the reduction in disease activity actually reduces other risk factors associated with infectious disease[435]. Our data[413,436] would suggest that the high levels of inflammatory cytokines in treatment-naïve patients would put them at increased risk of infection due to altered monocyte and macrophage dysfunction. The overall reduction in inflammation may enhance the ability of the myeloid compartment to clear bacterial infections in patients with RA.

Figures and Tables:

Figure 1. RA patients have an elevated inflammatory status. A) Z-scores for each cytokine was calculated based on log-transformed values and used to generate a heatmap of serum cytokines visualizing differences in inflammation between RA patients and controls. Black boxes indicate missing data. Generated using Heatmapper [PMID: 27190236]. B) Spearman correlations of cytokines in controls and RA patients show stronger synchronicity in RA. A black asterisk indicates correlations where p<0.05, a red asterisk indicates correlations where p<0.05 after FDR adjustment.



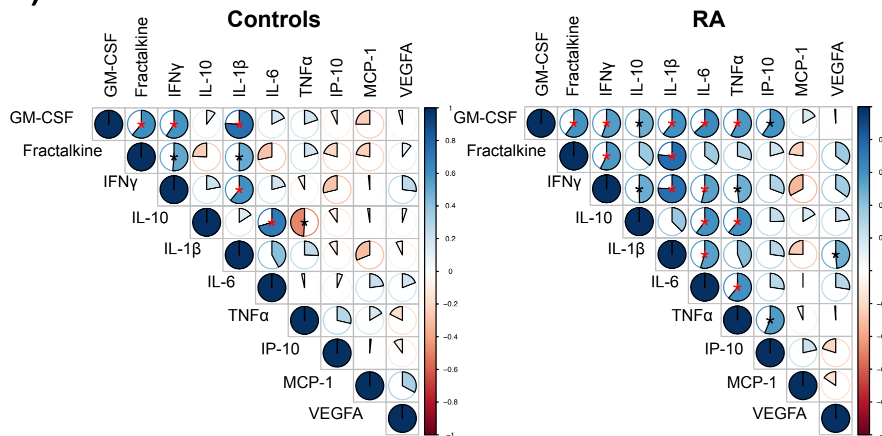


Figure 2. Soluble inflammatory markers and leukocyte numbers. Spearman correlations of cytokines with leukocyte numbers in RA patients. A black asterisk indicates correlations where p<0.05, a red asterisk indicates correlations where p<0.05 after FDR adjustment.

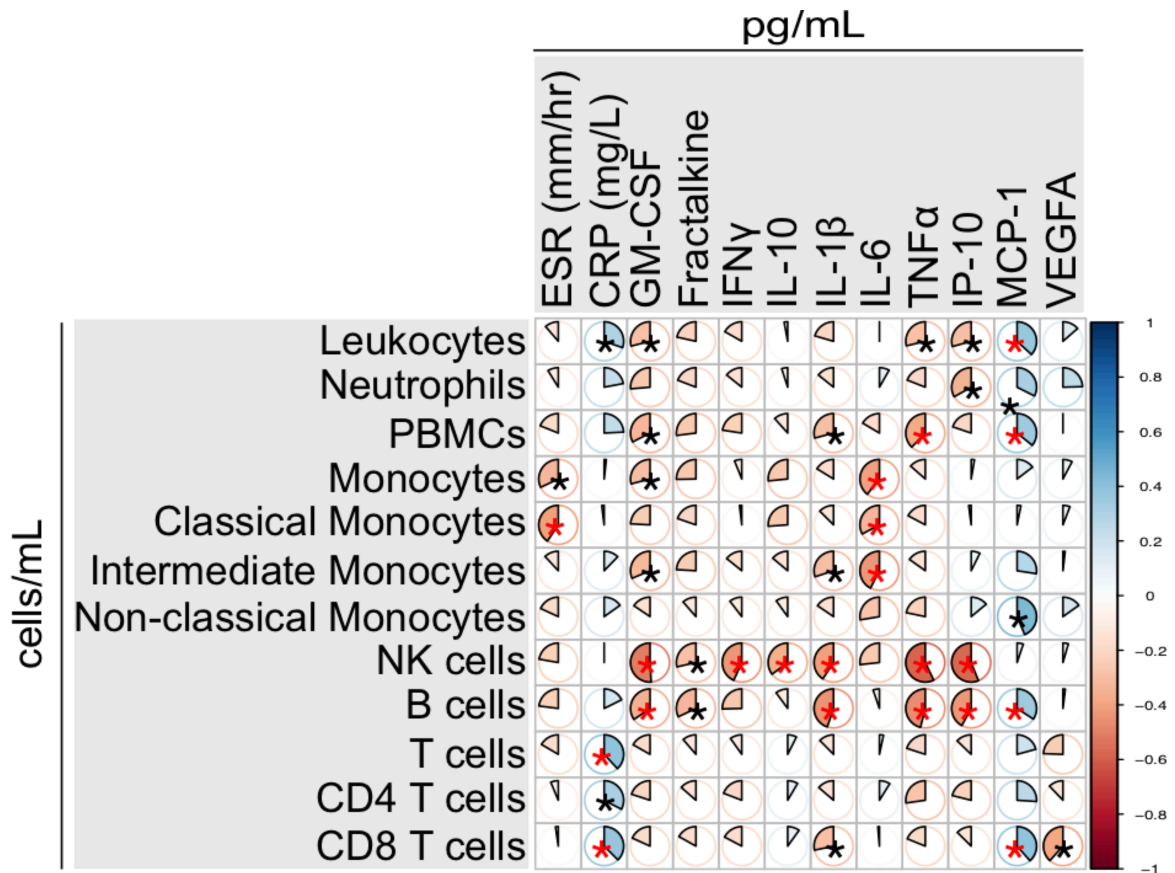
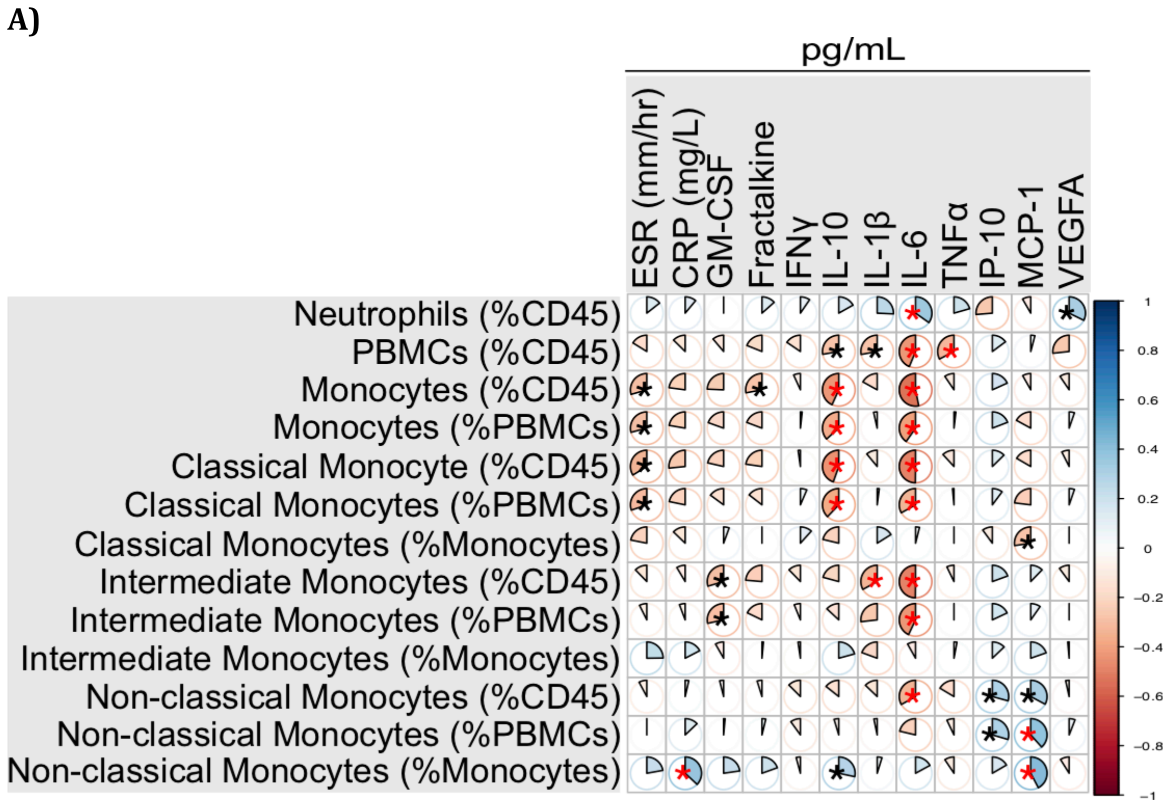


Figure 3. Soluble inflammatory markers and cell proportions. Spearman correlations of cytokines with myeloid(A) and lymphoid(B) populations in RA patients. A black asterisk indicates correlations where p<0.05, a red asterisk indicates correlations where p<0.05 after FDR adjustment.

A)



B)

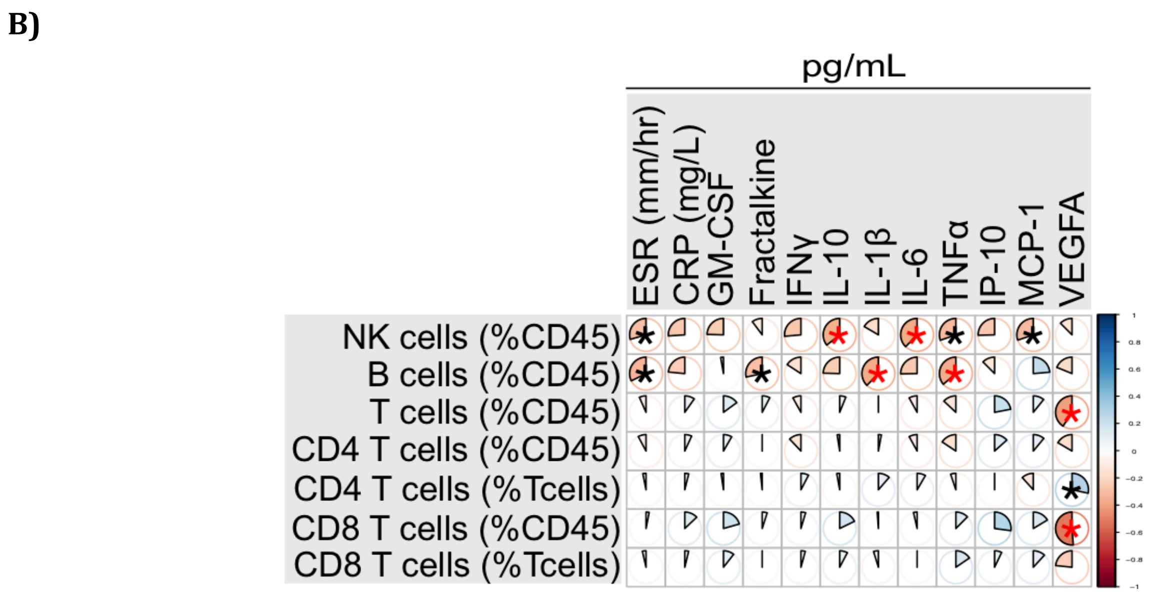


Figure 4. Soluble inflammatory markers and expression of monocyte activation markers. Spearman correlations of cytokines with monocyte activation markers in RA patients. A black asterisk indicates correlations where $p<0.05$, a red asterisk indicates correlations where $p<0.05$ after FDR adjustment.

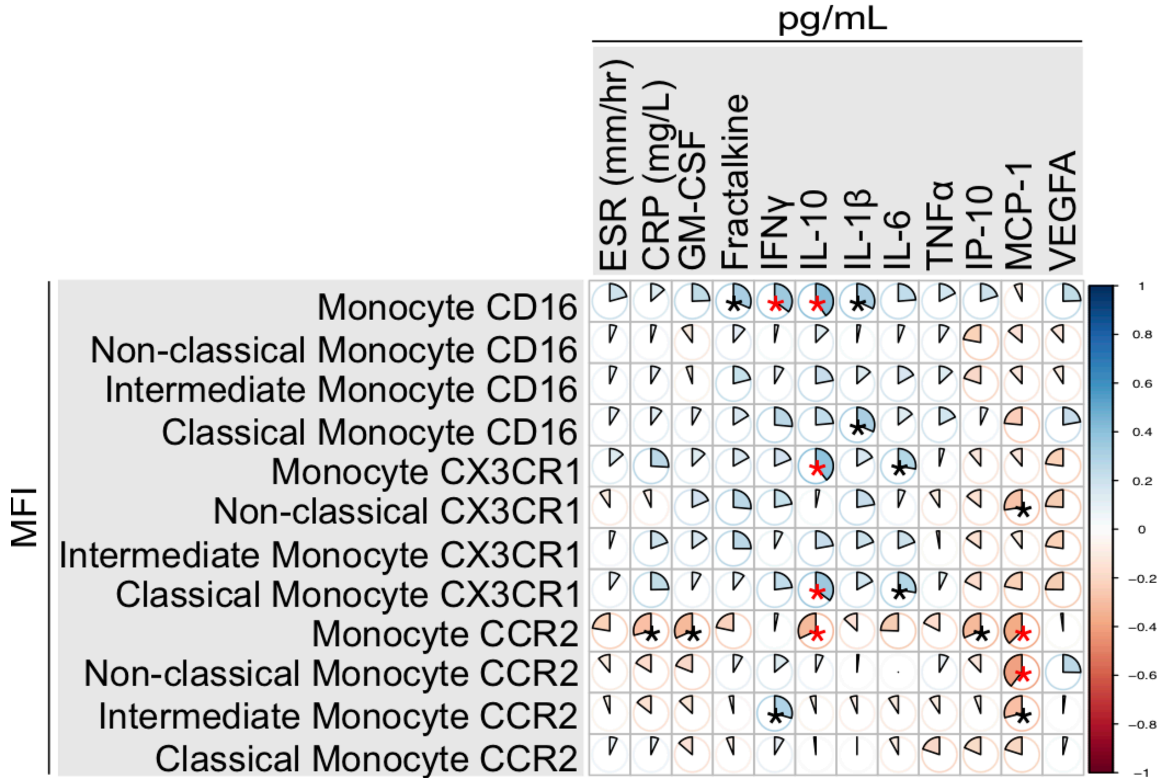


Table 1. Patient Description. Control and RA patients were age- and sex-matched. RA Patient characteristics and comorbidities are described in B & C.

A)

	Control						RA						t-test
	n	Mean	Median	SD	Min	Max	n	Mean	Median	SD	Min	Max	p
Age	19 (84.2% F; 15.8% M)	50.26	51	11.474	30	73	19 (84.2% F; 15.8% M)	49.58	52	11.212	27	76	0.854

B)

	n	Freq (%)
Sex		
Female	16	84.2
Male	3	15.8
Smoker		
Never	11	57.9
Prior	6	31.6
Current	2	10.5
Rheumatoid Factor		
+	8	42.1
-	11	57.9
anti-CPP		
+	4	21.1
-	13	68.4
No data	2	10.5
IBD/IBS, Ulcerative Colitis or Crohn's Disease	1	5.3

C)

Other conditions	n
Gallstones & Kidney stones	1
Malabsorption	1
Fibromyalgia	1
Depression	2
Asthma	2
Psoriasis	1
Anemia	1
Hyperlipidemia	2
Supraventricular tachycardia	1
Irritable Bowel Syndrome	1
Hypertension	1
Hypothyroidism	1

Table 2. Disease Activity Scores. The majority of the RA cohort had low-moderate disease activity. Disease activity decreased with DMARD treatment and scores were tested in a linear mixed models with patient set as a random effect. Statistical significance was tested by Analysis of deviance (Type II Wald chi square test).

A)

		Baseline		Visit 1		Visit 2	
Disease Activity	**CDAI Score**	n	%	n	%	n	%
Remission	0-2.8	0	0	0	0	1	7.7
Low Activity	2.9-10	1	10	5	38.5	6	46.2
Moderate Activity	10.1-22	4	40	3	23.1	4	30.8
High Activity	22.1-76	5	50	5	38.5	2	15.4

B)

	BASELINE						VISIT 1						VISIT 2						p
	n	Mean	Median	SD	Min	Max	n	Mean	Median	SD	Min	Max	n	Mean	Median	SD	Min	Max	
Tender Joint Count	17	11.06	9	7.14	4	33	17	8.41	3	10	0	33	16	6.06	3	6.87	0	20	**0.008**
Swollen Joint Count	17	6.41	6	4.86	0	22	17	5	4	6.01	0	20	16	3.06	2.5	3.26	0	12	**0.002**
Patient Global	11	5.64	5	2.29	2	8	15	3.67	3	2.44	0	8	13	3.38	3	1.8	0	7	**0.005**
Physician Global	11	5.36	5	2.62	1	10	13	4.08	4	2.75	1	9	12	3.25	3	1.66	1	7	**0.009**
CDAI	10	27.5	23.5	13.42	8	51	13	21.15	18	18.39	2	61	13	14.15	10	11.44	1	40	**0.005**

Table 3. Medication use and associated conditions. A) The proportion of RA patients taking 1, two or 3 DMARDs for treatment. B) The number of patients taking specified medications at baseline, visit 1 (3months), and visit 2 (6 months).

A)

Treatment	Baseline		Visit 1		Visit 2	
	n	Freq(%)	n	Freq(%)	n	Freq(%)
Single DMARD	2	10.5	14	73.7	11	57.9
Double DMARD			5	26.3	4	21.1
Triple DMARD				0	4	21.1

B)

Gallstones & Kidney stones
Malabsorption
Fibromyalgia
Depression
Asthma
Psoriasis
Anemia
Hyperlipidemia
SVT
IBS
Hypertension
Hypothyroidism

Medications		Baseline	Visit 1	Visit 2
Biologics	Etanercept			
	Enbrel			1
DMARDs	Hydroxychloroquine/Plaquenil	1	6	8
	Leflunomide/Arava		4	7
	Methotrexate	1	14	12
	Hydrochlorothiazide		1	2
NSAIDs	Meloxicam	2	2	2
	Naproxen	5	2	1
	Diclofenac	1		
	Advil		1	
Corticosteroids	Prednisone	1		
Proton Pump Inhibitors (GERD)	Pantoprazole/Tecta	3	2	1
	Rabeprazole	1	1	1
	Lansoprazole		1	1
Asthma	Symbicort	1		1
	Ventolin			2
Depression/Anxiety	Elavil	1	1	1
	Cymbalta	1	1	1
	Cipralex	1		
	Lorazepam	1		
	Oxazepam	1	1	1
Hyperlipidemia/Atherosclerosis	Lipitor	1	1	1
	Crestor/Rusavastatin	1	1	1
	Ramipril		1	1
	Adalat		1	1
Hypertension	Atenolol	1		
	Irbesartan	1		
Hypothyroid	Synthroid	1	1	1
Anemia/Iron Insufficiency	Venofer	1		
	Feramax	1	2	1
Pain Meds	Oxycocet	1	1	1
	Tramadol	1		
Supplements	Folic Acid	1	8	6
	Vitamin D	1		1
Bone density	Actonel		1	1
Antibiotics	Isoniazid		1	1

Table 4. Serum Cytokines in Healthy Controls and RA. Differences between healthy controls and RA patients were tested by ANCOVA while controlling for sex and age.

	Control						RA						ANCOVA						
													Control vs RA		Sex		Age		
	n	Mean	Median	SD	Minimum	Maximum	n	Mean	Median	SD	Minimum	Maximum	F	p	F	p	F	p	(Df_1, Df_{error})
ESR(mm/hr)							19	22.95	17	14.05	5	50							
CRP(mg/L)							18	23.98	3.4	43.95	0.5	150.9							
GM-CSF (pg/mL)	19	35.94	32.03	17.918	9	83	18	63.41	50.74	62.263	8	291	3.373	0.075	0.864	0.359	2.073	0.159	(1,33)
Fractalkine (pg/mL)	19	116.02	112.8	39.465	42	224	18	133.14	138.77	43.43	48	200	1.727	0.198	4.657	**0.039**	0.434	0.515	(1,33)
IFNγ (pg/mL)	19	25.88	22.66	11.344	10	51	18	29.39	31.78	11.375	11	50	0.865	0.359	0.919	0.345	0.085	0.772	(1,33)
IL-10 (pg/mL)	19	17.52	9.94	19.689	2	76	18	19.17	12.7	18.119	0	57	0.057	0.812	2.658	0.113	6.322	0.17	(1,33)
IL-1β (pg/mL)	19	2.41	2.02	1.591	1	8	18	3.28	3.5	1.471	1	6	2.956	0.095	1.588	0.216	0.354	0.556	(1,33)
IL-6 (pg/mL)	19	11.49	2.35	28.988	0	127	18	6.75	5.19	4.229	0	18	0.508	0.481	0.572	0.455	1.567	0.219	(1,33)
TNF (pg/mL)	19	10.67	9.7	3.718	7	22	18	12.78	12.18	3.226	8	19	3.865	0.058	0.505	0.482	3.701	0.063	(1,33)
IP-10 (pg/mL)	19	641.51	692.73	218.394	270	1068	18	1559.8	1025	1401.743	316	5838	7.975	**0.008**	1.643	0.209	0.004	0.951	(1,33)
MCP-1 (pg/mL)	19	1629.35	1657	396.826	783	2305	18	1536.18	1393.5	445.94	910	2386	0.425	0.519	0.003	0.956	0.002	0.964	(1,33)
VEGFA (pg/mL)	18	490.85	339.71	486.144	1	1643	18	553.49	370.39	639.827	68	2201	0.137	0.714	0.041	0.841	2.505	0.123	(1,33)
Inflammatory Index	19	1.58×10^{-10}	0.274	17.558	-41.4	37.2	19	11.626	20.59	25.779	-48.72	42.3	2.88	0.099	2.328	0.136	1.105	0.3	(1,34)

Table. 5. Leukocyte populations in Healthy Controls and RA. Differences between healthy controls and RA patients were tested by ANCOVA while controlling for sex and age.

	Control						RA						ANCOVA							
													Control vs RA			Sex		Age		
	N	Mean	Median	SD	Min	Max	N	Mean	Median	SD	Min	Max	F	p	Cohen's d[95%CI]	F	p	F	p	(df_1, df_{error})
Cell Counts(cells/mL blood)																				
Leukocytes	18	5.638×10^{6}	3.625×10^{6}	5.228×10^{6}	7.930×10^{5}	1.700×10^{7}	19	3.23×10^{6}	2.709×10^{6}	2.079×10^{6}	1.090×10^{6}	8.800×10^{6}	3.705	0.063		0.061	0.807	3.521	0.069	(1,33)
Neutrophils	18	3.195×10^{6}	2.42×10^{6}	2.753×10^{6}	3.94×10^{5}	7.81×10^{6}	19	2.097×10^{6}	1.660×10^{6}	1.589×10^{6}	8.350×10^{5}	6.93×10^{6}	2.507	0.123		0.408	0.527	4.431	**0.043**	(1,33)
PBMCs	18	2.255×10^{6}	1.304×10^{6}	2.428×10^{6}	3.060×10^{5}	9.2×10^{6}	19	9.46×10^{5}	7.950×10^{5}	6.865×10^{5}	1.650×10^{5}	3.12×10^{6}	5.448	**0.026**	-0.767[-1.4352- -0.0997]	0.103	0.751	2.978	0.094	(1,33)
Monocytes	18	2.285×10^{5}	1.075×10^{5}	2.883×10^{5}	9.89×10^{3}	1.1×10^{6}	19	8.921×10^{4}	6.420×10^{4}	8.23×10^{4}	1.040×10^{4}	2.58×10^{5}	4.436	**0.043**	-0.6925[-1.356 - -0.029]	0.518	0.477	2.748	0.107	(1,33)
Non-classical	18	2.157×10^{4}	7.65×10^{3}	2.500×10^{4}	1.71×10^{3}	8.34×10^{4}	19	1.008×10^{4}	5.260×10^{3}	1.450×10^{4}	1.700×10^{2}	5.75×10^{4}	3.515	0.07		0.000	0.987	6.338	**0.017**	(1,33)
Intermediate	18	1.122×10^{4}	6.804×10^{3}	9.956×10^{3}	1.400×10^{3}	3.2×10^{4}	19	6.724×10^{3}	4.660×10^{3}	6.477×10^{3}	3.680×10^{2}	1.87×10^{4}	3.318	0.078		0.275	0.603	6.981	**0.012**	(1,33)
Classical	18	1.657×10^{5}	7.66×10^{4}	2.236×10^{5}	5.44×10^{3}	8.54×10^{5}	19	6.435×10^{4}	4.820×10^{4}	5.687×10^{4}	6.11×10^{3}	1.99×10^{5}	3.990	0.054		0.501	0.484	2.343	0.135	(1,33)
NK cells	18	2.99×10^{5}	1.66×10^{5}	3.622×10^{5}	3.25×10^{4}	1.24×10^{6}	19	1.161×10^{5}	7.460×10^{4}	1.225×10^{5}	1.37×10^{4}	$4.24.\ \times10^{5}$	5.548	**0.025**	-0.7745[-1.4426 - -0.1063]	3.660	0.064	7.870	**0.008**	(1,33)
B cells	18	1.363×10^{5}	1.095×10^{5}	9.925×10^{4}	1.04×10^{4}	3.45×10^{5}	19	8.039×10^{4}	6.360×10^{4}	6.069×10^{4}	1.79×10^{4}	2.42×10^{5}	4.376	**0.044**	-0.6878[-1.351- -0.0246]	2.377	0.133	0.412	0.525	(1,33)
T cells	17	1.123×10^{6}	1.04×10^{6}	7.406×10^{5}	8.81×10^{4}	2.73×10^{6}	18	6.558×10^{5}	5.500×10^{5}	5.259×10^{5}	1.51×10^{5}	1.99×10^{6}	4.410	**0.044**	-0..7203[-1.414- -0.0266]	0.137	0.714	0.245	0.624	(1,31)
CD4 T cells	18	6.46×10^{5}	6.32×10^{5}	4.693×10^{5}	3.44×10^{4}	1.6×10^{6}6	18	4.494×10^{5}	3.840×10^{5}	4.081×10^{5}	7.12×10^{4}	1.46×10^{6}	1.822	0.187		0.075	0.786	1.306	0.262	(1,32)
CD8 T cells	18	3.205×10^{5}	2.735×10^{5}	2.734×10^{5}	2.03×10^{4}	9.75×10^{5}	18	1.656×10^{5}	2.205×10^{5}	1.215×10^{5}	3.65×10^{4}	4.21×10^{5}	4.585	**0.040**	-0.7138[-1.3876- -0.04]	0.084	0.774	0.157	0.696	(1,32)
% of Leukocytes																				
Neutrophils	19	56.63	55.60	13.82	13.50	81.80	19	63.87	64.20	10.00	51.60	86.30	3.234	0.081		0.445	0.509	0.577	0.453	(1,34)
PBMCs	19	40.68	42.20	15.26	17.30	86.40	19	28.78	29.60	9.95	13.40	46.20	8.192	**0.007**	-0.9286[-1.5979- -0.2593]	1.512	0.227	0.316	0.578	(1,34)
Monocytes	19	3.71	3.79	1.56	1.25	6.45	19	2.73	2.71	1.88	0.37	6.90	3.396	0.074		0.687	0.413	2.670	0.111	(1,34)
Non-classical	19	0.35	0.26	0.19	0.07	0.69	19	0.26	0.16	0.24	0.02	0.80	1.988	0.168		0.221	0.641	5.249	**0.028**	(1,34)
Intermediate	19	0.22	0.20	0.10	0.06	0.41	19	0.20	0.11	0.17	0.03	0.52	0.305	0.584		0.412	0.525	1.850	0.183	(1,34)
Classical	19	2.61	2.58	1.28	0.69	5.03	19	1.96	1.75	1.36	0.22	5.32	2.468	0.125		0.469	0.498	2.310	0.138	(1,34)
NK cells	19	5.30	4.66	2.97	2.56	14.30	19	2.87	2.50	1.50	0.39	6.24	11.016	**0.002**	-1.0768[-1.7573- -0.3964]	2.760	0.106	1.651	0.207	(1,34)
B cells	19	2.55	2.32	0.98	0.68	4.97	19	2.25	2.36	0.82	0.91	3.64	1.063	0.310		1.776	0.192	5.826	**0.021**	(1,34)
T cells	18	23.51	22.65	13.44	5.00	69.00	18	18.37	19.35	6.48	3.00	29.00	2.006	0.166		3.092	0.088	1.460	0.236	(1,32)
CD4 T cells	18	13.47	13.25	7.48	2.00	37.00	18	12.08	11.50	5.29	2.00	20.00	0.296	0.590		3.787	0.060	0.920	0.345	(1,32)
CD8 T cells	19	6.36	6.10	3.30	1.00	13.00	18	4.96	4.90	2.50	1.00	10.00	2.116	0.155		3.903	0.057	0.475	0.495	(1,33)
% of PBMCs																				
Monocytes	19	10.61	11.00	5.97	2.53	21.80	19	8.98	9.34	4.08	1.01	16.10	1.000	0.314		0.185	0.670	0.931	0.342	(1,34)
Non-classical	19	1.14	0.79	0.91	0.44	4.21	19	0.81	0.63	0.64	0.10	2.50	1.832	0.185		0.275	0.603	1.814	0.187	(1,34)
Intermediate	19	0.60	0.59	0.31	0.17	1.31	19	0.63	0.54	0.42	0.10	1.45	0.047	0.830		0.004	0.948	2.215	0.146	(1,34)
Classical	19	7.27	7.70	4.66	0.19	16.00	19	6.59	6.71	3.21	0.60	11.50	0.288	0.595		0.076	0.785	0.690	0.412	(1,34)
% of Monocytes																				
Non-classical	19	11.55	9.41	6.58	4.09	25.00	19	9.90	9.97	6.37	0.78	24.10	0.617	0.438		0.002	0.969	0.341	0.563	(1,34)
Intermediate	19	7.52	6.43	3.90	2.39	15.20	19	7.13	7.18	3.10	1.68	14.40	0.116	0.735		0.024	0.878	0.008	0.929	(1,34)
Classical	19	75.67	78.60	10.24	59.00	91.90	19	72.47	76.60	11.64	40.70	86.70	0.743	0.395		0.036	0.850	0.370	0.547	(1,34)
Ratio of Classical to Intermediate Monocytes	19	13.76	11.46	8.61	3.88	35.90	19	13.97	10.61	12.02	4.63	51.61	0.004	0.951		0.065	0.800	0.004	0.949	(1,34)
Ratio of Intermediate to Non-classical Monocytes	19	0.73	0.70	0.32	0.28	1.79	19	1.03	0.75	0.77	0.24	3.23	2.351	0.134		0.022	0.882	0.001	0.979	(1,34)
% of T cells																				
CD4 T cells	19	54.14	58.80	16.24	6.00	74.00	18	65.18	67.90	13.42	31.00	88.00	5.238	**0.029**	0.7525[0.0857-1.4194]	1.848	0.183	1.518	0.227	(1,33)
CD8 T cells	18	31.35	28.00	11.91	11.00	52.00	18	27.01	26.15	9.93	9.00	52.00	1.417	0.243		0.216	0.646	0.003	0.960	(1,32)
Ratio of CD4 to CD8 T cells	18	2.29	2.22	1.35	1.00	6.00	18	2.97	2.58	1.93	1.00	9.00	1.432	0.240		0.011	0.917	85.000	0.773	(1,32)

Table 6. Surface expression of monocyte activation markers in Healthy Controls and RA. Differences between healthy controls and RA patients were tested by ANCOVA while controlling for sex and age.

		Control						RA						ANCOVA						
														Control vs RA		Sex		Age		
		n	Mean	Median	SD	Min	Max	n	Mean	Median	SD	Min	Max	F	p	F	p	F	p	(df_1, df_{error})
CD16	Monocyte	19	172.3	141.0	107.9	66.3	506.8	19	164.0	131.8	151.1	30.3	719.3	0.027	0.870	0.018	0.893	0.727	0.400	(1,34)
	Non-classical	19	3166.2	2874.2	1914.0	778.0	8769.0	19	2627.4	2003.5	2487.5	641.8	9766.3	0.508	0.481	0.034	0.854	0.313	0.579	(1,34)
	Intermediate	19	1853.7	2177.3	898.2	633.0	4225.0	19	1453.9	1311.7	755.3	543.8	4079.3	2.061	0.160	0.092	0.764	0.150	0.701	(1,34)
	Classical	19	91.3	81.8	45.7	46.3	262.8	19	109.1	64.3	133.6	23.8	585.3	0.360	0.553	0.216	0.645	2.309	0.138	(1,34)
CX3CR1	Monocyte	19	1456.8	1450.2	704.3	342.0	3145.0	19	1678.8	1624.0	582.8	650.0	3101.0	1.191	0.283	0.376	0.544	1.460	0.235	(1,34)
	Non-classical	19	5080.5	4491.0	1390.6	3094.0	8032.4	19	5017.6	5512.0	1748.9	1851.0	7528.0	0.009	0.926	0.000	0.986	0.717	0.403	(1,34)
	Intermediate	19	3277.4	2840.2	1320.8	1408.0	5868.4	19	2979.7	3039.0	1222.1	768.0	5385.5	0.461	0.502	0.196	0.661	1.517	0.226	(1,34)
	Classical	19	1121.6	1082.2	603.3	264.0	2382.0	19	1399.4	1301.0	580.7	559.0	3238.0	2.198	0.147	0.337	0.565	1.645	0.208	(1,34)
CCR2	Monocyte	19	11276.1	10137.0	5193.4	4907.0	25835.0	19	12072.1	10316.0	6115.2	3281.0	24931.7	0.174	0.680	0.869	0.358	0.083	0.775	(1,34)
	Non-classical	19	268.5	39.0	808.7	0.0	3564.0	19	375.5	59.9	696.2	0.0	2560.7	0.177	0.676	0.002	0.963	0.014	0.906	(1,34)
	Intermediate	19	5141.4	4424.0	4425.3	1676.0	21968.0	19	7911.1	4847.0	7689.4	1079.0	29303.7	1.728	0.198	0.155	0.696	1.544	0.222	(1,34)
	Classical	19	21652.5	19934.0	4599.6	14900.0	30534.0	19	21523.5	20431.0	7381.8	10686.0	35382.0	0.007	0.936	0.635	0.431	0.310	0.582	(1,34)

Table 7. Changes in serum cytokines with treatment. The change in serum cytokines at visit 1 (3 months) and visit 2(6 months) of DMARD treatment were tested in a linear mixed model with patient set as a random effect. Statistical significance was tested by Analysis of deviance (Type II Wald chi square test).

	Baseline						Visit 1						Visit 2						p
	n	Mean	Median	SD	Minimum	Maximum	n	Mean	Median	SD	Minimum	Maximum	n	Mean	Median	SD	Minimum	Maximum	
ESR(mm/hr)	19	22.95	17	14.05	5	50	18	20.28	18.00	14.53	3.00	57.00	17	14.76	12.00	12.31	2.00	45.00	**0.002035**
CRP(mg/L)	18	23.98	3.4	43.95	0.5	150.9	18	6.79	3.45	9.84	0.40	41.30	17	5.21	2.50	6.35	0.40	20.00	**0.01523**
GM-CSF (pg/mL)	18	63.41	50.74	62.263	8	291	19	52.97	45.30	43.96	3.93	184.77	19	51.08	41.36	49.30	1.75	222.41	**0.006221**
Fractalkine (pg/mL)	18	133.14	138.77	43.43	48	200	19	124.86	137.17	45.07	43.43	202.68	19	117.92	132.09	50.64	4.04	195.15	0.151
IFNγ (pg/mL)	18	29.39	31.78	11.375	11	50	19	26.40	26.36	13.74	3.34	54.52	19	26.29	23.78	15.67	1.39	61.82	0.2044
IL-10 (pg/mL)	18	19.17	12.7	18.119	0	57	19	16.51	8.07	18.33	0.08	54.00	19	15.48	7.39	17.51	0.05	54.38	**0.007169**
IL-1β (pg/mL)	18	3.28	3.5	1.471	1	6	19	3.04	2.92	1.76	0.70	8.48	19	2.72	2.71	1.38	0.33	5.42	**0.0307**
IL-6 (pg/mL)	18	6.75	5.19	4.229	0	18	19	5.70	4.78	3.66	0.04	14.12	18	5.53	5.55	2.23	1.18	9.62	0.0924
TNF (pg/mL)	18	12.78	12.18	3.226	8	19	19	12.57	12.66	3.59	7.84	19.20	19	15.17	10.77	11.93	7.65	58.70	0.6551
IP-10 (pg/mL)	18	1559.8	1025	1401.743	316	5838	19	1620.11	1055.00	1774.46	157.85	7920.00	19	1503.89	1008.00	1262.44	210.56	4139.00	0.9304
MCP-1 (pg/mL)	18	1536.18	1393.5	445.94	910	2386	19	1751.19	1589.00	599.05	873.52	3140.00	19	1815.11	1522.00	886.80	712.97	4281.00	0.1031
VEGFA (pg/mL)	18	553.49	370.39	639.827	68	2201	19	408.72	288.09	343.58	28.60	1080.00	19	450.05	347.57	461.75	37.22	1556.00	0.1695
Inflammatory Index	19	11.626	20.59	25.779	-48.72	42.3	18	-8.36	-0.95	38.42	-117.58	42.55	17	-7.13	-3.38	31.50	-71.95	46.70	**0.01036**

Table 8. Changes in leukocyte populations with treatment.
The change in leukocyte populations at visit 1 (3 months) and visit 2 (6 months) of DMARD treatment were tested in a linear mixed model with patient set as a random effect. Statistical significance was tested by Analysis of deviance (Type II Wald chi square test).

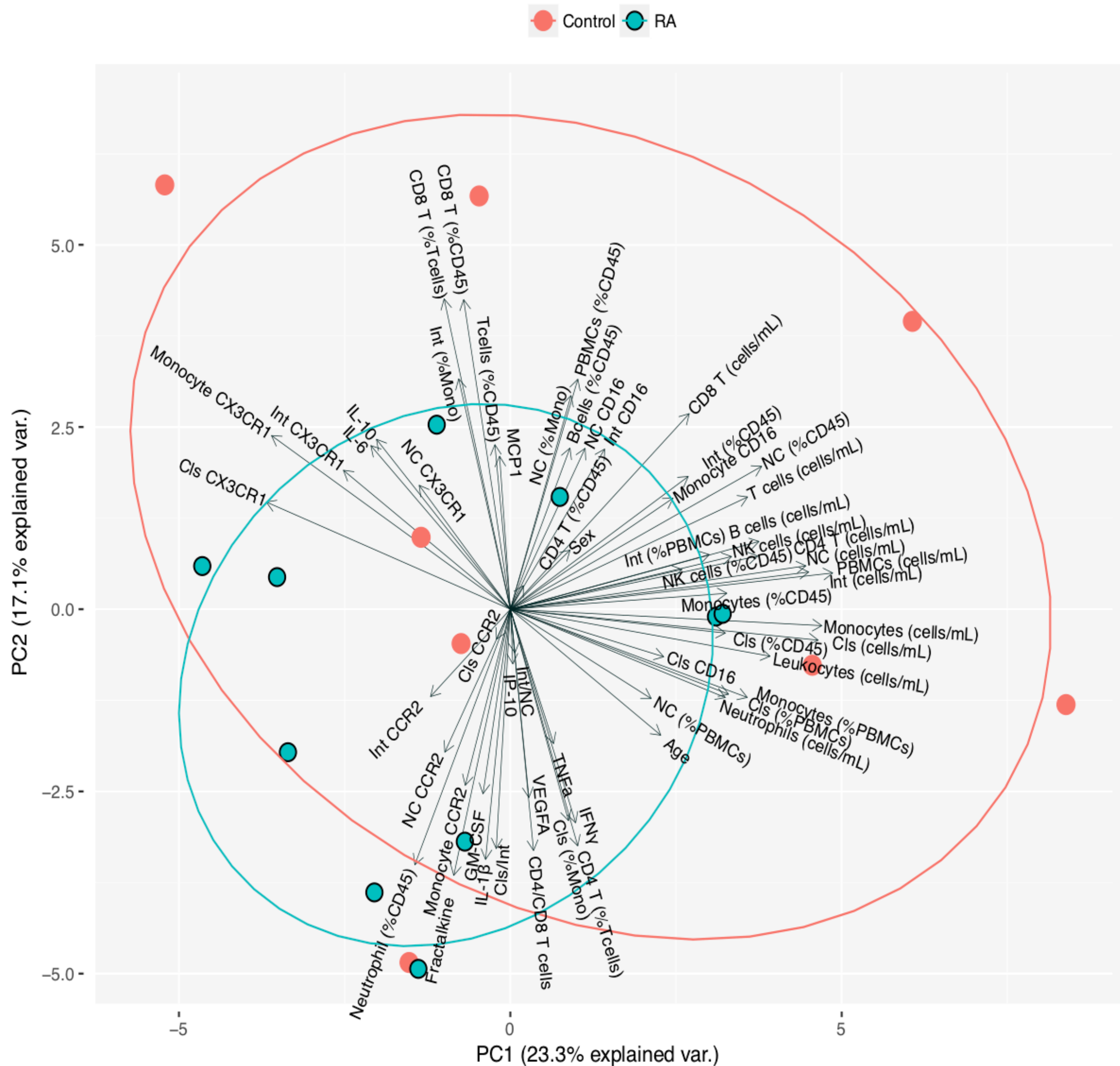
	Baseline						Visit 1						Visit 2						p
	n	Mean	Median	SD	Minimum	Maximum	n	Mean	Median	SD	Minimum	Maximum	n	Mean	Median	SD	Minimum	Maximum	
Cell Counts(cells/mL blood)																			
Leukocytes	19	$3.23x10^{6}$	$2.709x10^{6}$	$2.079x10^{6}$	$1.090x10^{6}$	$8.800x10^{6}$	18	$2.93x10^{6}$	$2.75x10^{6}$	$1.49x10^{6}$	$3.61x10^{5}$	$6.91x10^{6}$	18	$3.83x10^{6}$	$3.12x10^{6}$	$2.38x10^{6}$	$1.40x10^{6}$	$1.01x10^{7}$	0.226
Neutrophils	19	$2.097x10^{6}$	$1.660x10^{6}$	$1.589x10^{6}$	$8.350x10^{5}$	$6.93x10^{6}$	18	1.8610^{6}	$1.79x10^{6}$	$1.07x10^{6}$	$1.96x10^{5}$	$5.01x10^{6}$	18	$2.43x10^{6}$	$1.92x10^{6}$	$1.60x10^{6}$	$7.65x10^{5}$	$6.96x10^{6}$	0.3455
PBMCs	19	$9.46x10^{5}$	$7.950x10^{5}$	$6.865x10^{5}$	$1.650x10^{5}$	$3.12x10^{6}$	18	9.8010^{5}	$9.87x10^{5}$	$4.72x10^{5}$	$1.12x10^{5}$	$1.83x10^{6}$	18	$1.58x10^{6}$	$1.13x10^{6}$	$1.15x10^{6}$	$5.03x10^{5}$	$4.13x10^{6}$	**0.001762**
Monocytes	19	$8.921x10^{4}$	$6.420x10^{4}$	$8.23x10^{4}$	$1.040x10^{4}$	$2.58x10^{5}$	18	1.1410^{5}	$1.27x10^{5}$	$7.21x10^{4}$	$5.76x10^{3}$	$2.87x10^{5}$	18	$1.61x10^{5}$	$1.10x10^{5}$	$1.57x10^{5}$	$4.65x10^{4}$	$6.24x10^{5}$	0.07788
Non-classical	19	$1.008x10^{4}$	$5.260x10^{3}$	$1.450x10^{4}$	$1.700x10^{2}$	$5.75x10^{4}$	18	$1.15x10^{4}$	$9.16x10^{3}$	$8.65x10^{3}$	$2.40x10^{2}$	$2.78x10^{4}$	18	$1.44x10^{4}$	$1.37x10^{4}$	$9.93x10^{3}$	$1.04x10^{3}$	$3.38x10^{4}$	0.3131
Intermediate	19	$6.724x10^{3}$	$4.660x10^{3}$	$6.477x10^{3}$	$3.680x10^{2}$	$1.87x10^{4}$	18	$8.36x10^{3}$	$5.98x10^{3}$	$7.33x10^{3}$	$7.59x10^{2}$	$2.90x10^{4}$	18	$1.53x10^{4}$	$1.03x10^{4}$	$1.76x10^{4}$	$1.54x10^{3}$	$7.38x10^{4}$	**0.009267**
Classical	19	$6.435x10^{4}$	$4.820x10^{4}$	$5.687x10^{4}$	$6.11x10^{3}$	$1.99x10^{5}$	18	$7.67x10^{4}$	$8.41x10^{4}$	$5.17x10^{4}$	$3.92x10^{3}$	$1.72x10^{5}$	18	$1.22x10^{5}$	$8.14x10^{4}$	$1.26x10^{5}$	$3.47x10^{4}$	$4.83x10^{5}$	0.06013
NK cells	19	$1.161x10^{5}$	$7.460x10^{4}$	$1.225x10^{5}$	$1.37x10^{4}$	$4.24. x10^{5}$	18	$8.66x10^{4}$	$8.76x10^{4}$	$5x10^{4}$	$1.38x10^{4}$	$2.12x10^{5}$	18	$5.17x10^{5}$	$1.03x10^{5}$	$1.22x10^{5}$	$1.89x10^{4}$	$4.36x10^{6}$	0.106
B cells	19	$8.039x10^{4}$	$6.360x10^{4}$	$6.069x10^{4}$	$1.79x10^{4}$	$2.42x10^{5}$	18	$6.84x10^{4}$	$6.18x10^{4}$	$4.44x10^{4}$	$3.75x10^{3}$	$1.55x10^{5}$	18	$1.44x10^{5}$	$7.07x10^{4}$	$2.68x10^{5}$	$1.58x10^{4}$	$1.20x10^{6}$	0.2729
T cells	18	$6.558x10^{5}$	$5.500x10^{5}$	$5.259x10^{5}$	$1.51x10^{5}$	$1.99x10^{6}$	18	$5.82x10^{5}$	$5.33x10^{5}$	$3.19x10^{5}$	$4.03x10^{4}$	$1.13x10^{6}$	18	$5.97x10^{5}$	$4.47x10^{5}$	$4.08x10^{5}$	$8.80x10^{4}$	$1.58x10^{6}$	0.7741
CD4 T cells	18	$4.494x10^{5}$	$3.840x10^{5}$	$4.081x10^{5}$	$7.12x10^{4}$	$1.46x10^{6}$	18	$3.37x10^{5}$	$3.36x10^{5}$	$1.87x10^{5}$	$2.24x10^{4}$	$6.94x10^{5}$	18	$4.50x10^{5}$	$3.67x10^{5}$	$3.21x10^{5}$	$1.64x10^{4}$	$1.28x10^{6}$	0.2099
CD8 T cells	18	$1.656x10^{5}$	$2.205x10^{5}$	$1.215x10^{5}$	$3.65x10^{4}$	$4.21x10^{5}$	17	$1.66x10^{5}$	$1.35x10^{5}$	$1.29x10^{5}$	$1.44x10^{4}$	$4.68x10^{5}$	18	$1.82x10^{5}$	$1.60x10^{5}$	$1.20x10^{5}$	$4.91x10^{4}$	$5.28x10^{5}$	0.7034
% of Leukocytes																			
Neutrophils	19	63.87	64.20	10.00	51.60	86.30	19	61.36	61.00	8.63	46.10	77.00	18	61.49	60.75	8.89	41.70	78.30	0.576
PBMCs	19	28.78	29.60	9.95	13.40	46.20	19	34.90	34.80	8.26	21.70	49.00	18	36.14	36.25	8.14	21.20	53.00	**0.00579**
Monocytes	19	2.73	2.71	1.88	0.37	6.90	19	3.97	4.00	1.99	0.87	7.45	18	4.05	3.63	1.88	1.61	9.18	**0.001999**
Non-classical	19	0.26	0.16	0.24	0.02	0.80	19	0.39	0.40	0.25	0.07	0.90	18	0.41	0.39	0.24	0.05	0.95	**0.02701**
Intermediate	19	0.20	0.11	0.17	0.03	0.52	19	0.28	0.23	0.18	0.05	0.75	18	0.39	0.38	0.24	0.03	0.79	**0.0001995**
Classical	19	1.96	1.75	1.36	0.22	5.32	19	3.03	2.73	1.79	0.47	6.63	18	3.03	2.57	1.72	1.11	8.18	**0.00481**
NK cells	19	2.87	2.50	1.50	0.39	6.24	19	3.05	2.63	1.92	0.90	8.66	18	2.74	2.31	1.54	1.06	5.44	0.8314
B cells	19	2.25	2.36	0.82	0.91	3.64	19	2.30	2.04	1.21	0.79	4.56	18	3.16	2.31	4.06	0.86	19.10	0.4147
T cells	18	18.37	19.35	6.48	3.00	29.00	19	18.39	17.50	6.80	9.86	32.80	18	19.36	20.95	9.04	4.62	40.90	0.797
CD4 T cells	18	12.08	11.50	5.29	2.00	20.00	19	10.91	10.40	4.48	3.45	18.90	18	12.43	12.75	7.54	0.26	33.70	0.4019
CD8 T cells	18	4.96	4.90	2.50	1.00	10.00	18	5.33	4.18	3.57	1.16	13.40	18	5.38	5.45	3.68	1.08	15.80	0.9206
% of PBMCs																			
Monocytes	19	8.98	9.34	4.08	1.01	16.10	19	11.91	9.74	7.47	2.95	32.80	18	11.35	9.52	4.92	5.73	22.60	**0.02888**
Non-classical	19	0.81	0.63	0.64	0.10	2.50	19	1.09	1.07	0.60	0.22	2.16	18	1.14	1.04	0.65	0.11	2.58	**0.03579**
Intermediate	19	0.63	0.54	0.42	0.10	1.45	19	0.82	0.68	0.55	0.12	2.45	18	1.14	0.95	0.82	0.10	2.67	**0.0004576**
Classical	19	6.59	6.71	3.21	0.60	11.50	19	9.24	7.34	6.97	1.58	29.80	18	8.49	6.90	4.44	3.68	20.20	0.07098
% of Monocytes																			
Non-classical	19	9.90	9.97	6.37	0.78	24.10	19	11.64	11.00	8.93	2.01	34.00	18	11.30	10.70	7.02	1.21	26.40	0.5518
Intermediate	19	7.13	7.18	3.10	1.68	14.40	19	8.37	6.97	5.76	1.24	26.50	18	9.89	8.49	5.67	1.75	19.20	0.1253
Classical	19	72.47	76.60	11.64	40.70	86.70	19	72.23	74.80	13.20	43.10	91.00	18	73.28	73.70	11.84	50.30	94.50	0.961
Ratio of Classical to Intermediate Monocytes	19	13.97	10.61	12.02	4.63	51.61	19	15.35	9.16	16.29	2.02	72.02	18	12.39	8.79	11.54	2.99	48.91	0.7664
Ratio of Intermediate to Non-classical Monocytes	19	1.03	0.75	0.77	0.24	3.23	19	1.01	0.62	0.83	0.23	3.16	18	1.18	0.79	0.92	0.32	3.24	0.5854
% of T cells																			
CD4 T cells	18	65.18	67.90	13.42	31.00	88.00	19	59.83	61.80	14.72	32.90	93.00	18	62.01	65.45	21.36	5.25	91.00	0.5346
CD8 T cells	18	27.01	26.15	9.93	9.00	52.00	18	28.10	26.75	12.85	6.00	53.90	18	29.38	24.40	17.57	7.59	77.60	0.8923
Ratio of CD4 to CD8 T cells	18	2.97	2.58	1.93	1.00	9.00	17	3.12	2.33	3.42	0.63	15.51	18	3.31	2.69	2.79	0.07	11.98	0.4283

Table 9. Changes in surface expression of monocyte activation markers with treatment. The changes in monocyte markers at visit 1 (3 months) and visit 2 (6 months) of DMARD treatment were tested in a linear mixed model with patient set as a random effect. Statistical significance was tested by Analysis of deviance (Type II Wald chi square test).

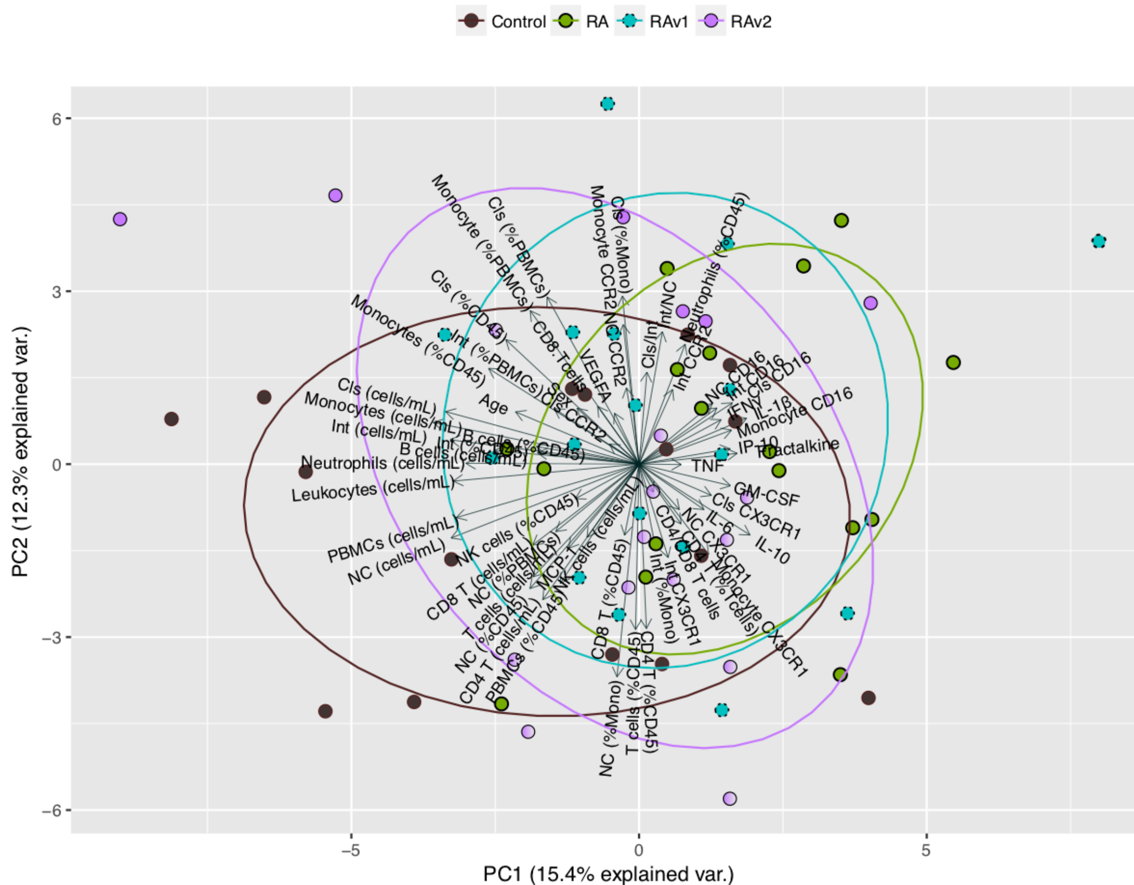
		Baseline						Visit 1						Visit 2						P
		n	Mean	Median	SD	Minimum	Maximum	n	Mean	Median	SD	Minimum	Maximum	n	Mean	Median	SD	Minimum	Maximum	
CD16	Monocyte	19	164.0	131.8	151.1	30.3	719.3	19	244.99	79.62	617.70	22.50	2763.48	18	181.06	95.73	195.35	47.71	741.71	0.3232
	Non-classical	19	2627.4	2003.5	2487.5	641.8	9766.3	19	3305.68	2320.02	3586.07	583.78	15591.48	18	2711.21	1477.73	2757.54	425.36	10490.71	0.2658
	Intermediate	19	1453.9	1311.7	755.3	543.8	4079.3	19	2309.26	1524.52	2857.88	552.78	13391.48	18	1845.33	1381.56	1939.19	26.00	8951.71	0.1908
	Classical	19	109.1	64.3	133.6	23.8	585.3	19	147.33	30.90	478.20	12.40	2119.48	18	89.49	35.39	154.47	19.60	658.71	**0.02023**
CX3CR1	Monocyte	19	1678.8	1624.0	582.8	650.0	3101.0	19	1497.69	1481.00	784.68	250.00	3774.50	18	1879.85	1644.30	799.62	820.00	3576.00	0.09123
	Non-classical	19	5017.6	5512.0	1748.9	1851.0	7528.0	19	4566.56	4592.00	1819.98	23.60	7249.50	18	5163.29	5088.00	1816.94	2813.00	8932.00	0.4159
	Intermediate	19	2979.7	3039.0	1222.1	768.0	5385.5	19	2788.58	2598.10	1327.49	793.00	4958.50	18	3517.96	3024.05	1340.78	1878.00	6019.00	0.05677
	Classical	19	1399.4	1301.0	580.7	559.0	3238.0	19	1117.69	1053.00	563.70	187.00	2258.50	18	1468.63	1255.35	725.98	545.00	3330.00	**0.02666**
CCR2	Monocyte	19	12072.1	10316.0	6115.2	3281.0	24931.7	19	9128.08	8589.40	4420.81	2532.00	20539.00	18	10619.96	9047.00	6541.37	1938.00	28899.00	**0.01562**
	Non-classical	19	375.5	59.9	696.2	0.0	2560.7	19	158.16	48.00	306.30	0.00	1291.10	18	597.63	36.75	1348.18	0.00	5214.00	0.8848
	Intermediate	19	7911.1	4847.0	7689.4	1079.0	29303.7	19	4814.76	4114.00	2566.86	1520.00	10487.00	18	4660.34	3962.50	3312.03	708.00	13883.80	**0.06014**
	Classical	19	21523.5	20431.0	7381.8	10686.0	35382.0	19	18202.07	17526.00	3736.66	13928.30	29197.00	18	19879.08	19017.00	5172.03	13202.00	30816.00	0.1189

Supplementary Figure 1. RA patients are immunologically distinct from healthy controls. Principal component analysis comparing RA patients and age- and sex-matched controls based on serum cytokines and immunological parameters. RA patients are significantly different from controls (p=0.043,R^2=0.119), tested by pairwise Adonis with a Bonferonni multiple comparison adjustment.

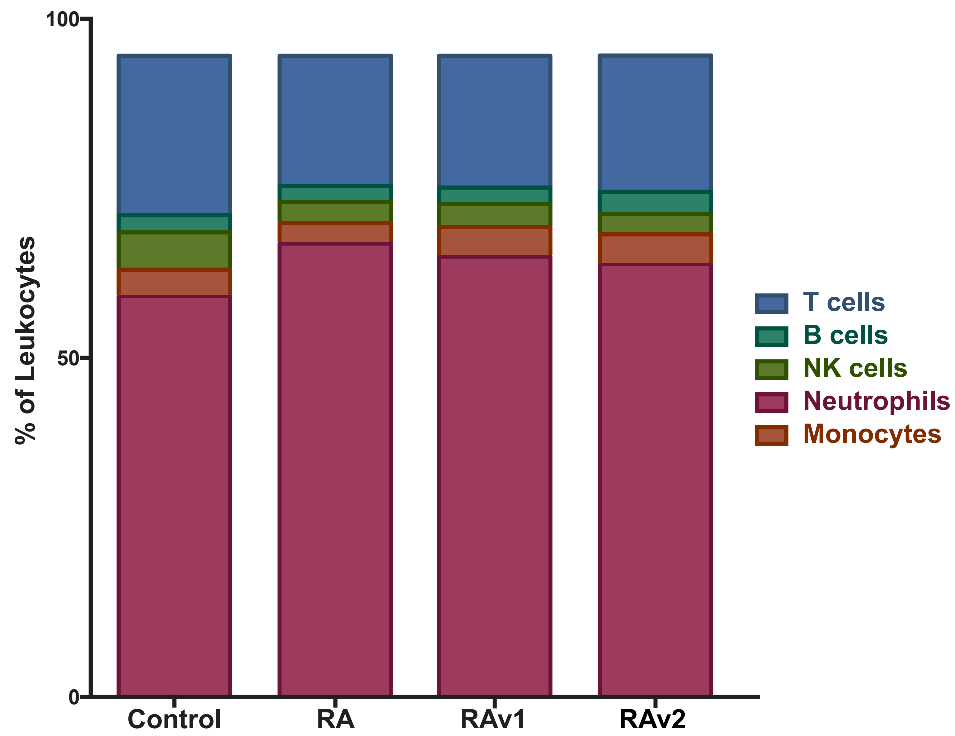
-



Supplemental Figure 2. RA patient immunological profiles change with DMARD treatment.
Principal component analysis comparing RA patients and age- and sex-matched controls based on serum cytokines and immunological parameters, and RA patients after 3 months (RAv1) and 6 months (RAv2) of DMARD treatment.



Supplemental Figure 3. Leukocyte composition. The proportions of leukocytes are different between healthy controls and RA patients, due to higher neutrophils and lower PBMCs in RA patients.



Supplementary Table 1. Disease Activity Decreases with Treatment. Tested in a linear mixed model with patient set as a random effect. Statistical significance was tested by Analysis of deviance (Type II Wald chi square test).

Tender Joint Count~ Visit + (1|Pt.ID)						
n	50					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	11.059	1.979	5.588	9.6252	2	**0.008127**
Baseline-Visit1	-2.647	1.695	-1.562			
Baseline-Visit2	-5.371	1.731	-3.102			
Random Effects	**Variance**	**SD**				
Patient	42.16	6.493				
Residual	24.42	4.942				

Swollen Joint Count~ Visit + (1|Pt.ID)						
n	50					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	6.412	1.186	5.406	12.492	2	**0.001938**
Baseline-Visit1	-1.412	1.01	-1.398			
Baseline-Visit2	-3.627	1.032	-3.515			
Random Effects	**Variance**	**SD**				
Patient	15.245	3.904				
Residual	8.674	2.945				

Patient Global~ Visit + (1|Pt.ID)						
n	39					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	5.3527	0.6357	8.421	10.463	2	**0.005344**
Baseline-Visit1	-1.6860	0.6009	-2.806			
Baseline-Visit2	-1.8581	0.6321	-2.940			
Random Effects	**Variance**	**SD**				
Patient	2.813	1.677				
Residual	2.168	1.472				

Physician Global~ Visit + (1|Pt.ID)						
n	36					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	4.534	0.6323	7.791	9.4748	2	**0.008761**
Baseline-Visit1	-0.6185	0.4627	-1.337			
Baseline-Visit2	-1.4886	0.4921	-3.025			
Random Effects	**Variance**	**SD**				
Patient	4.534	2.129				
Residual	1.077	1.038				

CDAI~ Visit + (1|Pt.ID)						
n	36					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	23.933	3.892	6.149	10.559	2	**0.005094**
Baseline-Visit1	-3.337	2.961	-1.127			
Baseline-Visit2	-9.758	3.132	-3.115			
Random Effects	**Variance**	**SD**				
Patient	-0.446	2.129				
Residual	-0.468	1.038				

Supplementary Table 2. Systemic Inflammation decreases with treatment. Tested in a linear mixed model with patient set as a random effect. Statistical significance was tested by Analysis of deviance (Type II Wald chi square test).

Inflammatory Index ~ Visit + (1|Pt.ID)						
n	52					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	0.459	8.1448	0.056	9.1397	2	**0.01036**
Baseline-Visit1	-10.426	4.6875	-2.224			
Baseline-Visit2	-12.964	4.7898	-2.916			
Random Effects	Variance	SD				
Patient	20.19	4.49				
Residual	57.94	7.61				

ln(GM-CSF(pg/mL))~ Visit + (1|Pt.ID)						
n	56					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	3.827	0.2159	17.725	10.16	2	**0.006221**
Baseline-Visit1	-0.20948	0.09724	-2.514			
Baseline-Visit2	-0.30402	0.09724	-3.127			
Random Effects	Variance	SD				
Patient	0.79252	0.8902				
Residual	0.08629	0.2937				

ln((ESR(mm/hr)) ~ Visit + (1|Pt.ID)						
n	54					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	2.935	0.1857	15.808	12.395	2	**0.002035**
Baseline-Visit1	-0.205	0.1794	-1.143			
Baseline-Visit2	-0.634	0.1829	-3.467			
Random Effects	Variance	SD				
Patient	0.362	0.602				
Residual	0.293	0.541				

ln(IL-10(pg/mL))~ Visit + (1|Pt.ID)						
n	56					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	2.2615	0.4199	5.386	9.876	2	**0.007169**
Baseline-Visit1	-0.3949	0.2180	-1.811			
Baseline-Visit2	-0.6836	0.2180	-3.135			
Random Effects	Variance	SD				
Patient	2.8810	1.6973				
Residual	0.4339	0.6587				

ln(CRP(mg/L))~ Visit + (1|Pt.ID)						
n	53					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	1.756	0.329	5.435	8.368	2	**0.01523**
Baseline-Visit1	-0.5734	0.289	-1.982			
Baseline-Visit2	-0.8324	0.295	-2.820			
Random Effects	Variance	SD				
Patient	1.262	1.123				
Residual	0.714	0.855				

ln(IL-1β(pg/mL))~ Visit + (1|Pt.ID)						
n	56					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	1.0755	0.14138	7.607	6.967	2	**0.0307**
Baseline-Visit1	-0.1233	0.0903	-1.326			
Baseline-Visit2	-0.2455	0.0903	-2.639			
Random Effects	Variance	SD				
Patient	0.294	0.542				
Residual	0.079	0.281				

ln(IL-6(pg/mL))~ Visit + (1|Pt.ID)						
n	55					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	1.6440	0.2489	6.604	4.7632	2	0.0924
Baseline-Visit1	-0.2760	0.1348	-2.048			
Baseline-Visit2	-0.2330	0.1375	-1.694			
Random Effects	Variance	SD				
Patient	0.998	0.999				
Residual	0.166	0.407				

Supplementary Table 3. Cellular inflammation decreases with treatment. Tested in a linear mixed model with patient set as a random effect. Statistical significance was tested by Analysis of deviance (Type II Wald chi square test).

Monocyte CCR2(MFI)~ Visit + (1|Pt.ID)						
n	56					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	12072	1317	9.169	8.3188	2	**0.01562**
Baseline-Visit1	-2944	1024	-2.875			
Baseline-Visit2	-1263	1044	-1.210			
Random Effects	Variance	SD				
Patient	2.297×10^7	4793				
Residual	9.96×10^6	3156				

ln(Intermediate Monocyte CCR2(MFI))~ Visit + (1|Pt.ID)						
n	56					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	8.5712	0.1736	49.36	5.622	2	0.06014
Baseline-Visit1	-0.2258	0.1467	-1.54			
Baseline-Visit2	-0.3479	0.1495	-2.33			
Random Effects	Variance	SD				
Patient	0.3683	0.6069				
Residual	0.2045	0.4522				

ln(Classical Monocyte CD16(MFI))~ Visit + (1|Pt.ID)						
n	56					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	4.3198	0.2175	19.86	7.8007	2	**0.02023**
Baseline-Visit1	-0.6309	0.2332	-2.705			
Baseline-Visit2	-0.4589	0.2374	-1.933			
Random Effects	Variance	SD				
Patient	0.3823	0.6183				
Residual	0.5166	0.7188				

Classical Monocyte CX_3CR_1(MFI)~ Visit + (1|Pt.ID)						
n	56					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	1399.43	143.05	9.783	7.2491	2	**0.02666**
Baseline-Visit1	-281.74	137.47	-2.049			
Baseline-Visit2	-72.13	140.02	0.515			
Random Effects	Variance	SD				
Patient	2.09×10^5	457.4				
Residual	1.79×10^5	423.7				

Monocytes(%CD45)~ Visit + (1|Pt.ID)						
n	56					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	2.7282	0.4425	6.804	12.431	2	**0.001999**
Baseline-Visit1	1.2428	0.4357	2.852			
Baseline-Visit2	1.4226	0.4438	3.206			
Random Effects	Variance	SD				
Patient	1.916	1.384				
Residual	1.804	1.343				

PBMCs(%CD45)~ Visit + (1|Pt.ID)						
n	56					
Fixed Effects	Estimate	SE	t	X^2	df	p
(Intercept)	28.784	2.028	14.194	10.303	2	**0.00579**
Baseline-Visit1	6.116	2.47	2.476			
Baseline-Visit2	7.523	2.511	2.996			
Random Effects	Variance	SD				
Patient	20.19	4.49				
Residual	57.94	7.61				

Supplementary Table 4. Cytokine correlates of CD16 and CX_3CR_1.

Partial Correlation controlling for age, sex, and patient					
Variable 1	Variable 2	n	r	95% CI	p
Classical Monocyte CD16	IL-1β	54	0.293	0.028 - 0.520	**0.0348**
Classical Monocyte CX_3CR_1	IL-10	54	0.386	0.131 - 0.592	**0.0048**
	IL-6	54	0.297	0.031 – 0.522	**0.0330**
Intermediate Monocyte CCR2	MCP-1	54	-0.278	-0.508- -0.010	**0.0464**

Supplementary Table 5. The inflammatory index predicts changes in CD16 and PBMCs. Tested in a linear mixed model with patient, age, sex and visit set as a random effect. Statistical significance was tested by Analysis of deviance (Type II Wald chi square test).

ln(Monocyte CD16) ~ Inflammatory Index + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)						
n	52					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	4.792178	0.148603	32.25	8.5681	1	**0.003421**
Inflammatory Index	0.01182	0.004038	2.92			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	20.19	0.0005213	0	0	0.47	
SD	4.49	0.02283	0	0	0.68	

ln(PBMCs(%CD45)) ~ Inflammatory Index + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)						
n	52					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	3.390761	0.134116	25.28	4.3284	1	**0.03748**
Inflammatory Index	-0.002929	0.001408	-2.08			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	0	0.01504	0.021431	0.009344	0.06858	
SD	0	0.12264	0.14639	0.09666	0.26188	

Supplementary Table 6. VEGFA, IL-6, IL-1β and IL-10 influence neutrophil proportions. Tested in a linear mixed model with statistical significance tested by Analysis of deviance (Type II Wald chi square test).

Neutrophils (%CD45) ~ ln(VEGFA) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)						
n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	44.563	7.067	6.306	6.5238	1	**0.01064**
ln(VEGFA)	3.146	1.232	2.554			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	7.01	1.34	9.23×10^{-14}	0	67.11	
SD	2.65	1.16	3.04×10^{-7}	0	8.19	
Neutrophils (%CD45) ~ IL-6 + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)						
n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	55.7241	3.3478	16.645	12.706	1	**0.0003644**
IL-6	1.2513	0.3511	3.565			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	11.527	15.317	7.594	0	49.139	
SD	3.395	3.914	2.756	0	7.01	
Neutrophils (%CD45) ~ IL-1β + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)						
n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	57.107	4.4002	12.978	8.0347	1	**0.004589**
IL-1β	2.376	0.8381	2.835			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	2.569	0	24.009	0	69.431	
SD	1.603	0	4.9	0	8.333	
Neutrophils (%CD45) ~ ln(IL-10) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)						
n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	60.4544	3.2933	18.36	3.8409	1	0.05002
ln(IL-10)	1.6089	0.8209	1.96			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	18.71	0	11.51	0	62.74	
SD	4.326	0	3.933	0	7.921	

Supplementary Table 7. TNF, IL-1β, IP-10 and MCP are associated with monocyte proportions and phenotype. Tested in a linear mixed model with statistical significance tested by Analysis of deviance (Type II Wald chi square test).

Monocytes (%CD45) ~ ln(TNF) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	0.3192	1.5789	0.202	5.9178	1	**0.01499**
ln(TNF)	1.3838	0.5688	2.433			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	0.11057	3.98389	0.01344	0.24756	0.94552	
SD	0.3325	1.996	0.1159	0.4976	0.9724	

Classical Monocytes (%CD45) ~ ln(TNF) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	0.2252	1.334	0.169	4.4787	1	**0.03432**
ln(TNF)	1.0248	0.4842	2.116			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	0	2.8221	0	0.1162	0.764	
SD	0	1.6799	0	0.3408	0.8741	

Intermediate Monocytes (%CD45) ~ ln(TNF) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	-0.13505	0.21097	-0.64	4.8537	1	**0.02759**
ln(TNF)	0.17265	0.07836	2.203			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	0.021621	0.006939	0	0.00808	0.016499	
SD	0.14704	0.0833	0	0.08989	0.12845	

Non-classical Monocytes (%CD45) ~ ln(TNF) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	0.07456	0.2608	0.286	1.2103	1	0.2713
ln(TNF)	0.10915	0.09922	1.1			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	0.024644	0.002632	0	0.007299	0.032218	
SD	0.15698	0.0513	0	0.08543	0.17949	

Monocytes (%PBMCs) ~ IL-1β + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	9.0614	2.0819	4.353	7.0945	1	**0.00773**
IL-1β	0.8715	0.3272	2.664			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	2.26×10^{-15}	36.20	1.32	0.627	8.30	
SD	4.75×10^{-8}	6.02	1.15	0.792	2.88	

Classical Monocytes (%Monocytes) ~ IL-1β + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	67.074	4.514	14.858	3.645	1	0.05624
IL-1β	2.073	1.086	1.909			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	1.20×10^{-14}	28.8	12.4	0	113	
SD	1.09×10^{-7}	5.37	3.52	0	10.6	

Intermediate Monocytes (%Monocytes) ~ IL-1β + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	11.4891	1.7991	6.386	4.1888	1	**0.04069**
IL-1β	-0.9897	0.4836	-2.047			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	11.727	0	0	0.5393	14.8345	
SD	3.4245	0	0	0.7344	3.8516	

Non-classical Monocytes (%Monocytes) ~ IL-1β + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	12.0158	3.0662	3.919	0.9337	1	0.3339
IL-1β	-0.6418	0.6642	-0.966			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	33.028	0	5.803	0	24.011	
SD	5.747	0	2.409	0	4.9	

Monocytes (%CD45) ~ ln(IP-10) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	0.3921	1.6921	0.232	5.1253	1	**0.0235**
ln(IP-10)	0.5127	0.2265	2.264			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	0	3.8652	0.2132	0.2613	1.0071	
SD	0	1.966	0.4618	0.5112	1.0035	

Classical Monocytes (%CD45) ~ ln(IP-10) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	0.7467	1.423	0.525	2.5049	1	0.1135
ln(IP-10)	0.3023	0.191	1.583			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	5.32×10^{-15}	2.685	0.029	0.122	0.811	
SD	7.29×10^{-8}	1.639	0.169	0.350	0.900	

Intermediate Monocytes (%CD45) ~ ln(IP-10) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	-0.23584	0.25675	-0.919	5.0551	1	**0.02455**
ln(IP-10)	0.07908	0.03517	2.248			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	0.016033	0.007805	0.003034	0.00933	0.017344	
SD	0.12662	0.08835	0.05509	0.09659	0.1317	

Non-classical Monocytes (%CD45) ~ ln(IP-10) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	-0.29305	0.30619	-0.957	4.6163	1	**0.03167**
ln(IP-10)	0.09121	0.04245	2.149			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	0.0185	0	0	0.008004	0.033	
SD	0.13601	0	0	0.08947	0.18166	

Monocyte CCR2 (MFI) ~ ln(MCP-1) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	43280	15001	2.885	4.7313	1	**0.02962**
ln(MCP-1)	-4379	2013	-2.175			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	13239880	6473219	2284691	1664575	7357602	
SD	3639	2544	1512	1290	2712	

Classical Monocytes (%Monocytes) ~ ln(MCP-1) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	152.594	31.403	4.859	6.5166	1	**0.01069**
ln(MCP-1)	-10.812	4.235	-2.533			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	0	6.737	6.272	0	120.127	
SD	0	2.596	2.504	0	10.96	

Intermediate Monocytes (%Monocytes) ~ ln(MCP-1) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	-19.854	15.953	-1.245	3.1707	1	0.07497
ln(MCP-1)	3.848	2.161	1.781			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	9.274	3.74×10^{-14}	0.000	0.876	15.900	
SD	3.045	1.93×10^{7}	0.000	0.936	3.980	

Non-classical Monocytes (%Monocytes) ~ ln(MCP-1) + (1|Pt.ID) + (1|Age) + (1|Sex) + (1|Visit)

n	54					
Fixed Effects	**Estimate**	**SE**	**t**	**X^2**	**df**	**p**
(Intercept)	-42.152	21.438	-1.966	6.0023	1	**0.01429**
ln(MCP-1)	7.048	2.877	2.45			
Random Effects	**Patient**	**Age**	**Sex**	**Visit**	**Residual**	
Variance	17.6473	0.6298	8.7504	0	26.3355	
SD	4.2009	0.7936	2.9581	0	5.1318	

Appendix:

Changes in the anti-bacterial response and upper respiratory tract microbiota with chronic inflammation

Baseline Visit

Introduction

Consent received? Yes No	Visit Date: yyyy/mm/dd
Participant's age:	Participant's sex: Male Female

Inclusion Criteria (proceed only if "yes" to 1)

1. ≥ 18 years? No Yes

Part A: Does the participant have any chronic medical problems (other than RA)?

(e.g. cardiovascular disease, autoimmune conditions, lupus, dementia, type 2 diabetes)

1.	**6.**
2.	**7.**
3.	**8.**
4.	**9.**
5.	**10.**
Crohn's disease? No Yes **Ulcerative colitis?** No Yes	**Smoker?** No Yes Prior **If yes, how many pack years?** ________________ **If prior, when did patient give up, and how many pack years?** ________________

Part B: Clinical Blood Work Results

Rheumatoid Factor Positive? No Yes	**Anti-CPP Positive?** No Yes

Erythrocyte Sedimentation Rate (ESR, mm/hr): ___________________ Date of test result: yyyy/mm/dd	**C-Reactive Protein (CRP, mg/L):**___________________ Date of test result: yyyy/mm/dd

Part C: Current medication profile (regular medications only)

1. Start Date: yyyy/mm/dd	**6.** Start Date: yyyy/mm/dd
2. Start Date: yyyy/mm/dd	**7.** Start Date: yyyy/mm/dd
3. Start Date: yyyy/mm/dd	**8.** Start Date: yyyy/mm/dd
4. Start Date: yyyy/mm/dd	**9.** Start Date: yyyy/mm/dd
5. Start Date: yyyy/mm/dd	**10.** Start Date: yyyy/mm/dd
Antibiotics (within the last 2 weeks)?	**Antibiotics (current)?**

Part D: Select vaccination history

Has the participant ever been vaccinated against Influenza? (e.g. Annual Influenza vaccine) Yes No Unsure
Has the participant ever been vaccinated against *Streptococcus pneumoniae*? (e.g. pneumococcal polysaccharide vaccine, 23-valent polysaccharide vaccine) Yes No Unsure

Part E: Contact with children

Does the participant have contact with children? No Yes If **yes,** list ages of children: ________________________

CHAPTER 4

Monocyte activation is elevated in women with knee-osteoarthritis and associated with inflammation, BMI and pain

Preface:

This chapter contains a published manuscript from *Osteoarthritis and Cartilage**. The purpose of this manuscript was to determine if chronic inflammation independent of an autoimmune condition, like RA in chapter 3, contributed to changes in the monocyte compartment. Osteoarthritis (OA) is an age-associated, chronic inflammatory disease resulting in articular cartilage breakdown, bone erosions, swelling and ultimately joint degradation. Disease activity is strongly correlated with monocytes and macrophages in circulation and in affected synovial joints. This study examined the differences in inflammation and monocyte numbers, phenotype and function between older adult women (51-84yrs) with osteoarthritis and healthy age- and sex-matched controls. Age-matching controls with our OA participants allowed us to test the specific effect of inflammation on the monocyte compartment. We found elevated inflammation in women with OA compared to controls. Markers of monocyte activation were elevated and monocytes produced more inflammatory cytokines following LPS-stimulation compared to healthy controls. Furthermore, monocyte expression of CCR2 correlated with inflammation, body mass index and pain.

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Monocyte activation is elevated in women with knee-osteoarthritis and associated with inflammation, BMI and pain

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SUMMARY

Objective: Monocytes contribute to synovitis and disease pathogenesis in osteoarthritis (OA). Low-grade inflammation occurs in OA and correlates with disease severity and progression. Since monocyte development and function is altered by systemic inflammation, we analyzed monocyte numbers and function between individuals with knee OA and healthy age- and sex-matched controls.
Design: We analyzed markers of soluble and cellular inflammation in peripheral blood of women with knee OA and compared them to healthy age- and sex-matched controls. Soluble inflammatory mediators (TNF, IL-6, IL-10 and CRP) in the serum were measured by high-sensitivity ELISA. Leukocyte numbers, surface expression of monocyte activation markers, and monocyte production of pro-inflammatory mediators (TNF and IL-1β) following stimulation were measured by flow cytometry.
Results: Women with knee OA ($n = 15$) had elevated levels of serum c-reactive protein (CRP) and a lower proportion of circulating monocytes. Monocytes from OA participants had elevated expression of the activation markers CD16, CCR2, and HLA-DR and induced greater production of tumor necrosis factor (TNF) and IL-1β compared to healthy controls. Higher serum TNF and BMI were correlated with increased monocyte expression of CCR2. Additionally monocyte CCR2 expression and serum TNF were correlated with worse pain on a validated questionnaire.
Conclusions: Our findings suggest monocytes are activated prior to their entry into the synovium. Modulating systemic inflammation and monocyte recruitment to the synovium could be of therapeutic benefit.
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Introduction

Although the root cause of osteoarthritis (OA) remains unknown, there are multiple factors that contribute to disease risk, progression and severity (i.e., genetics, age, obesity, smoking, joint injury, metabolic dysfunction)[1]. The immune system plays a pivotal role in the pathogenic mechanisms of OA. Both soluble (e.g., cytokines, chemokines, complement) and cellular (e.g., monocytes, macrophages) mediators of immunity contribute to cartilage destruction, abnormal bone remodeling, synovitis and joint effusion[2]. Cartilage destruction results from chondrocyte activation, which stimulates the release of matrix metalloproteinases (MMPs), disintegrin and metalloproteinases with thrombospondin motifs (ADAMTSs), and pro-inflammatory cytokines (tumor necrosis factor (TNF), IL-1β, IL-6). Activated chondrocytes undergo hypertrophy and cease to produce new cartilage matrix[3]. The loss of cartilage, concurrent with other pathological joint tissue changes such as abnormal subchondral bone remodeling, subchondral cysts and osteophytes, destabilizes the joint.

Mechanical injury is thought to trigger the disease by activating joint tissues to release proinflammatory mediators[4]. While the temporal sequence of OA initiation remains unclear, some animal models suggest that damage to subchondral bone and articular cartilage initiates a cascade of events, including the

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origination of inflammatory signals that incite synovitis[2,5,6]. Inflammatory signals in the subchondral bone influence the development and activation of osteoclasts, which contribute to bone resorption and joint destruction in OA[7]. Osteoclasts can be derived from recently recruited monocytes in the appropriate microenvironment[8], and can contribute to cartilage degradation[9]. Synovitis is propagated by synovial macrophages and results in an inflammatory edema and joint effusion[4]. Synovial macrophages are activated by the pro-inflammatory cytokines and danger-associated molecular patterns (DAMPs) released by cartilage and bone breakdown (i.e., proteoglycans, glycosaminoglycan, hyaluronan, calcium pyrophosphate, sodium urate)[10,11]. They respond by releasing vascular endothelial growth factor (VEGF), TNF, IL-1β, IL-6, and chemokines (i.e., CCL2), stimulating the vascularization of the synovium and recruitment of circulating leukocytes[10,11]. The thickening of the synovial membrane is driven by an influx of monocytes, which differentiate into synovial macrophages[10,11]. Inflammatory mediators produced in the synovial membrane diffuse into the joint via the synovial fluid, and bathe the cartilage, increasing chondrocyte apoptosis[5]. Synovial fluid may additionally access and activate the subchondral bone through osteochondral lesions[5]. Although the relative importance and temporal sequence of subchondral bone and synovium activation in OA pathogenesis remains to be determined, monocyte involvement is central to both.

Monocyte chemokines and cytokines are found at increased concentrations in the synovial fluid of osteoarthritic joints[12]. In humans, soluble monocyte and macrophage markers (CD14, CD163) in the serum and synovial fluid are correlated with the number of activated synovial macrophages, joint-space narrowing and osteophytes[13]. Depletion of synovial macrophages in animal models decreases osteophyte formation[14,15] and cartilage destruction[16]. Many depletion studies additionally deplete monocytes, and thus likely reduce osteoclast numbers and bone resorption. Subchondral bone remodeling and innate immune infiltration are highest during early OA[17]. This body of data suggests that monocytes and their downstream progeny (i.e., osteoclasts and synovial macrophages) drive OA disease pathogenesis. Thus we believe reducing monocyte activation and recruitment to the joint may be of therapeutic benefit to individuals with OA.

Monocytes can be subdivided into three subsets based on their expression of two surface markers (CD14 and CD16): classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$). Intermediate monocytes, also referred to as inflammatory monocytes, produce more pro-inflammatory cytokines on a per cell basis and are recruited to sites of inflammation. These cells have been used as biomarkers in cancer, atherosclerosis and colitis[18]. However there is a paucity of data on circulating monocyte numbers, phenotype or cytokine production in individuals with OA compared to healthy controls.

Hematopoietic stem cells (HSCs) are finely tuned to even subtle changes in systemic levels of inflammation. Slight increases in inflammation trigger HSCs to produce monocytes to provide a rapid innate response to infection or injury[19]. People with OA have elevated circulating inflammatory mediators including c-reactive protein (CRP), TNF, IL-6[20–22]. The magnitude of systemic inflammation strongly correlates with OA disease severity and progression[21,22]. We have shown that age-associated increases in systemic inflammation can remodel the monocyte compartment by increasing their output and activating them to produce higher levels of pro-inflammatory cytokines[23]. There is some evidence of innate immune activation in OA, as circulating monocytes from individuals with OA have greater osteoclastogenic potential and resorptive activity compared to healthy controls[24]. Thus we hypothesized that low-grade inflammation in OA, similar to what occurs with age or other chronic inflammatory conditions, activates circulating monocytes, increasing their surface expression of trafficking and activation markers and their production of pro-inflammatory cytokines.

Materials and methods

Participants and ethics

The study population included 22 community-dwelling women with clinical (symptomatic) knee OA over the age of 50 who were enrolled in a larger randomized controlled trial (clinical trial number NCT02370667). Women were included if they answered yes to three or more of the following American College of Rheumatology clinical criteria[25]: (a) Have knee pain in most days of the week (b) Have fewer than 30 min of morning stiffness (c) Have crepitus with active range of motion (d) Have a bony enlargement (e) Have bony tenderness with palpation (f) Have signs of inflammation and/or have been diagnosed with radiographic osteoarthritis. Participants were excluded if they had any other forms of arthritis (e.g., rheumatoid, psoriatic), active non-arthritic knee disease (e.g., bursitis), patellofemoral symptoms, knee surgery (e.g., high tibial osteotomy, joint replacement, ligament repair), history of an osteoporotic fracture, planned surgery in the next 6 months, lower extremity trauma in the last 3 months, an unstable heart condition or neurological conditions (e.g., stroke), recent or current exposure to radiation, or other health conditions that might be exacerbated by the protocol. Following consent, weight-bearing radiographs of the affected knee in a fixed flexion posture were obtained to confirm disease (i.e., Kellgren–Lawrence grade ≥2). Each OA participant was age-matched to a control, of the same sex, within 3–4 years of their age. Control participants without OA were recruited from the community and submitted to the same exclusion criteria listed above and did not answer yes to any of the American College of Rheumatology clinical criteria cited above.

Self-reported knee pain intensity was reported using the Knee injury and Osteoarthritis Outcome Score (KOOS) pain subscale, which is a five-point Likert scale (0 = no pain, 4 = intense pain) rating pain during nine different activities[26]. The score was normalized to a score out of 100, where higher scores represent less pain[27]. The 6 min walk test (6MWT) was used to capture mobility, by measuring the furthest distance a participant can walk in 6 min in a well-lit, rectangular hallway[28]. This study was approved by the Hamilton Integrated Research Ethics Board. All participants provided written, informed consent (Controls: REB# 1949; OA

Table I
Participant description

	Control		OA		*t*-test *P*-value
	n	Mean (Min–Max)	*n*	Mean (Min–Max)	
Age	15	65.40 (51–84)	22	66.9 (51–84)	0.683
BMI(kg/m^2)	11	27.35 (19.16–36.35)	22	30.00 (20.7–44.6)	0.211

OA patient characteristics	*n*	Mean (SD) or Median (Min–Max)*
Radiographic Score	20	3 (1–4)
KOOS pain score	22	54.14 (15.03)
KOOS Symptom	22	51 (15.50)
KOOS ADL	22	61.05 (17.52)
KOOS Sport/Rec	22	30 (0–75)
KOOS QOL	22	31 (13–75)
6 min walk test distance (m)	22	461.92 (65.41)

[*] Median shown where variable does not follow a Gaussian distribution.

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Table II
Soluble inflammatory mediators in serum

	Control		OA		Mann–Whitney *U* test *P* value
	n	Median (Min–Max)	*n*	Median (Min–Max)	
IL-10 (pg/mL)	8	3.49 (0.18–21.57)	19	7.63 (0.09–31.08)	0.0697
IL-6 (pg/mL)	8	1.56 (0.49–2.38)	20	1.67 (0.74–4.63)	0.2425
TNF (pg/mL)	8	7.98 (2.89–10.07)	20	8.27 (4.33–16.78)	0.4448
CRP (mg/L)	5	3.468 (2.794–6.599)	18	5.552 (2.971–12.12)	**0.0352**

Bold value indicates statistical significance.

participants: REB #15-021). Characteristics of the OA and control participants are summarized in Table I.

Immunophenotyping

Venous blood was collected in heparinized tubes and 100 μL was stained for 30 min with monoclonal antibodies of the following specificities: CD45-BV510, CCR2-PE, CD15-BV610, CD14-BV421, CD56-AF700 (BioLegend); CD16-PE-Cy7, HLA-DR-PerCP-Cy5.5, CD19-AF700 (eBioscience); CD11b-APC, CD3-AF700 (BD Pharmingen) CX3CR1-FITC (MBL Life Sciences). Samples were then incubated with 1X Fix/Lyse Buffer (eBioscience) for 10 min with frequent inversion and centrifuged at room temperature, washed and resuspended in FACS Wash (5 mM EDTA, 0.5% BSA in PBS). Samples were then run on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo 10 software (Treestar). Total cell counts were determined with 123 count eBeads (eBioscience). For gating strategy, see Supplemental Figures 2 and 3. Fluorescence intensities of the proteins were detected by flow cytometry and follow a logarithmic-normal distribution in the cell populations analyzed, thus geometric means were calculated using the FlowJo 10 software to quantify the mean fluorescence intensity (intra-assay CV: 2%

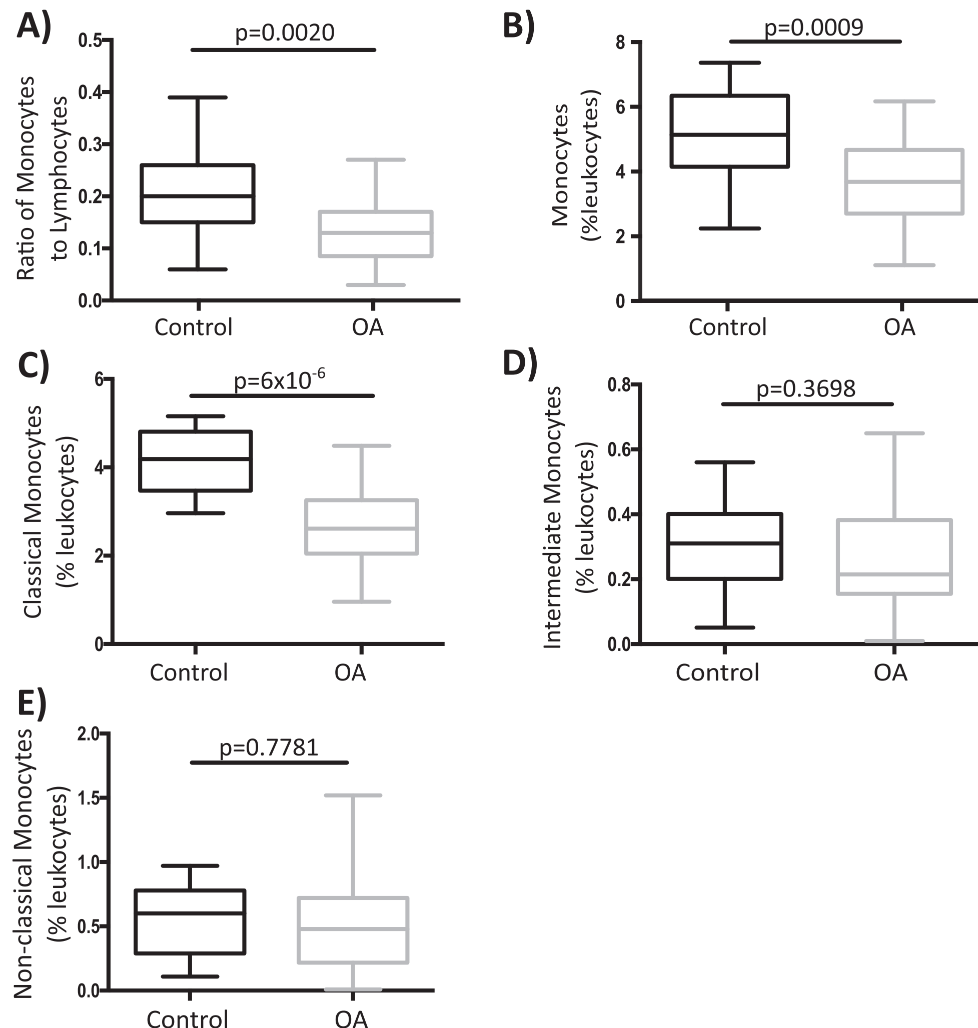

Fig. 1. Women with OA have altered proportions of circulating leukocyte compared to healthy controls. A) The proportion of circulating monocytes to leukocytes was lower in individuals with OA ($n = 15$) compared to controls ($n = 22$). B–E) Total monocytes and classical monocytes as a percentage of circulating leukocytes, were lower in individuals with OA, but there was no difference in intermediate or non-classical monocytes. Two-tailed Mann–Whitney test used except in A & C) where an Student's *t*-test was applied.

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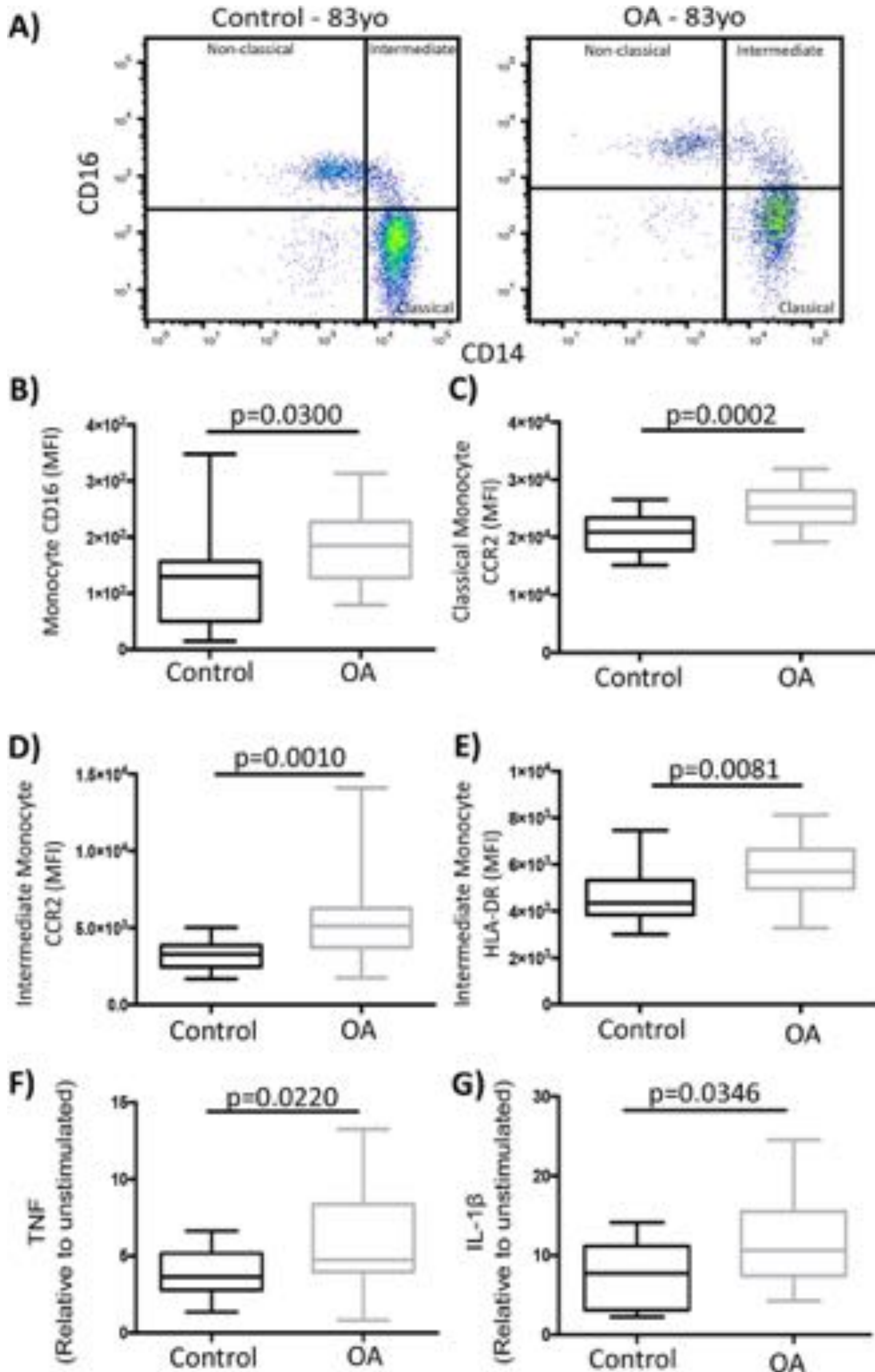

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inter-assay CV: 12%). Background fluorescence intensity was measured in isotype controls and was subtracted from antibody specific fluorescence intensity.

Intracellular cytokine staining

Intracellular cytokine staining was performed on cryopreserved peripheral blood mononuclear cells (PBMCs) isolated from whole blood by Ficoll density-gradient centrifugation and Leucosep tubes (Grenier Bio-one) stored at −150°C in human AB serum (Lonza) with 10% dimethyl sulfoxide until use. Briefly, PBMCs from participants were later cultured in two conditions (1×10^6 PBMCs per condition) for 4 h at 37°C/5% CO_2: complete media (RPMI, Invitrogen, ON, CA) supplemented with 10% FBS and 1X Protein Transport Inhibitor (eBioscience) and complete media with 50 ng/mL lipopolysaccharide (LPS) (Invivogen). Surface staining was performed for 30 min at room temperature with the conjugated antibodies CD14-PE-Dazzle, CD3-APC-Cy7 fixed with 1X Fix/Lyse buffer (eBioscience) for 10 min. Cells were permeabilized with 1X Permeabilization Buffer (eBioscience) at room temperature for 30 min. Cells were then stained at room temperature for 30 min with the following antibodies diluted in 1X Permeabilization Buffer: TNF-Alexa Fluor 700, IL-1β-PE (eBioscience), IL-6-FITC (BioLegend). Cells were washed and resuspended in FACS Wash prior to analysis. Monocytes were defined by size, granularity and expression of CD14(+) and CD3(−). Flow cytometry analysis was performed as described above.

Serum cytokines

Venous blood was collected and centrifuged at 1.5 × g for 10 min at 25°C and serum was stored at −140°C until processed. Serum cytokines IL-6, IL-10 and TNF were measured using Milliplex MAP 60K cytokine panel as per manufacturer's protocol (EMD Millipore, HSTCMAG-28SK; Intra-assay CV: <5%, Inter-assay CV: <15%). CRP was analyzed by an in-house ELISA. In short, the wells were coated with capture antibody (1 ug/mL, ab8279, Abcam) overnight, blocked with blocking buffer (PBS with 10% FBS) for 2 h. Samples were diluted 1/10,000 and added to the wells, incubated for 1 h before being washed with washing buffer (PBS with 0.05% Tween-20). Plate was incubated for 1 h with HRP-conjugated detection antibody (1ug/mL, ab24462, Abcam) diluted in blocking buffer for 1 h before being washed. Plates were developed with TMB as per manufacturer's protocol. The serum values were measured in duplicate and an average value was reported (intra-assay CV: 1.5%, inter-assay CV: 7.2%, 90.1% recovery).

Statistical analysis

The Student *t* test was used to evaluate differences between OA and Controls for normally distributed variables, tested using the Kolmogorov–Smirnov Test. The Mann–Whitney *U* test was used to evaluate the difference between OA and controls for non-normally distributed variables. Correlation analyses between monocyte activation markers, inflammation, age, BMI, pain and mobility were performed using Spearman's rank correlation rho or Pearson correlation depending on normality of variables. Statistical analysis was performed using SPSS (Version 21; IBM, Armonk, NY, USA), Prism (Version 6; GraphPad, San Diego, CA, USA).

Results

Women with OA have higher serum CRP and altered circulating leukocyte proportions compared to healthy controls

Serum CRP was elevated in the OA group (median: 5.55 mg/L) compared to controls (median: 3.468 mg/L) (Cohen's *d* [95%CI]: 0.925 [−0.101–1.952], $P = 0.0352$, Table II); however, levels of serum cytokines did not differ between the two groups (Table II). To evaluate differences in cellular inflammation, we quantitated leukocyte populations using flow cytometry. The ratio of circulating monocytes to lymphocytes was lower in women with OA compared to healthy controls (mean difference [95%CI]: −0.07533 [−0.1211 to −0.02958], Cohen's *d* [95%CI]: −1.119 [−1.823 to −0.415], $P = 0.002$; Fig. 1(A)). This was due to both a lower number of circulating monocytes and a higher number of circulating lymphocytes in the OA group (Fig. 1; Supplementary Table 1). The lower number of circulating monocytes was primarily driven by the decrease in classical monocytes (mean difference [95%CI]: −1.52 [−2.099 to −0.9477] % leukocytes, Cohen's *d* [95%CI]: −1.84 [−2.634 to −1.047], $P = 6 \times 10^{-6}$; Fig. 1(B)–(E)).

Circulating monocytes express higher levels of activation markers in OA

We measured surface expression of monocyte trafficking (CCR2) and activation (CD16 and HLA-DR) markers with flow cytometry by analyzing mean fluorescence intensity (MFI). The median expression of CD16 on all monocytes was higher in OA group compared to controls (median difference [95%CI]: 55.08 [7.790–105.8] MFI, Cohen's *d* [95%CI]: 0.712 [0.029–1.395], $P = 0.03$, Fig. 2(A),(B)). This occurred on all monocytes, rather than a specific subset, as can be seen by the shift of the total monocyte population in a representative flow cytometry plot of an individual with OA compared to their age-matched control [Fig. 2(A)]. Expression of CCR2 was elevated in classical and intermediate monocytes in women with OA compared to controls (mean difference [95%CI]: 4863 [2451–7275]MFI, Cohen's *d* [95%CI]: 1.371 [0.681–2.12], $P = 0.0002$; median difference [95%CI]: 1830 [885–3045]MFI, Cohen's *d* [95%CI]: 1.049 [0.344–1.755], $P = 0.001$; Fig. 2(C)–(D)), as was the expression of HLA-DR on intermediate monocytes (mean difference [95%CI]: 1350 [324–1965] MFI, Cohen's *d* [95%CI]: 0.887 [0.194–1.581], $P = 0.0081$; Fig. 2(E)).

Monocytes from women with OA produce more inflammatory cytokines following stimulation

It has been reported that peripheral blood mononuclear cells (e.g., lymphocytes and monocytes) from individuals with OA produce higher levels of IL-6, TNF and IL-1β compared to healthy controls, however the particular cell type producing these cytokines was not identified[29]. We performed intracellular staining to identify whether monocytes were responsible for the increased production of IL-6, TNF and IL-1β, in unstimulated conditions and following stimulation with lipopolysaccharide (LPS). LPS, a TLR-4 agonist, was chosen as many of the DAMPs released in OA have been shown to activate macrophages through TLR-4[12]. There was no difference in basal production of TNF, IL-1β or IL-6 by monocytes (Supplemental Fig. 1), however monocytes from women with OA had a higher induction of TNF and IL-1β production following

Fig. 2. Circulating Monocytes are activated in women with OA compared to controls. Age-matched control and OA participant flow cytometry plots show a global increase in CD16 expression on monocytes in the individual with OA. (B). Expression of CCR2 on classical and intermediate monocytes (C,D) and HLA-DR on intermediate monocytes were higher in women with OA (E). Monocyte production of tumor necrosis factor (TNF) and IL-1β following stimulation with LPS relative to unstimulated was greater in the OA group (F,G). Two-tailed Mann–Whitney test used except in C where an Student's *t*-test was applied. $n = 15, 22$ (A–E); $n = 13, 17$ (F–G); MFI-Geometric Mean fluorescence intensity.

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stimulation with LPS compared to healthy controls (median difference [95%CI]: 1.102 [0.222–3.712] TNF production (relative to unstimulated), Cohen's *d* [95%CI]: 0.843 [0.09–1.596], $P = 0.022$; median difference [95%CI]: 2.925 [0.1064–8.143] IL-1β production (relative to unstimulated), Cohen's *d* [95%CI]: 0.834 [0.081–1.586], $P = 0.0346$; Fig. 2(F)–(G)). However there was no difference in the production of IL-6 basally or in response LPS (data not shown).

Age and inflammation alter monocyte characteristics and function. We performed correlation analysis to determine if these factors were related to monocyte activation in OA. Age did not correlate with any of the monocyte activation markers (Supplementary Table 2), however TNF was positively associated with the expression of CCR2 on intermediate monocytes (Spearman's rho [95%CI] = 0.5092 [0.07201–0.7823], $P = 0.0218$, $n = 20$; Fig. 3(A)). Additionally, BMI was positively associated with the expression of CCR2 on classical monocytes (Pearson coefficient [95%CI] = 0.5064 [0.1077–0.7648], $P = 0.0162$, $n = 22$; Fig. 3(B)).

We next determined whether the monocyte activation markers correlated with pain or mobility. Intermediate monocyte CCR2 expression and TNF were both inversely correlated with the KOOS Pain Score (Spearman's rho [95%CI] = −0.4884 [−0.7603 to −0.07077], $P = 0.0211$, $n = 22$; Pearson coefficient [95% CI] = −0.4926 [−0.7678 to −0.6391], $P = 0.0273$, $n = 20$; Fig. 3(C)–(D)). Mobility, measured by the 6MWT distance, did not correlate with monocyte activation markers (Supplementary Table 2), but inversely correlated with BMI (Pearson coefficient [95%CI] = −0.511 [−0.7728 to −0.2007], $P = 0.0151$, $n = 22$; Fig. 3(E)).

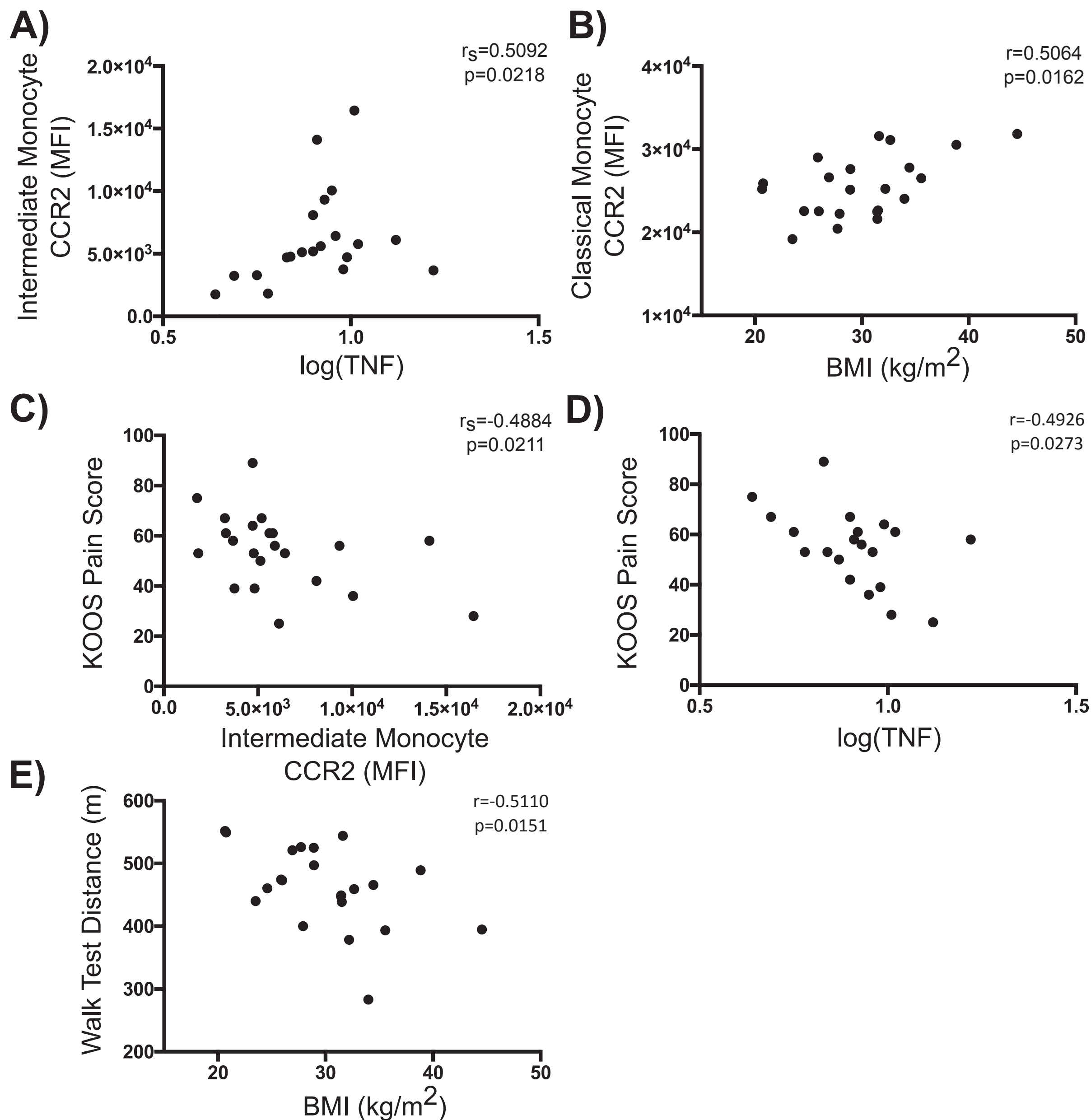

Fig. 3. Monocyte activation correlates with inflammation, BMI and pain in OA. A) Log transformed serum TNF(pg/mL) correlates with intermediate monocyte CCR2 expression ($n = 20$). B) BMI correlates with classical monocyte CCR2 expression ($n = 22$). Intermediate monocyte CCR2 expression (C, $n = 22$) and log transformed serum TNF(pg/mL) (D, $n = 20$) are inversely correlated to the Knee injury and Osteoarthritis Outcome Score (KOOS) Pain Score. E) BMI is inversely correlated to the 6 min walk test distance ($n = 22$). Spearman's correlation coefficient was reported for A & C, while Pearson's correlation coefficient was reported for B, D and E. BMI-Body Mass Index; MFI-Geometric Mean fluorescence intensity.

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Discussion

Soluble inflammation has been well characterized in both the synovial fluid and serum of people with OA, however the changes to monocyte populations in OA have not. Consistent with previous reports[30], we found elevated levels of circulating CRP in women with OA compared to their age- and sex-matched controls. The median and maximum values of other cytokines measured (IL-6, TNF and IL-10) were elevated in the OA group compared to controls, however due to the low sample size and large variation, they were not statistically different. To our knowledge this is the first report demonstrating lower numbers of circulating monocytes relative to leukocytes in individuals with OA compared to healthy controls. Our quantitation of monocytes (mean (SD): 3.02 (0.39) $\times$ 10^5 cells/mL; 3.66 (0.28)%leukocytes) was similar to a previous report (mean [SD]: 3.79 [0.37] $\times$ 10^5 cells/mL; 3.7 (0.4)%leukocytes[31]), however this group did not include healthy controls in order to demonstrate that these counts were lower in OA (mean [SD]: 4.97 (1.02) $\times$ 10^5 cells/mL; 5.25 (0.34)%leukocytes; Fig. 1; Supplementary Table 1). This decrease in circulating monocytes may be due to increased infiltration into synovial tissue since a key trafficking receptor, CCR2, was elevated on classical and inflammatory monocytes from individuals with OA. The decrease in circulating monocytes was most pronounced in the classical monocytes, which express the highest levels of CCR2 of the three monocyte subsets. The ligand for CCR2, CCL2/MCP-1, is elevated in the intimal lining of individuals with joint disease[32]. Furthermore, CCR2 has been shown to be critical to monocyte trafficking and infiltration of the synovium as CCR2 knockout animals have less synovial macrophages and experience less cartilage damage and osteophyte formation[33]. Whether the elevated expression of CCR2 on monocytes of individuals with OA increases the capacity to traffic to and invade synovial tissues warrants further investigation.

In addition to the increased invasive potential, circulating monocytes from individuals with OA were more activated. We found elevated expression of CD16 and HLA-DR on monocytes and intermediate monocytes, respectively, in women with OA. The expression of CD16 increases during the differentiation of monocytes into osteoclasts[7], which aligns with the increased resorptive activity of monocyte-derived osteoclasts from individuals with OA[24]. Additionally, the increased expression of CD16 can increase the sensitivity of monocytes to stimulation by immune complexes, which are found at elevated concentrations in OA joints and in circulation[34]. The expression of HLA-DR is elevated on inflammatory monocytes, which allows them to present antigen and activate T cells more efficiently. The elevated expression of CD16 and HLA-DR on monocytes in individuals with OA did not correlate with serum cytokines or BMI (data not shown). This suggests these differences are unique to the OA disease process and independent of systemic inflammation and BMI.

We have previously shown that CCR2 levels on monocytes are increased during chronic inflammation and specifically in the presence of TNF[35]. Levels of serum TNF in individuals with OA correlated with increased expression of CCR2 on intermediate monocytes. Furthermore, serum TNF and intermediate monocyte CCR2 expression correlated with increased pain in women with knee OA. This supports previous findings that serum and synovial fluid levels of the CCR2 ligand, CCL2/MCP-1, correlate with pain and physical disability in OA[36]. Not only will monocytes expressing CCR2 traffic to areas with high CCL2/MCP-1 levels, but they are also major producers of CCL2 and may thus increase cellular recruitment and pain. Since pain is one of the most significant causes of immobility and health care utilization in people with OA, reducing monocyte recruitment to the joint may be of therapeutic benefit.

We found monocytes in individuals with OA produce more cytokines when they encounter a TLR4 agonist (e.g., LPS). Although basal production of IL-1β and TNF by monocytes were not different between the OA and control group, the induction of both cytokines following stimulation with LPS was greater in women with OA. This indicates that monocytes in OA may produce more inflammatory cytokines on a per cell basis following stimulation with DAMPs, which are found in high abundance in the synovial fluid[12]. Together these findings confirm circulating monocytes are more activated and have greater inflammatory potential in individuals with OA compared to controls.

Obesity increases the risk of developing OA[37,38]. This association is not solely due to increased mechanical load, as the risk also exists for non-weight-bearing joints[39]. Adipose tissue and altered lipid metabolism contribute to increased systemic inflammation[40]. We found BMI was positively correlated with the expression of CCR2 on classical monocytes, which is consistent with previous data showing BMI and fat mass correlate with increased expression of CCR2 on monocytes[41]. Thus, adiposity may accelerate the CCR2-mediated infiltration of monocytes to the synovium where they contribute to pathogenesis (i.e., pain, cartilage erosion). In animal models of OA, blockade of CCL2/CCR2 leads to decreased inflammation, macrophage accumulation in the joint and cartilage damage[33]. This, in conjunction with controlling weight and adiposity, may be able to slow the progression of OA.

Although our study is limited by a small sample size and cross-sectional design, it is the first study, to our knowledge, to specifically characterize changes in circulating monocytes in individuals with OA compared to healthy controls. We have shown that monocytes in OA display an activated phenotype with increased expression of activation markers as well as increased production of pro-inflammatory cytokines. Monocyte activation may contribute to the development of multiple comorbidities that commonly co-exist with OA, such as depression, obesity, metabolic syndrome and dyslipidemia[4,39,42]. Activation and homing of inflammatory monocytes has been associated with many chronic inflammatory conditions including dementia, atherosclerosis and type II diabetes[18,43]. As in OA, these conditions are characterized by slight but measurable changes in soluble and cellular inflammation and similar changes in leukocyte phenotype and function[23]. Since having one chronic inflammatory condition is associated with a higher chance of developing multiple co-morbidities[44], it is believed that there are common immunological changes that predispose to multiple diseases. As an example, in mice, OA increases neuroinflammation and accelerates Alzheimer's disease pathology[45]. Changes in monocytes contribute to the development of chronic inflammatory conditions[46]. Monocyte trafficking mediated by CCR2/MCP-1 is essential for the development of atherosclerotic plaques[47] and has been shown to contribute to inflammation in the brain[48] and adipose tissue[49]. Further studies are warranted to explore the contribution of monocyte activation and trafficking to the risk of developing comorbidities in OA. Therapeutically targeting monocytes in OA may slow disease progression or decrease the risk of other chronic inflammatory diseases with monocyte involvement.

Contributions

All authors were involved in conception and design. Participants with OA were recruited by SK and MRM. DL performed experiments, DM, DMEB, and MRM analyzed and interpreted data, and drafted the article. DMEB and MRM obtained funding.

Competing interest statement

The authors do not have competing interests to declare.

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Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.joca.2017.10.018.

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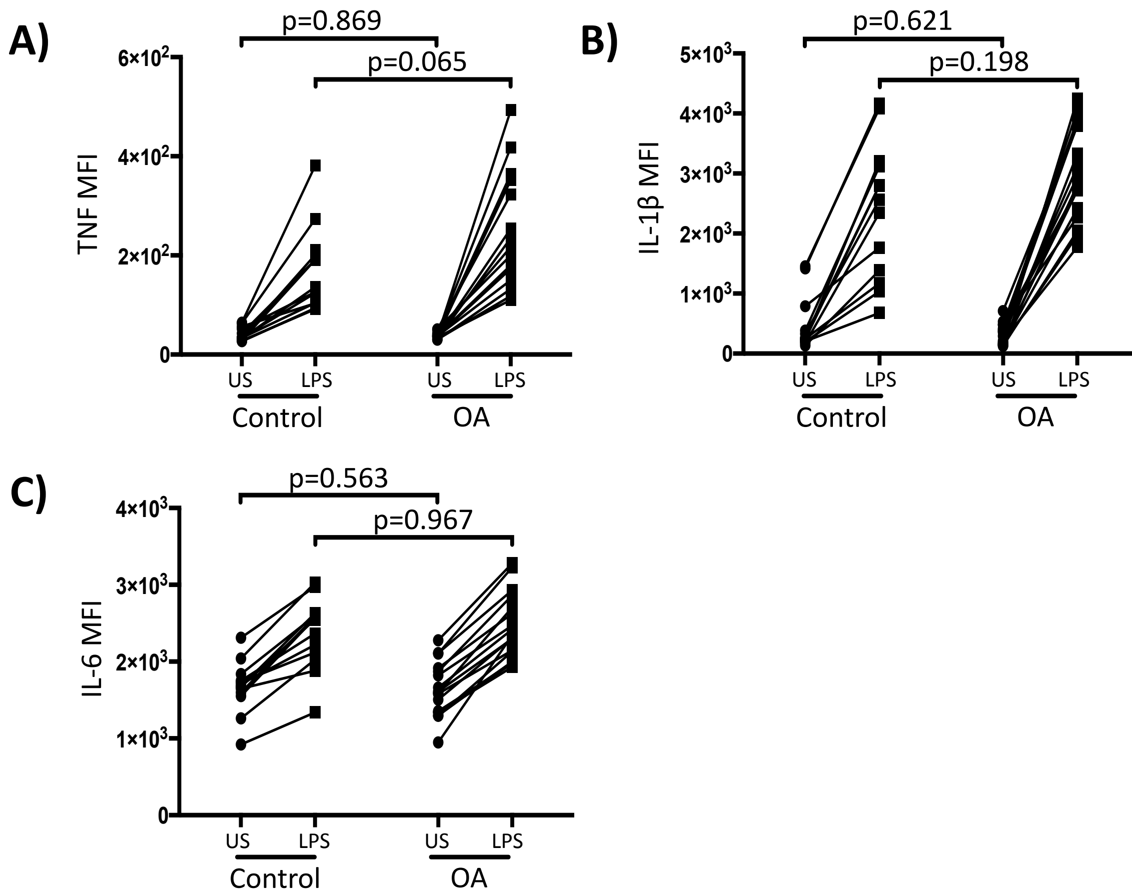
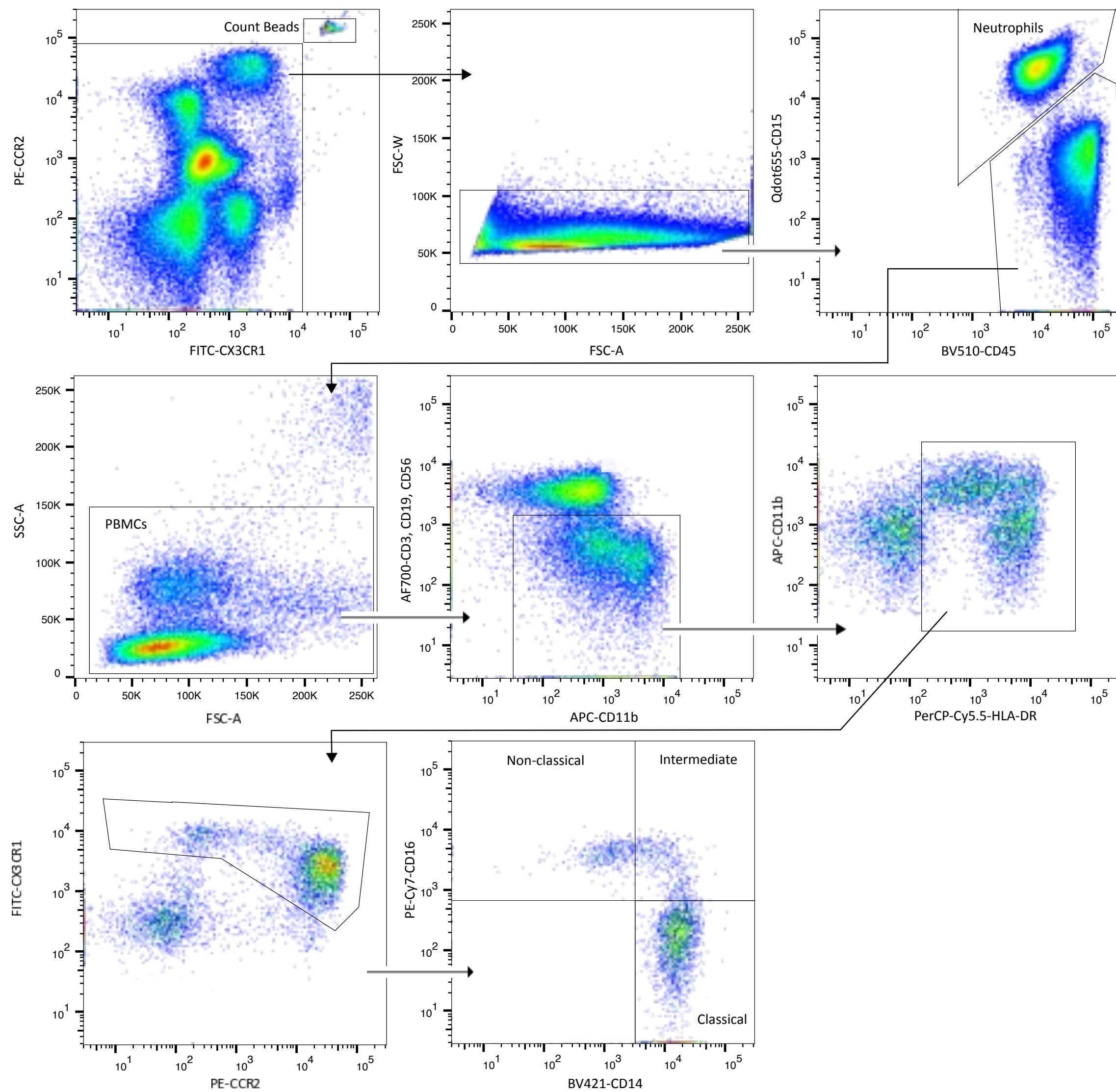
Supplementary Figure 1. Monocyte intracellular cytokine production. Monocyte production of TNF (A), IL-1β (B) and IL-6 (C) was measured by flow cytometry using intracellular cytokine staining. Production of all cytokines per cell (measured by MFI) were not different between Control and OA basally (US) or following stimulation with LPS. Two-tailed Mann–Whitney test used; (A–C) n = 13, 17 . US – unstimulated; LPS – lipopolysaccharide; MFI-Geometric Mean fluorescence intensity.



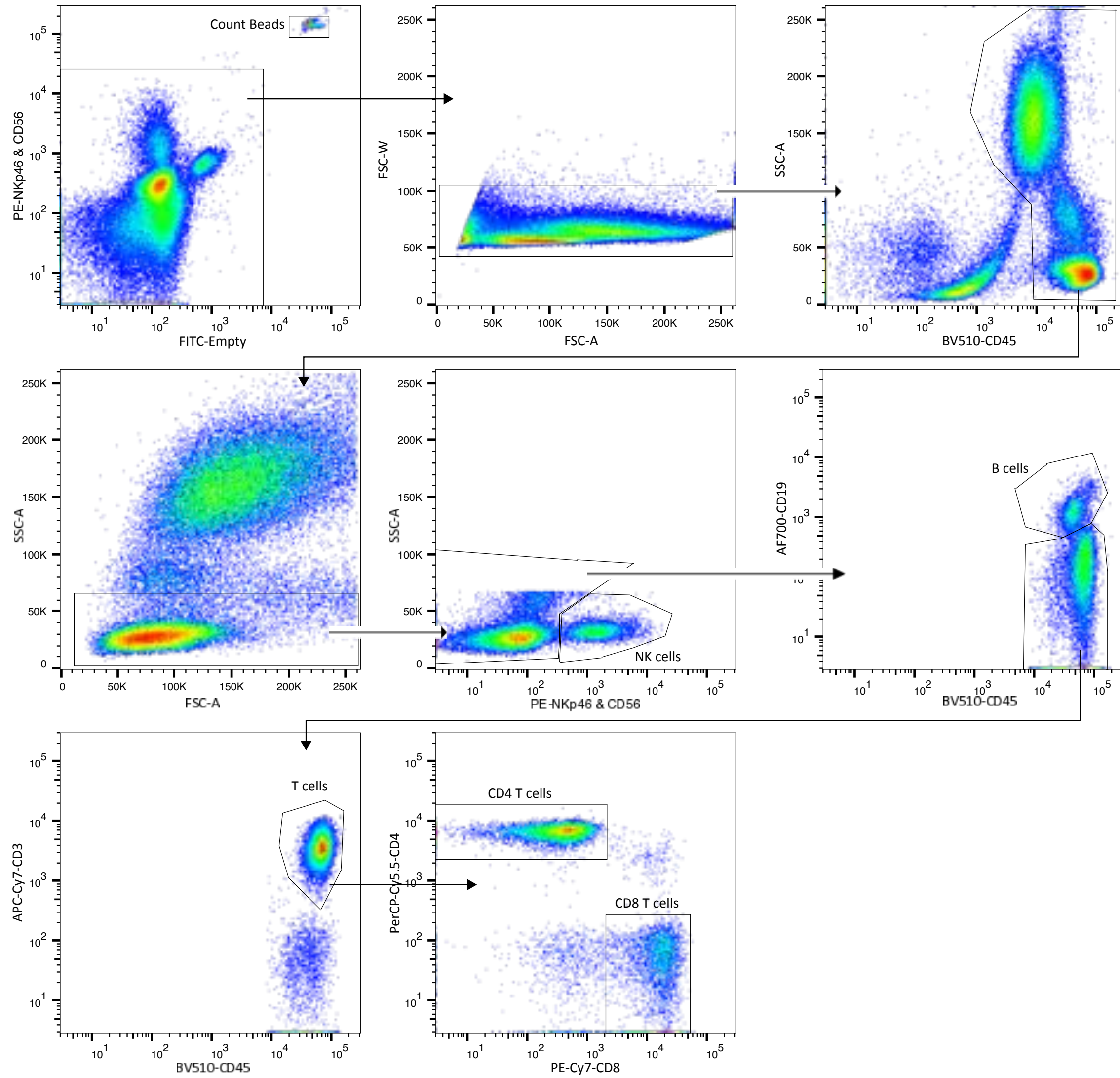
Loukov, D., Karampatos, S., Maly, M. R. & Bowdish, D. M. E. Monocyte activation is elevated in women with knee-osteoarthritis and associated with inflammation, BMI and pain. Osteoarthr. Cartil. doi:10.1016/j.joca.2017.10.018. Copyright © 2017 by Osteoarthritis Research Society International, published by Elsevier Ltd.

Supplementary Figure 2. Whole blood monocyte gating strategy. Analysis of flow cytometry data on whole blood was done using FlowJo9. Following exclusion of count beads, singlet cells are selected and CD45 is used to select leukocytes. Neutrophils are selected based on their expression of CD15. Lymphocytes are eliminated (NK cells: $CD56^{+}$, B cells: $CD19^{+}$ and T cells: $CD3^{+}$). Monocytes are selected by their expression of CD11b, HLA-DR, CX_3CR_1 and CCR2 and are further subdivided into their 3 subsets based on their expression of CD14 and CD16.



Loukov, D., Karampatos, S., Maly, M. R. & Bowdish, D. M. E. Monocyte activation is elevated in women with knee-osteoarthritis and associated with inflammation, BMI and pain. Osteoarthr. Cartil. doi:10.1016/j.joca.2017.10.018. Copyright © 2017 by Osteoarthritis Research Society International, published by Elsevier Ltd.

Supplementary Figure 3. Whole blood lymphocyte gating strategy. Analysis of flow cytometry data on whole blood was done using FlowJo9. Following exclusion of count beads, singlet cells are selected and CD45 is used to select leukocytes. Lymphocytes are selected based on size(FSC) and granularity(SSC) and subdivided into NK cells ($CD56^+Nkp46^+$), B cells ($CD19^+$) and T cells($CD3^+$). T cells are then divided based on their expression of CD4 and CD8.



Loukov, D., Karampatos, S., Maly, M. R. & Bowdish, D. M. E. Monocyte activation is elevated in women with knee-osteoarthritis and associated with inflammation, BMI and pain. Osteoarthr. Cartil. doi:10.1016/j.joca.2017.10.018. Copyright © 2017 by Osteoarthritis Research Society International, published by Elsevier Ltd.

CHAPTER 5

Myeloid Development:

Monocyte and Macrophage Maturity and Function

Introduction:

As mentioned in the introduction, HSCs are quiescent in the steady state and only start proliferating and differentiating in response to stress or inflammation[437]. Inflammation can act on the stromal compartment to trigger mobilization of HSCs, by downregulating adhesion molecules, decreasing chemokine production, and secreting hematopoiesis-supporting factors[438]. Cytokines like IFNγ and TNF, as well as TLR-ligands, have been shown to trigger proliferation and drive lineage commitment of HSCs[246]. This is also referred to as emergency hematopoiesis, which favours the production of myeloid cells over lymphoid cells[246]. Chronically elevated inflammation has been shown to lead to alterations in hematopoiesis[439–441]. TNF is chronically elevated in diseases where there are significant changes to myelopoiesis (e.g. MDS[442] and RA[390]); however, the mechanisms by which it influences hematopoiesis are still unclear.

The role of TNF in myeloid development

There have been many studies on the effect of TNF on HSCs and hematopoiesis with conflicting results. Some have shown that TNF suppresses HSCs. For example, low dose TNF exposure inhibits HSC cycling but maintains the viability and the mitotic response in human marrow explants when co-administered with other hematopoietic factors (e.g. FL, IL-3, IL-6 and GM-CSF)[443]. Another group demonstrated that TNFR1$^{-/-}$ and TNFR2$^{-/-}$ progenitors show no significant differences in proliferation in the steady state; however, in competitive and serial bone marrow transplantation models, TNFR double KO bone marrow proliferates at a greater rate than WT bone marrow, indicating that TNF suppresses the proliferative capacity of HSCs[444]. This phenomenon is not specific to either of the two TNFRs and is not due to TNF-mediated apoptosis, but is a result of the effect of TNF on cycling rather than quiescent HSCs. Consistent with this, it has been observed that TNF signalling through TNFRI compromises the reconstitution capacity of cycling HSCs *in vivo*, independent of apoptosis and the cell cycle, but rather by promoting myeloid differentiation, which was demonstrated *in vitro* [445]. In contrast to these findings, TNFR1-/- progenitors were shown to have impaired reconstitution capacity upon serial transplantation[446]. These differences were not due to changes in proliferative response and were proposed to be due to differential homing of HSCs to the bone marrow; however, in the steady state, the HSC compartment of TNFRI$^{-/-}$ mice was not different compared to WT until they were older than 6 months of age[446]. Since there is a concomitant increase in TNF and the

number of cycling HSCs with age, the group suggested that HSCs might be more sensitive to TNF with age. This is supported by findings that 20 ng/mL TNF maximally induces colony formation of HSCs, but does not at higher concentrations[447]. The same group showed that HSCs cultured in SCF and IL-7 preferentially form granulocytes, but following the addition of TNF, more than 95% of the resultant progeny cells are macrophages. Although the data on how TNF influences HSC self-renewal remains unclear, TNF has consistently been shown to drive differentiation of monocytes and macrophages over other lineages. In fact, TNF is expressed throughout macrophage differentiation, increasing proliferation in the presence of M-CSF[447]. It is possible that TNF differentially influences early hematopoietic progenitors compared to those committed to the myeloid lineage. Consistent with this, another study demonstrated that exogenous TNF inhibits proliferation of early progenitors, while endogenous TNF is instrumental in driving differentiation of macrophages *in vitro*[448]. As such, the dosage and the timing of exposure are likely critical to how TNF influences myeloid development. Our previous work demonstrates that age-associated TNF significantly increases circulating inflammatory monocytes in humans and in mice[39], but we do not know how TNF mediates this effect.

Hypothesis and Aims:

We hypothesize that the increased monopoiesis with age is a result of a chronic state of emergency hematopoiesis stimulated by TNF.

We aim to determine if age-associated TNF drives:

a) Increased myelopoiesis
b) Differential surface expression of myeloid maturity markers on myeloid progenitors
c) Differential expression of genes involved in myeloid development
d) Altered function in monocytes and macrophages

CHAPTER 5.1

Inflammation alters hematopoiesis:

TNF drives splenic monopoiesis in old mice

Preface:

This chapter contains a published manuscript from *The Journal of Leukocyte Biology**. We found that the splenomegaly in our old mice was a result of extramedullary hematopoiesis (EMH). EMH occurs in response to inflammatory insults like myocardial infarctions, as well as in bacterial infection, but it had not previously been described in the context of an aging host. In this paper we demonstrated that TNF increases the number of proliferating myeloid progenitors in the bone marrow and spleens of old mice, contributing to the increase in Ly6C^{high} monocytes with age. We were the first to show that TNF is involved in the increase in monopoiesis with age. There have been many studies to investigate intrinsic changes of hematopoietic progenitors with age; however, through bone marrow chimeras and TNF neutralization, we were able to demonstrate the importance of inflammation in the environment shaping hematopoiesis with age.

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JLB

Article

Tumor necrosis factor drives increased splenic monopoiesis in old mice

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ABSTRACT

Aging is accompanied by changes in hematopoiesis and consequently in leukocyte phenotype and function. Although age-related changes in bone marrow hematopoiesis are fairly well documented, changes in extramedullary hematopoiesis are less well described. We observed that 18–22-mo-old mice had larger spleens than young controls and found that the enlargement was caused by increased monopoiesis. Because extramedullary hematopoiesis is often driven by inflammation, we hypothesized that the chronic, low-level inflammation that occurs with age is a causal agent in splenomegaly. To test this theory, we compared the number of monocytes in 18-mo-old tumor necrosis factor–knockout mice, which are protected from age-associated inflammation, and found that they did not have increased extramedullary monopoiesis. To determine whether increased splenic monopoiesis is caused by intrinsic changes in the myeloid precursors that occur with age or by the aging microenvironment, we created heterochronic bone marrow chimeras. Increased splenic monopoiesis occurred in old recipient mice, regardless of the age of the donor mouse, but not in young recipient mice, demonstrating that these cells respond to signals from the microenvironment. These data suggest that decreasing the inflammatory microenvironment with age would be an effective strategy for reducing inflammatory diseases propagated by cells of myeloid lineage, which increase in number with age. *J. Leukoc. Biol.* 100: 000–000; 2016.

Introduction

The primary niche for hematopoiesis in the body is the bone marrow; however, EMH occurs at sites such as the spleen and liver [1, 2]. EMH is a result of HSPCs that migrate from the bone marrow and seed peripheral tissues, where they proliferate locally [3, 4]. This phenomenon occurs during normal fetal development in the liver and spleen [5] but is maintained at a lower level in adulthood [6]. HSPCs are highly responsive to cues in their microenvironment (e.g., hormones [7], metabolic products [8, 9], inflammatory mediators [10]). EMH occurs primarily in the spleen during infectious or inflammatory insult to the host and favors myelopoiesis over lymphopoiesis [11–13]. This process results in rapid production of myeloid cells, which have both antimicrobial and regenerative properties [1, 6]. For example, during infection with *Leishmania major* myeloid progenitors increase in the spleen [14], whereas during early infection with *Listeria monocytogenes* inflammatory monocytes ($CCR2^{high}Ly6C^{high}$) accumulate in the spleen [15–17]. In contrast, during inflammatory events such as myocardial infarction, splenic EMH produces a reservoir of monocytes that are rapidly mobilized in response to myocardial infarction and facilitate healing [12, 13]. Likewise, splenic progenitors transferred from normal mice differentiate into functional islet cells when adoptively transferred into nonobese diabetic mice with type I diabetes, rescuing this autoimmune condition when accompanied by immune therapy [18, 19].

Splenic EMH is not solely a response to inflammatory insult but has been shown to contribute to many inflammatory diseases including atherosclerosis, myelofibrosis, colitis, and cancer [12, 20–25]. Myeloid cells, particularly monocytes and macrophages of splenic origin, are major propagators of inflammatory diseases and have been investigated as potential biomarkers in these diseases [25, 26]. In mice there are 2 monocyte subsets: $Ly6C^{low}$ and $Ly6C^{high}$. The $Ly6C^{low}$ monocytes are long lived and patrol the vasculature and promote tissue healing by preferentially differentiating into macrophages with regenerative properties [27]. Conversely, $Ly6C^{high}$ monocytes are short lived and accumulate at inflammatory sites where they produce reactive oxygen species, nitric oxide, TNF, IL-1β, and type 1 IFN in response to bacterial and viral ligands. These "inflammatory" monocytes can differentiate into dendritic cells or macrophages that are capable of secreting high levels of proinflammatory cytokines TNF and IL-6 in tissue [27]. In fact, splenic EMH preferentially generates $Ly6C^{high}$ monocytes that localize to affected tissues and contribute to the pathologic course of diseases, including cancer and

Abbreviations: CMP = common myeloid progenitor, GMP = granulocyte–macrophage progenitor, EMH = extramedullary hematopoiesis, HSPC = hematopoietic stem/progenitor cell, KO = knockout, LSD = least-significant difference, WT = wild type

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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atherosclerosis by increased production of inflammatory mediators [20, 21].

The documented myeloid bias in splenic EMH is believed to be a result of the dominant presence of myeloid growth factors (e.g., GM-CSF) in extramedullary sites [28, 29]. However, proinflammatory cytokines, including type I IFN and TNF, have been shown to stimulate myelopoiesis [30–35]. In these studies, researchers have examined only short-term exposure, and the effects of sustained inflammation, as observed during chronic inflammatory disease, remain to be explored. Aging is an excellent system for the study of long-term exposure to inflammation, as it is often described as a chronic inflammatory condition [36]. With age, there is an increase in proinflammatory cytokines (i.e., TNF, IL-6, IL-1β, and IFN-γ) in circulation and in tissues, referred to as "inflammaging" [37]. There is a concomitant increase in age-associated diseases (i.e., cardiovascular disease, type II diabetes, cancer, Alzheimer's disease, and chronic lower respiratory disease) driven by increased inflammatory monocytes and macrophages [25, 38]. We have found that there is a TNF-dependent increase in circulating inflammatory monocytes in old mice [39]. Whether the age-associated increase in inflammation drives myelopoiesis and leads to the development of these chronic diseases or the inflammatory diseases themselves establish this chronically inflamed state remains to be explored.

We observed significant splenomegaly in our old mice (>18 mo old), which led us to hypothesize that is a result of EMH. We quantified the cell types, which were increased in the spleens of old mice and found that the increase in myeloid cells and proliferation in the splenic red pulp were consistent with EMH. Because high levels of TNF are associated with myelodysplastic syndrome [31, 40, 41], which closely mirrors the hematopoietic changes documented with age [42], we hypothesized that age-associated increases in TNF drive extramedullary myelopoiesis. To explore this possibility, we examined splenic hematopoiesis in TNF-knockout mice and found that prolonged exposure to TNF contributed to increased splenic myelopoiesis, which was reversible after 3 wk of anti-TNF treatment. Furthermore, using age-discordant bone marrow chimeras, we were able to determine that the increased splenic myelopoiesis was a result of the aging microenvironment.

MATERIALS AND METHODS

Animals

Female WT (C57BL/6) and TNF-KO (B6;129S-*Tnf*^{tm1Gkl}/J) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and aged in-house. All mice were housed in pathogen-free conditions in accordance with Institutional Animal Utilization protocols approved by McMaster University's Animal Research Ethics Board, as per the recommendations of the Canadian Council for Animal Care. All animals used were sex matched to their controls and were maintained in the same animal room. The pathogen-free status of mice within the aging colony was confirmed in mice through constitutive monitoring of sentinel mice and specific testing of fecal samples for common mouse pathogens.

Heterochronic bone marrow chimeras

Groups of 10 old (18 mo, C57Bl/6; The Jackson Laboratory) and 10 young (6–8 wk, B6.SJL-*Ptprca Pepcb*/BoyJ C57Bl/6; Jackson Laboratories) recipient mice were used. Of these, 5 per group received bone marrow from age-matched congenic CD45.1 donors as a control, and 5 per group received CD45.1 age-discordant marrow (e.g., old→young and young→old). Recipients were treated with prophylactic antibiotics for 3 d (e.g., sulfamethoxazole/trimethoprim; Septra, Pfizer, New York, NY, USA) before receiving 2 doses of 550 rads of γ irradiation (1100 rads total). Within 2 h of a second irradiation, mice were injected intravenously with 1×10^7 cells of T-cell-depleted donor bone marrow (CD90.2 Positive Selection Kit II; cat. no. 18951; StemCell, Vancouver, BC, Canada). Engraftment was determined by ratios of CD45.1 to CD45.2 determined by flow cytometry and was determined to be >95% in all recipients.

In vivo anti-TNF treatment

Adalimumab (Humira; Abbott Laboratories, Arlington Heights, IL, USA), a fully humanized anti-TNF antibody, was used, with human IgG (BioLegend, San Diego, CA, USA) administered to control animals in sterile saline. A dose of 50 ng/g of body weight was given intraperitoneally every other day for 3 wk to young and old WT mice. In total, each mouse received 10 doses of adalimumab or an equal dose of control hIgG.

Histology and immunohistochemistry

Histologic analysis was performed on samples from spleens of old WT and TNF KO mice and the young controls. Upon collection, spleens, livers, and kidneys were fixed in 10% neutral buffered formalin for a minimum of 24 h and were embedded in paraffin. Resultant tissue blocks were cut into 3 μm sections stained with H&E or Ki67 (1:100 dilution; Dako, Carpinteria, CA, USA) at the Core Histology Facility, McMaster Immunology Research Centre. For Ki67 staining, paraffin-embedded sections were deparaffinized, and heat-induced antigen retrieval was performed with Target Retrieval Solution (Dako). Detection was performed with an anti-rat antibody detection system (EnVision+; Dako). Images were acquired with a DM LB2 microscope at a magnification of ×10, ×20, and ×40 and a DFC 280 camera (both from Leica, Bannockburn, IL, USA). The magnification of the eyepiece remained at ×10, achieving total magnification of ×100, ×200, and ×400.

Flow cytometry

Single-cell suspensions were prepared by homogenizing spleen tissue through a 40 μm cell strainer or by passing bone marrow from the femurs through an 18-gauge needle. Cells were incubated for 1 h at room temperature with a direct application of antibodies. Monoclonal antibodies with the following specificities were used for monocyte subset differentiation: CCR2 (PE; R&D Systems, Minneapolis, MA, USA) and F4/80 (APC), Ly-6C (FITC), CD45 (eFluor 450), CD11b (PE-Cy7), MHC II (PerCP eFluor 710), and CD3, B220, NK1.1 (Alexa Fluor 700) , all from eBioscience (San Diego, CA, USA). Samples were then incubated with 1× Fix/Lyse Buffer (eBioscience) for 10 min with frequent inversion and centrifuged at room temperature. Samples were washed with sterile PBS and resuspended in 2% paraformaldehyde before flow cytometric analysis and were then run on an LSRII flow cytometer (BD Biosciences, Franklin Lake, NJ, USA) and analyzed with FlowJo 9 software (Treestar, Ashland, OR, USA). Total cell counts were determined with 123 count eBeads (eBioscience). For monocyte gating strategy see Puchta et al. [43]. Monoclonal antibodies with the following specificities were used for analysis of hematopoietic progenitors: Sca-1 (AF700), CD117 (FITC), CD34 (APC), CD16 (PerCP eFluor 710), and Gr-1, CD3, Ter119, B220, and CD11b (PE-Cy7), all from eBioscience, and Ki67 (BV605) from BioLegend. For progenitor gating strategy, see Challen et al. [44]. Intracellular staining for Ki67 was performed after permeabilization with Perm Buffer II (BD Biosciences), as per manufacturer's instructions. The positive gate for Ki67 was set based on isotype control.

mRNA expression

Single-cell suspensions of bone marrow and spleen were lysed with GENEzol reagent (Geneaid, New Taipei City, Taiwan), and RNA was extracted with the GENEzol TriRNA Pure Kit (Geneaid). The RNA was reverse transcribed to cDNA with M-MULV reverse transcriptase (New England Biolabs, Ipswich, MA, USA), and qPCR was performed with Go*Taq* qPCR Master Mix (Promega, Madison, WI, USA) and the ABI 7900HT Fast Real-time PCR System (Thermo Fisher Scientific Life Sciences, CA, USA), all according to the manufacturers'

instructions. Cycle threshold (Ct) values relative to the internal reference dye were transformed by a standard curve, followed by normalization to the housekeeping gene GAPDH. Normalized results are presented as relative to an internal calibrator sample. Primer sequences are as follows (F, forward; R, reverse): *gapdh*, F-TGTGTCCGTCGTGGATCTGA, R-CCTGCTTCACCACCTTCTTGA; *vcam-1*, F-TGACAAGTCCCCATCGTTGA, R-ACCTCGCGACGGCATAATT; *m-csf*, F-GGCTTGGCTTGGGATGATTCT, R-GAGGGTCTGGCAGGTACTC; *g-csf*, F-ATGGCTCAACTTTCTGCCCAG, R-CTGACAGTGACCAGGGGAAC; *gm-csf*, F-GGCCTTGGAAGCATGTAGAGG, R-GGAGAACTCGTTAGAGACGACTT.

Serum TNF

Serum TNF was measured by using Milliplex Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA, USA), according to the manufacturer's instructions.

Statistical analysis

Unless otherwise mentioned in the figure legend, statistical significance was determined by 2-way analysis of variance with Fisher's *post hoc* test and unpaired 2-tailed t tests. Statistical significance was defined as a $P < 0.05$. All data were analyzed with Prism (Version 6; GraphPad, San Diego, CA, USA).

RESULTS

Splenomegaly in old mice is a result of extramedullary hematopoiesis

One of the striking phenotypes in our old mice (>18 mo) is splenic enlargement/splenomegaly (**Fig. 1**A). We observed distinct changes to the microarchitecture and composition of the spleen in old mice compared to their young counterparts. The size and cellularity of the red pulp, which is the site of hematopoiesis of myeloid precursors, increased, as did the number of megakaryocytes in the subcapsular region of the red pulp, a hallmark of extramedullary hematopoiesis [45] (Fig. 1B). To confirm that active proliferation was occurring,

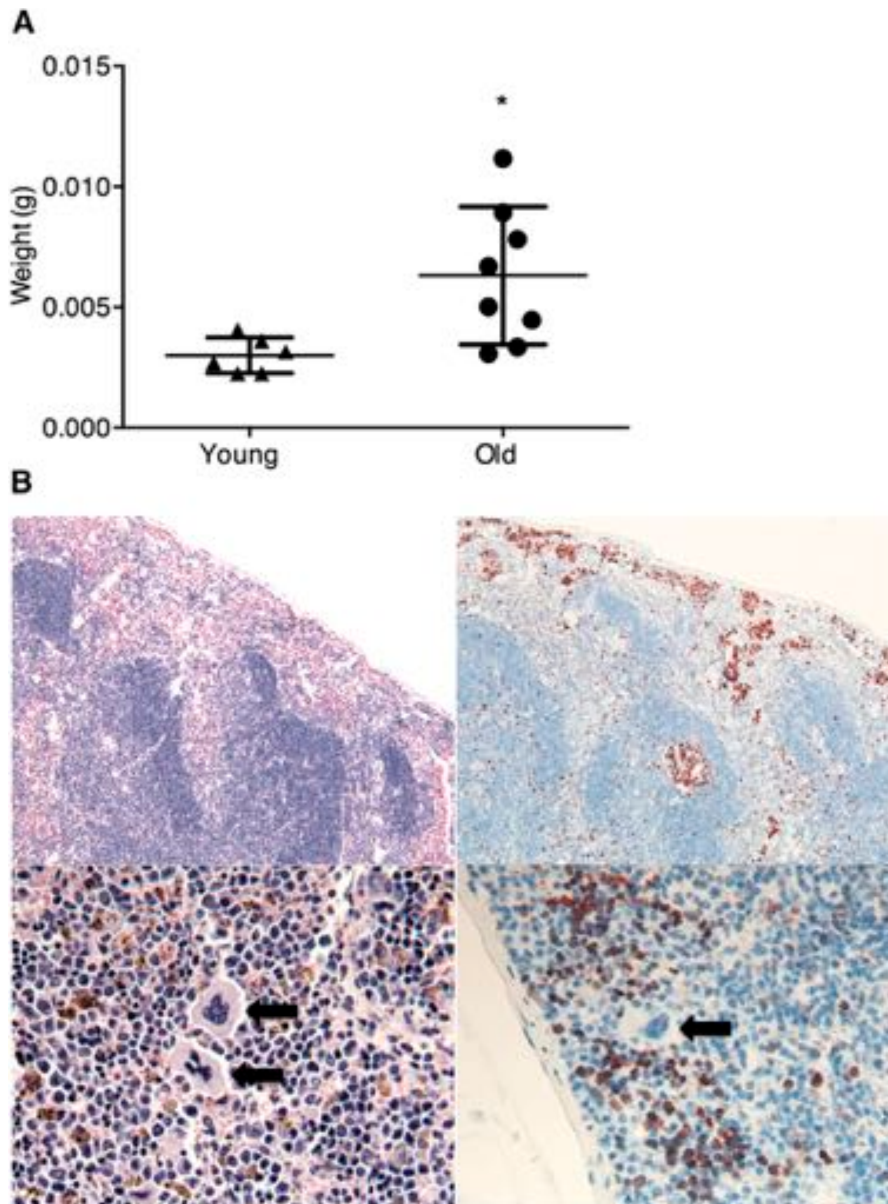

Figure 1. Extramedullary hematopoiesis occurs in spleens of old mice. (A) Old mice (18–22 mo) exhibited splenomegaly, as seen by increased splenic weight (normalized to body weight) compared to young mice (6–8 wk old). Means ± SEM (n = 6–8). (B) Representative histologic sections from an old mouse stained with H&E and Ki67 revealed markers of EMH (increased cellularity in the subcapsular red pulp, increased megakaryocytes, and Ki67 positive cells). Arrows: megakaryocytes. *$P < 0.05$, 2-tailed Mann-Whitney-Wilcoxon test.

immunohistochemistry for Ki67, a marker of cellular proliferation, was performed. Cells staining for Ki67 were localized in clusters in the subcapsular regions of the splenic red pulp, in proximity to the megakaryocytes. No signs of extramedullary hematopoiesis were seen in the kidneys or liver (Supplemental Fig. 1).

Extramedullary myelopoiesis driven by life-long exposure to TNF

Because proliferation was primarily localized to the red pulp, where monocytosis has been demonstrated to occur, splenic leukocytes were quantified, to determine whether extramedullary hematopoiesis is skewed toward monopoiesis. The total number of the various lymphocytes (B cells, T cells, and NK cells) was the same between young and old mice, whereas myeloid cells (megakaryocytes and monocytes) were significantly increased (**Fig. 2**). Because TNF is linked to myeloproliferative conditions and increased in our old mice (young: 1.397 ± 0.1931 pg/ml, n = 14; old: 3.447 ± 0.7372 pg/ml, n = 30), we examined the splenic phenotype of old TNF KO mice and found that they did not have increased spleen weight with age, nor did the number of megakaryocytes or monocytes increase. There were no differences in spleen size or number of monocytes between young TNF KO and WT mice (Fig. 2A).

TNF drives increases in the number and proliferation of splenic myeloid progenitors

The hematopoietic progenitors in the bone marrow and spleens of young and old WT and TNF KO mice were quantified by flow cytometry. Old mice had significantly more CMPs and GMPs than young mice had in the spleen and bone marrow (**Fig. 3A–D**). The fold change in number of cells was significantly higher between young and old WT mice in the spleen (GMP, 7-fold increase; CMP, 14-fold increase), in comparison to the bone marrow (GMP, 2.7-fold increase; CMP, 1.78-fold increase). Old TNF KO mice did not have increased

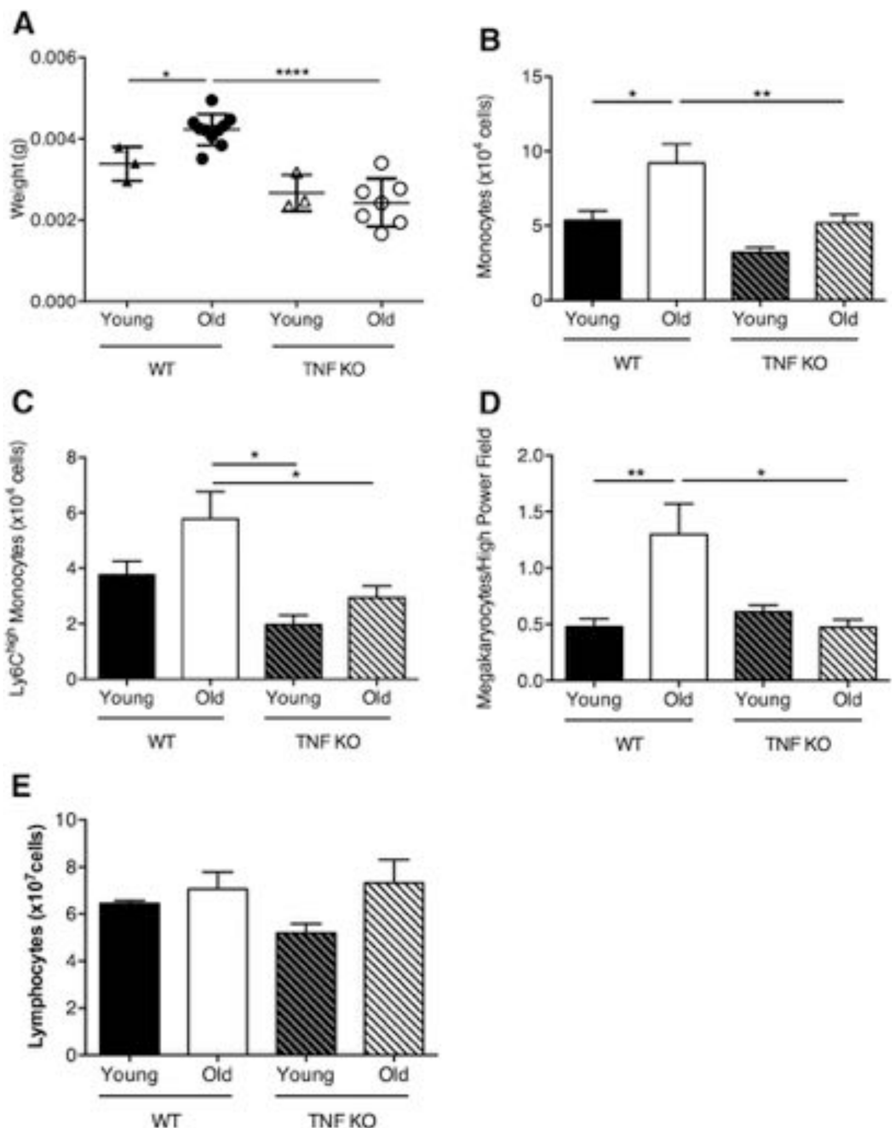

Figure 2. Lifelong exposure to TNF drives splenic myelopoiesis. Data are means ± SEM. (A) Spleen weights (normalized to body weight) of young (6–8 wk) and old (18–22 mo) WT and TNF KO mice revealed that old TNF KO mice were protected from splenomegaly. The total number of splenic monocytes (B) (n = 3–9) and splenic $Ly6C^{high}$ monocytes (C) (n = 3–9) and enumeration of megakaryocytes in the subcapsular red pulp (D) (n = 10 high-power fields) showed increased production of myeloid cells with age, which is dependent on TNF. The total number of lymphocytes (E) (n = 3–9) indicate that lymphopoiesis was unchanged in old TNF KO mice. Data represent 1 of 2 independent experiments. $*P < 0.05$, $**P < 0.005$, $****P < 0.00005$, 1-way ANOVA with Fisher's LSD *post hoc* test, when appropriate.

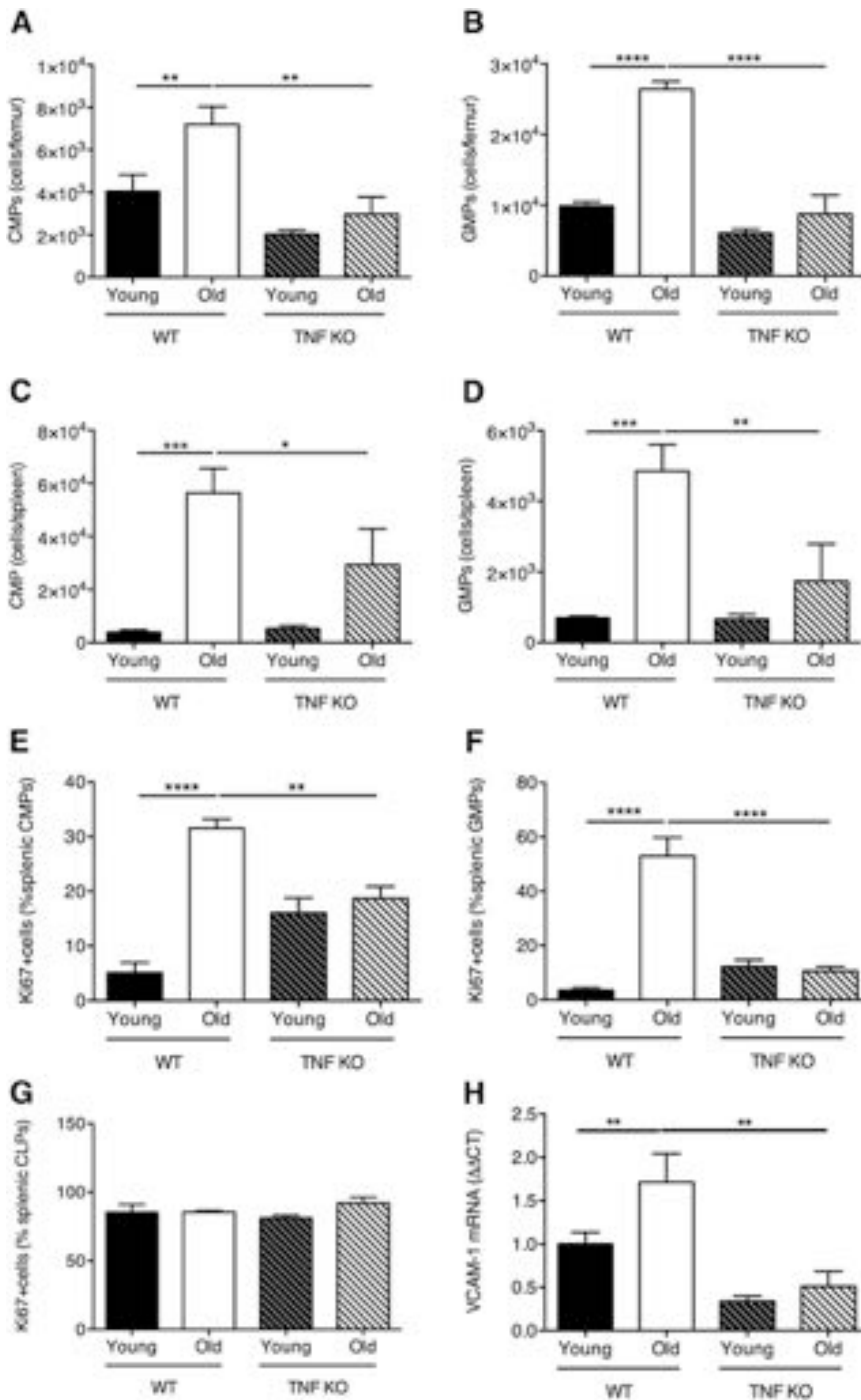

Figure 3. Lifelong exposure to TNF drives increased proliferation and retention of myeloid progenitors in the spleen. Data are means ± SEM. (A–D) Total number of CMPs and GMPs in the bone marrow and spleen of young (8 wk) and old (22 mo) WT and TNF KO mice showed an age-associated increase in myeloid progenitors that did not occur in the old TNF KO mice. (E–G) Intracellular staining for Ki67 showed that the proportion of CMPs and GMPs actively dividing were higher in the old WT than in the other groups. No differences were seen in the CLPs (n = 3–5). (H) Levels of VCAM-1 transcript in the spleen were measured by quantitative PCR. Expression was normalized to GAPDH and shown relative to the average young WT expression (n = 3–5). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$, $^{****}P < 0.00005$, 1-way ANOVA with Fisher's LSD *post hoc* test, when appropriate.

CMPs and GMPS with age. In addition, intracellular staining revealed a higher proportion of splenic CMPs, and GMPs were positive for Ki67 in old mice; however, no differences were seen in the CLPs (Fig. 3E–G). Although we found no differences in the growth factors G-CSF, M-CSF, or GM-CSF to explain the increased number or proliferation of these progenitors (Supplemental Fig. 2), we found greater expression of the adhesion molecule VCAM-1 in the spleens of old mice (Fig. 3H).

TNF neutralization reverses extramedullary myelopoiesis and reduces the number of Ly6C^{high} monocytes in old mice

We noted that, of the 2 monocyte populations (Ly6C^{high} and Ly6C^{low}) that have been reported in the spleen, Ly6C^{high} "inflammatory" monocytes were more numerous with increasing age. Because we observed that chronic TNF drives extramedullary monopoiesis in our old mice (Fig. 2A), we were curious about whether its neutralization would reduce the

number of inflammatory monocytes and thus reduce extramedullary monopoiesis. TNF was ablated with anti-TNF, and human IgG was used as a control. Antibodies were administered to young and old mice every other day for 3 wk. As predicted, old mice receiving the anti-TNF treatment displayed complete reversal of splenomegaly and showed decreased monocyte and megakaryocyte counts, indicating decreased extramedullary myelopoiesis (**Fig. 4**).

The aging microenvironment drives extramedullary monopoiesis

To determine whether intrinsic changes in the myeloid progenitors themselves or changes in the aging microenvironment drive splenic monopoiesis, age-discordant bone marrow chimeras were created. Bone marrow from either young or old donor mice was transplanted into both young and old recipients. The number of splenic monocytes was higher in old recipient mice, regardless of the age of the bone marrow donor, suggesting that the aging microenvironment drives monopoiesis (**Fig. 5A**). Of these monocytes, the proportion of Ly6C^{high} cells increased (Fig. 5B) in a microenvironment-dependent manner.

DISCUSSION

Our results indicate that, with age, there is increased splenic extramedullary hematopoiesis, giving rise primarily to myeloid cells. Aging is accompanied by many changes to the hematopoietic compartment. Mesenchymal stem cells are instrumental in directing hematopoiesis by differentiating into cell types, such as bone and fat precursors that make up the bone marrow stroma. In the elderly, decreased osteoblast differentiation and red marrow with a concomitant enrichment of adipocytes lead to a distorted niche less conducive to hematopoiesis [46, 47]. It has been proposed that EMH compensates for compromised hematopoiesis in the bone marrow [1], which could explain the increased splenic EMH in our old mice. What remains to be determined is whether an increase in circulating HSPCs from the bone marrow seed the spleen, or those that have seeded the spleen throughout the lifetime of these animals respond to factors in their microenvironment [48]. Our data indicate there is an age-associated increase in myelopoiesis, because of the greater number of myeloid progenitors (CMPs and GMPs) in the bone marrow and spleens of our old mice. The fold change in the number of progenitors between young and old mice was more pronounced in the spleen. In addition, the proportion of splenic progenitors undergoing proliferation was significantly higher in the old mice. This increased division likely gave rise to the increase in monocytes in the old mice. Our heterochronic chimera study suggests that the splenic progenitors are newly seeded cells, in that native HSPCs are destroyed during irradiation. Mice aged in the absence of TNF were protected from increased monopoiesis, and we concluded from the data that, regardless of the source of these progenitors, TNF from the aging microenvironment supports production and proliferation of monocytes.

Levels of TNF in the circulation and tissues increase with age [36, 49]; however, its source is unknown. The strongest evidence to date is that stimulation by chronic viral infections (i.e., Epstein Barr virus and cytomegalovirus) results in the expansion of terminally differentiated, virus-specific effector T cells that produce proinflammatory cytokines [50]. Alternatively, in animal [51] and *Drosophila* [52] models, microbial translocation caused by impaired intestinal barrier function has been proposed to drive "inflammaging," and this is consistent with observations of increased circulating bacterial products in the elderly [53]. In fact, long-term exposure to low-dose LPS in young mice simulates many of the age-associated changes in hematopoiesis [54]. Furthermore, the chronic inflammation that occurs with age has been shown to alter the MSC compartment and promote the formation of adipocytes [55–60], which secrete high amounts of TNF [60].

TNF is known to inhibit lymphopoiesis [61], while promoting myelopoiesis [35, 62]. TNF could support monopoiesis by promoting the recruitment, proliferation, or retention of progenitor cells in the spleen. TNF stimulation increases production

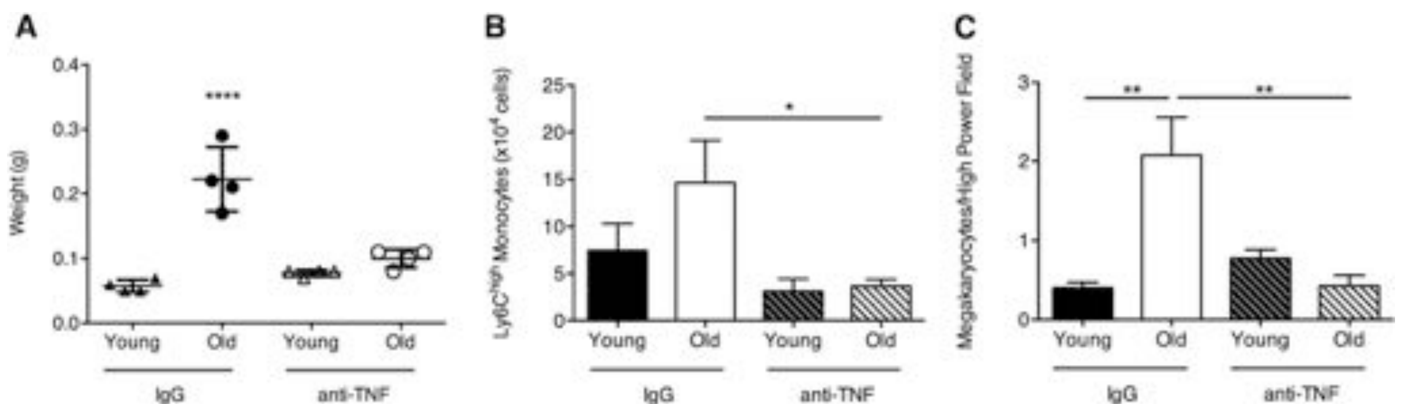

Figure 4. TNF neutralization reverses splenic monopoiesis in old mice. Young (6–8 wk) and old (18–22 mo) mice were treated with human IgG or anti-TNF (adalimumab) at 50 ng/g body weight every other day for 3 wk. Data are means ± SEM. Spleen weight (normalized to body weight) (A) (n = 4–5) and total splenic Ly6C^{high} monocytes (B) (n = 5) and megakaryocytes (C) in the subcapsular red pulp (n = 10 high-power fields) showed a decrease in splenomegaly and splenic monopoiesis in old mice receiving anti-TNF therapy. *$P < 0.05$, **$P < 0.005$, ****$P < 0.00005$, 1-way ANOVA with Fisher's LSD *post hoc* test, when appropriate.

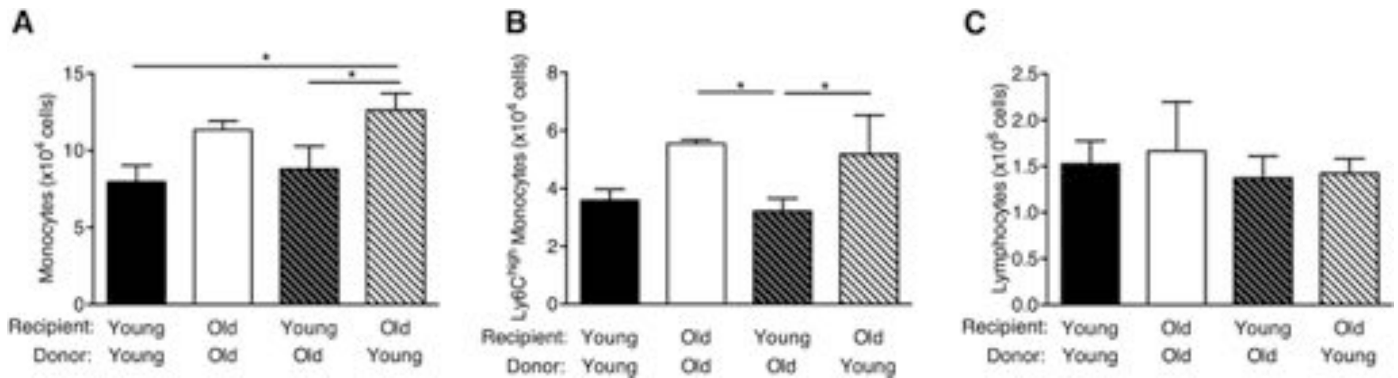

Figure 5. The TNF that drives splenic monopoiesis is derived from the aging microenvironment. Heterochronic bone marrow chimeras were generated with young (6–8 wk) and old (18 mo) mice. Data are means ± SEM ($n = 5$). The total number of splenic Ly6C^{high} (A) and Ly6C^{low} (B) monocytes showed that increased monopoiesis occurred in the old host, independent of the age of donor bone marrow. Data represent 1 of 2 independent experiments. *$P < 0.05$, 1-way ANOVA with Fisher's LSD *post hoc* test, when appropriate.

of growth factors (e.g., G-CSF, GM-CSF, and CSF-1) by fibroblasts, endothelial cells, and monocytes and upregulates receptors for these growth factors [63]. Alternatively, TNF may upregulate growth factor receptors on HSPCs directly. TNF has been shown to act in synergy with G-CSF and IFN-γ to upregulate GM-CSFR on DC progenitors [63] and human acute myeloid leukemia cells [64]. Other possible mechanisms could be driven by increased recruitment and retention of monocytes and HSPCs in the spleen. MSCs have been shown to be present in the adult spleen and facilitate EMH in these tissues [65]. When splenic MSCs are stimulated with TNF, they increase production of MCP-1 [66]. Even more striking is that these MSCs do not become refractory upon restimulation with TNF, but produce increased levels of MCP-1 [66], which could lead to increased recruitment of monocytes to the spleen. We investigated whether the increase in splenic monopoiesis was related to the higher production of colony-stimulating factors (M-CSF, G-CSF, or GM-CSF), but saw no differences in transcript levels in the spleen that could explain the phenotype. However, there was an increase in the expression of VCAM-1 in our old mice. VCAM-1 is expressed on splenic macrophages to retain HSPCs [67] and is known to be upregulated by TNF through NFκB activation [68, 69]. Our data are consistent with an age-associated increase in TNF that drives greater expression of VCAM-1 in the spleen, resulting in higher retention of HSPCs and subsequent proliferation under the influence of the local myeloid-polarizing environment [28].

Monocytes are key to the propagation of many inflammatory diseases [26]. CCR2^{high}Ly6C^{high} monocytes are particularly involved in propagating inflammation and have been the target of therapeutic modulation and ablation in these diseases [70, 71]. Most strategies have been to reduce the mobilization and recruitment of monocytes by targeting the chemokine MCP-1 or its receptor CCR2 [70]. Systemic apoptosis-mediated ablation of CCR2^{high}Ly6C^{high} monocytes after their selective uptake of polystyrene microparticles improved the symptoms and progression of colitis, experimental autoimmune encephalomyelitis, and myocardial infarction [71]. This approach has shown promise; however, concerns have been raised with regard to increasing the risk of infection and impairing the resolution of inflammation and tissue repair [72]. What remains unknown is whether monocytes arising in the spleen are different from those generated in the bone marrow or other extramedullary sites in their potential to instigate and maintain the pathogenesis of chronic inflammatory disease. Modulating the splenic monocyte compartment may have some merit as a potential therapeutic, given that they have been shown to mobilize to affected tissues and contribute to the pathogenesis in atherosclerosis and cancer [20, 21]. For example, Ly6C^{+} splenic monocytes and macrophages were preferentially targeted with lentivirus and resulted in decreased incidence and severity of autoimmune arthritis [73]. In our old mice, 3 wk of treatment with anti-TNF significantly reduced the number of Ly6C^{high} monocytes. The current work highlighted the need to better understand how TNF drives monopoiesis and the potential to modulate TNF to control systemic inflammation and to curb the development of chronic inflammatory diseases.

AUTHORSHIP

D.M.E.B. and D.L. designed the experiments. D.L., A.P., and A.N. performed the experiments. D.L., A.N., J.A., and D.M.E.B. analyzed the data. D.L. and D.M.E.B. wrote the paper.

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DISCLOSURE

The authors declare no conflicts of interests.

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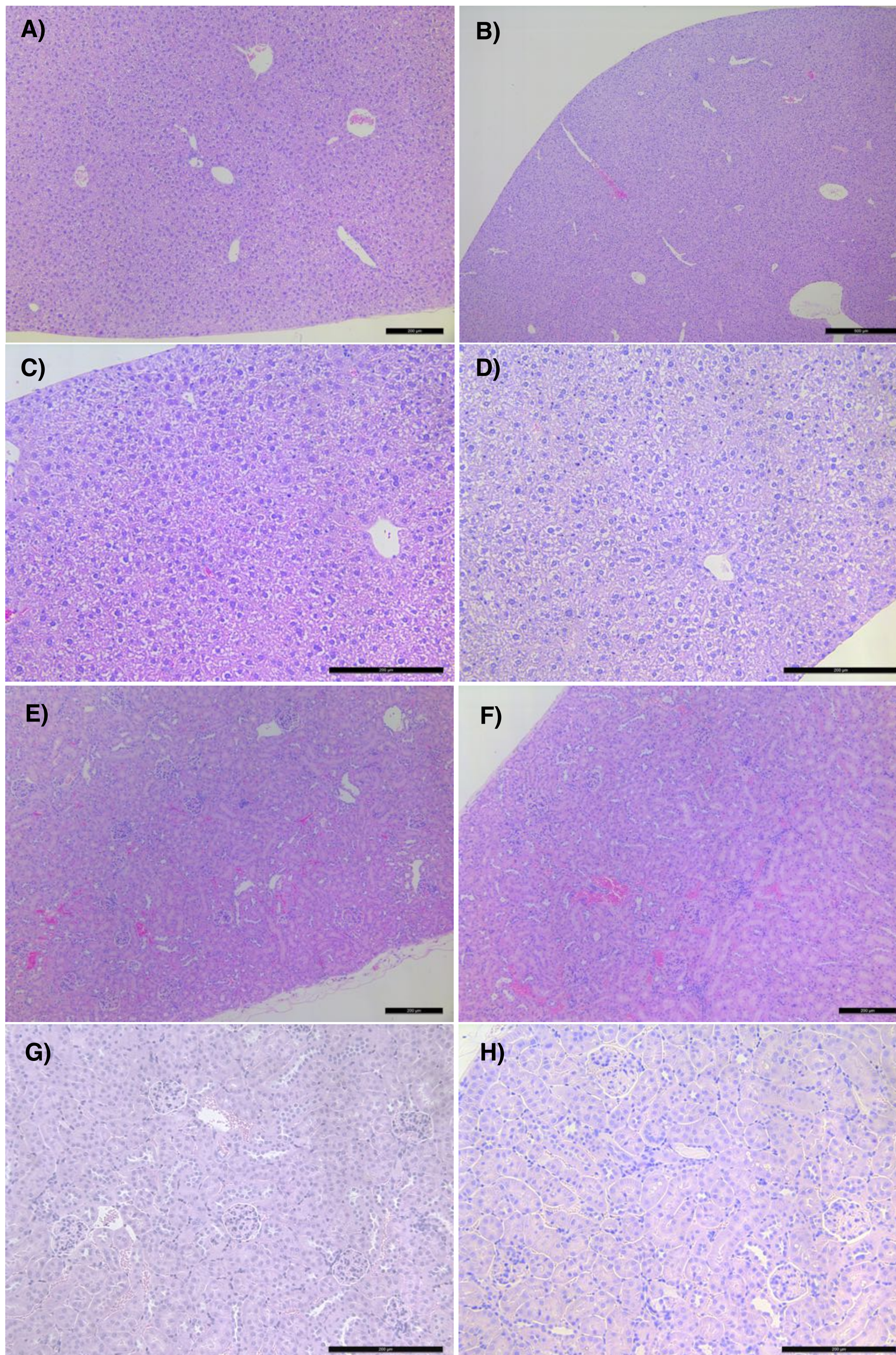
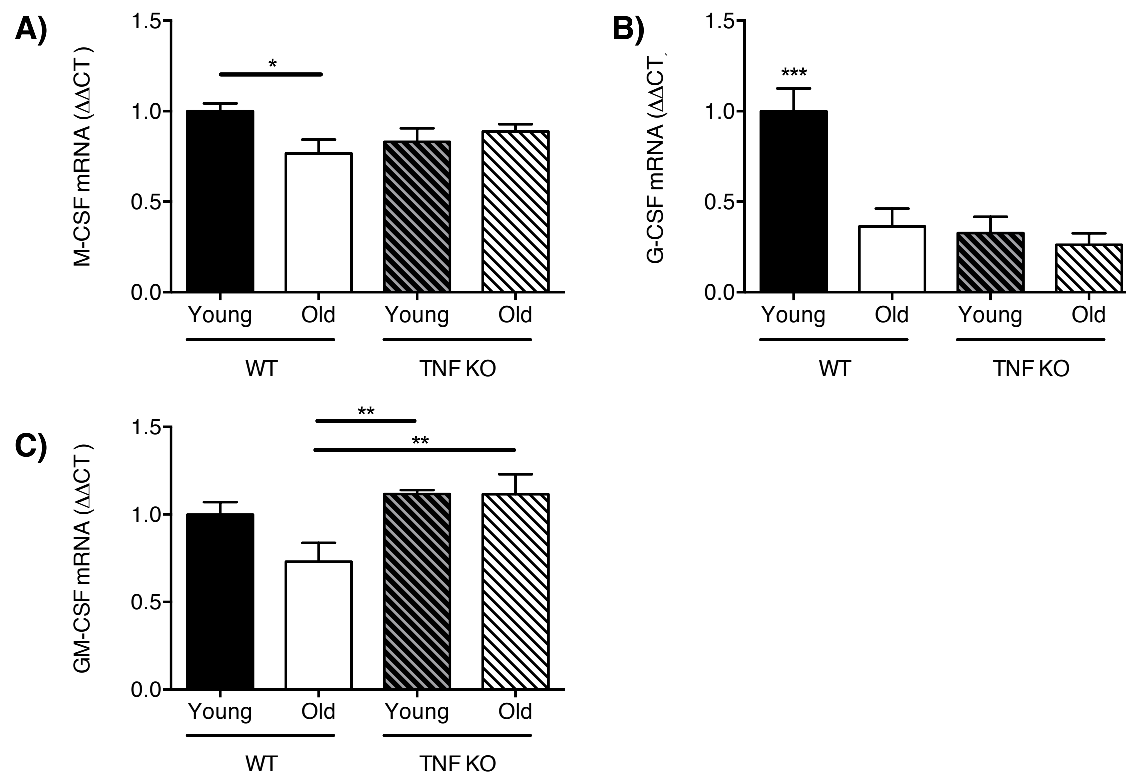
KEY WORDS:
aging/elderly · monocyte · hematopoiesis · splenomegaly · age-associated inflammation

Supplementary Figure 1



Supplementary Figure 1. No signs of extramedullary hematopoiesis in the liver or kidneys of old mice. Representative histological sections from the liver (A-D) and kidney(E-H) of a young (8wo; A,C,E,G) and old mouse (22mo; B,D,F,H) stained with hematoxylin and eosin show no signs of extramedullary hematopoiesis.

Supplementary Figure 2



Supplementary Figure 2. Differential expression of myeloid growth factors in the spleen of young and old mice. Levels of M-CSF (A), G-CSF (B) and GM-CSF (C) transcripts in the spleen were measured by quantitative PCR. Expression was normalized to GAPDH and shown relative to the average young WT expression. (mean ± SEM, n=3-5). Statistical significance was determined by one-way ANOVA with Fisher's LSD post-test where appropriate. * indicates $p < .05$, ** indicates $p < 0.005$, *** indicates $p < 0.0005$.

CHAPTER 5.2

Inflammation and monocyte development

Introduction:

As discussed in the introduction, myeloid development from an HSC to a macrophage is regulated by epigenetic and transcriptional modifications, which are influenced by growth factors and cytokines in the environment. In mice, the surface markers cKit, Flt-3, and CD115 are used to delineate distinct myeloid progenitors and their signalling is essential to the progression of myeloid differentiation (Supplemental Figure 1).

cKit (also referred to as KIT) is a receptor tyrosine protein kinase that is expressed on HSCs and downstream progenitors. cKit is a cytokine receptor that binds stem cell factor (SCF) to promote cell survival and synergizes with other cytokines (i.e. IL-3, IL-7, GM-CSF) to promote proliferation of HSCs[449]. cKit is expressed at higher levels on myeloid progenitors compared to lymphoid progenitors and is lost upon differentiation to monocytes. Mast cells and dendritic cells retain high expression of cKit which they require for proliferation, survival and function[449]. KIT is additionally a proto-oncogene; activating mutations or overexpression of the gene have been found in 70% of myeloid leukemias[450]. As

such, we posited that increased expression on myeloid progenitors could contribute to the increased proliferation we found in the previous section.

FMS-like tyrosine kinase 3 (Flt-3) belongs to the same receptor tyrosine kinase family as cKit. It is expressed on CD34^{+} HSCs and immature hematopoietic progenitors[451]. It is additionally expressed at lower levels on non-hematopoietic cells in multiple organs including the spleen, liver, lymph nodes, thymus, kidney lung, colon. The Flt-3 ligand (FL) activates Flt-3 and stimulates proliferation and differentiation of HSCs[451]. Most tissues express FL, suggesting Flt-3 is the limiting step in activation and tissue-specificity. Mononuclear cells express FL on their surface, potentially providing a pathway for paracrine or autocrine regulation. Like cKit, Flt-3 synergizes with cytokines to induce proliferation and survival, which we believe could be driving monopoiesis with age.

CD115 has many names: c-fms, colony stimulating factor 1 receptor (CSF-1R), macrophage colony stimulating factor receptor (M-CSFR). It belongs to the same family of receptor tyrosine kinases as cKit and Flt-3. It binds IL-34 and CSF-1 (M-CSF) and leads to proliferation, survival and differentiation of macrophages[108]. Ligand binding results in dimerization, autophosphorylation and downstream activation of MAPK, PI3K and Akt[345,346]. CD115 is primarily expressed by myeloid and monocytic progenitors, monocytes and macrophages. The receptor is additionally expressed on trophoblasts during embryonic development and on osteoclasts, but the gene is under regulation from a separate promoter than is used

in myeloid cells[347]. Cells of mesenchymal origin (i.e. blood vessel endothelial cells, fibroblasts, bone marrow stromal cells), macrophages, and tumour cells produce CD115's ligand, CSF-1[95]. CSF-1 is found at concentrations of 10 ng/mL in circulation and in the pg/mL range in tissues[96]; without the protein, macrophages and their precursors undergo cell death, as it is needed to enter cell cycle[94].

In old mice, the myeloid compartment is expanded with an increase in Ly6C^{high} monocytes in circulation as well as in the bone marrow and spleen[452]. We have demonstrated that extramedullary hematopoiesis contributes to this with an increased number of proliferating myeloid progenitors (i.e. common myeloid progenitor) in the murine spleen[453]. To determine if this was due to altered sensitivity to hematopoietic growth factors, we quantified the expression of cKit, Flt-3, and CD115 at different stages of myeloid development from the common myeloid progenitor (CMP), to the macrophage and dendritic cell progenitor (MDP), to the common monocyte progenitor (cMoP) and finally to monocytes. We hypothesize that progenitors from old WT mice will differentially express growth factor receptors compared to those from young and old TNF KO mice.

Furthermore, old mice have an elevated number of monocytes, primarily the inflammatory subset (Ly6C^{high}CCR2^{+})[453]. These monocytes have higher expression of CCR2 and produce higher levels of pro-inflammatory cytokines on a per cell basis[413]. Bone marrow derived macrophages from old mice retain their phenotype *in vitro* (i.e. decreased phagocytosis and bacterial killing, increased production of pro-

inflammatory cytokines). Mice aged in the absence of age-associated inflammation (TNF KOs) are protected from these changes to the myeloid compartment[39]. This age-associated phenotype is reversible by decreasing circulating TNF and decreasing microbiota-driven inflammation[413,453,454]. These data suggest that stable but reversible changes in myeloid cell differentiation and function that are consistent with epigenetic modification. TNF has been shown to prime cells by affecting their chromatin structure[455]. Thus, we hypothesize that chronic exposure to TNF with age drives differential expression of genes central to myeloid development.

Materials and Methods:

Animals

WT (C57BL/6) and TNF KO (B6;129S-Tnftm1Gkl/J) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred and aged in-house. All mice were female and housed in specific pathogen-free conditions in accordance with Institutional Animal Utilization protocols approved by McMaster University's Animal Research Ethics Board, as per the recommendations of the Canadian Council for Animal Care. All animals used were sex matched to their controls and were maintained in the same animal room. The pathogen-free status of mice within the aging colony was confirmed in mice through constitutive monitoring of sentinel mice and specific testing of fecal samples for common mouse pathogens.

Tissue isolation and immunophenotyping

Single-cell suspensions were prepared by homogenizing spleen tissue and spinal bone marrow through a 40 μm cell strainer. Cells were incubated for 1 h at 4°C with a direct application of antibodies diluted in FACS Wash (5 mM EDTA, 0.5% BSA in PBS). Monoclonal antibodies with the following specificities were used for monocyte subset differentiation: CCR2 (PE; R&D Systems, Minneapolis, MA, USA), CD115(AF488), Ly-6C (eFluor 450), CD45 (PerCP-Cy5.5), CD11b (PE-Cy7), and CD3, B220, NK1.1 (Alexa Fluor 700), all from eBioscience (San Diego, CA, USA). The following antibodies were used for myeloid progenitor phenotyping: Sca-1 (PE-Dazzle 594), CD117 (BV421), Flt-3 (PE), CD127(PerCP-Cy5.5), CD34 (AF647), CD16/32 (BV711), CD11b (APC-Cy7), CD115(AF488), Ly6G(AF700) and CD3, Ter119, B220 (PE-Cy7) and all from BioLegend. Samples were then incubated with 1X Fix/Lyse Buffer (eBioscience) for 10 min at room temperature with frequent inversion and centrifuged at 4°C, washed and resuspended in FACS Wash. Samples were run on a Fortessa flow cytometer (BD Biosciences, Franklin Lake, NJ, USA) and analyzed with FlowJo 10 software (Treestar, Ashland, OR, USA). For monocyte gating strategy see Puchta et al. [39]. For myeloid progenitor gating see Supplemental Figure 2, which was adapted from published methods[92,456].

Cell sorting and RNA sequencing

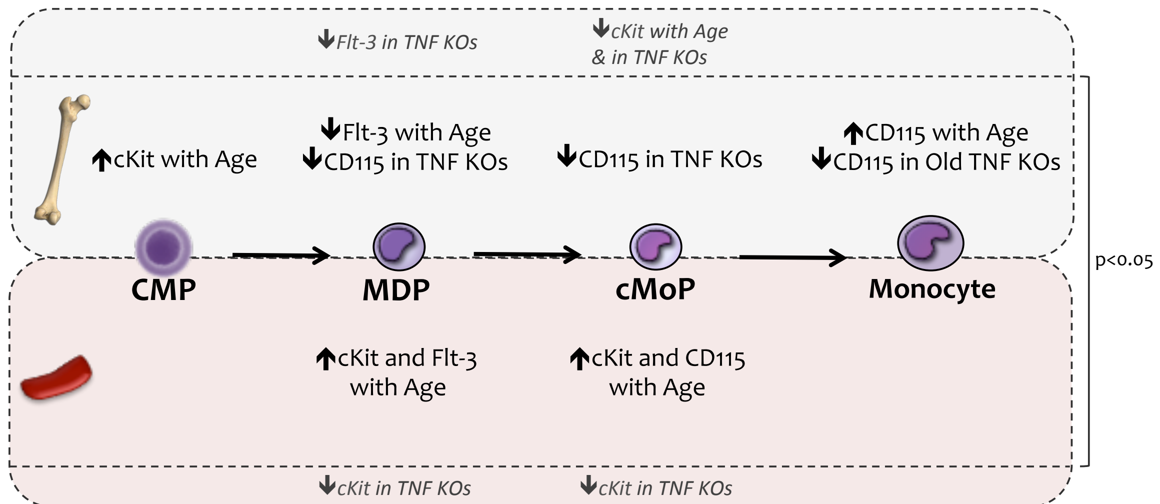
Spinal bone marrow was filtered through a 40 μm cell strainer and incubated in 1X RBC lysis buffer (BioLegend) for 5 mins at 4°C. Cells were washed and stained

for 30 mins at 4°C with monoclonal antibodies of the following specificities CD45 (eFluor 450), Ly-6C (FITC), CD11b (PE-Cy7), and Ly6G, CD3, B220, NK1.1 (Alexa Fluor 700), all from eBioscience (San Diego, CA, USA). Cells were washed and resuspendend in FACS Wash, filtered through a 40 μm cell strainer and 7AA-D (BD Pharmingen) was added at 1/250(V/V) 10 mins prior to sorting. For each mouse, 50 000 cells were sorted per Ly6C^{high} and Ly6C^{low} monocyte population, per mouse using a BD FACSAria III Cell Sorter according to the gating strategy in Supplemental Figure 3. Viability and purity of sorted cells was >95%. Monocytes were resuspended in Qiazol (QIAGEN) and sent to Dr. Deborah Winter's group at Northwestern University, who performed RNA sequencing analysis of gene expression. PBS-perfused lungs were collected and sent to by Dr. Alexander Misharin's group Northwestern University for single cell alveolar cell sorting, performed using their published protocol[457]. RNA extraction and library preparation as well as data analysis were performed as previously described[458], by both groups at Northwestern University.

Statistical Analysis

Unless otherwise stated in the figure captions, data were analyzed using a two-way ANOVA with a Fisher's LSD post-test to test the effect of age and TNF. Data were analyzed with Prism (Version 6; GraphPad, San Diego, CA, USA).

Results:



Summary of changes in growth factor receptor expression on myeloid progenitors with age and TNF. Top- and bottom-most changes are patterns observed in the data that were not statistically significant.

cKit and Flt-3 are elevated in myeloid progenitors in the bone marrow and spleens of old mice

CMPs in the bone marrow of old mice expressed higher levels of cKit than young mice; however, this difference was lost after differentiation to the MDP (Figure 1). It is not clear whether age-related differences in cKit are TNF-dependent; however, old TNF KO CMPs trended towards lower expression of cKit, and young and old TNF KO cMoPs trended towards lower expression of cKit than age-matched

WT controls. Bone marrow MDPs in old mice had lower expression of Flt-3 compared to young controls, which was independent of TNF.

Patterns of cKit and Flt-3 expression on splenic myeloid precursors differ from those in the bone marrow. Splenic MDPs from old WT and TNF KO mice expressed higher levels of cKit than their young controls. In contrast to the bone marrow, cMoPs in the spleens of old mice expressed higher levels of cKit (Figure 1). Although not statistically significant, the expression of cKit on splenic MDPs and cMoPs trended lower in TNF KO progenitors compared to WT, suggesting that TNF stimulates cKit expression. Splenic MDPs from old mice had higher expression of Flt-3 compared to young controls, which was opposite to what was seen in the bone marrow MDPs (Figure 1).

CD115 is elevated on splenic cMoPs and circulating monocytes in old mice

In the bone marrow, MDP and cMoP expression of CD115 is regulated by TNF but does not change with age (Figure 1). No age- or TNF-dependent changes in CD115 expression were found in splenic MDPs; however, splenic cMoPs had an age-associated increase in CD115 expression (Figure 1). CD115 was expressed at higher levels on monocytes from old WT mice, particularly Ly6C^{low} monocytes, but was not on monocytes from old TNF KO mice (Figure 2A). Bone marrow derived macrophages, alveolar macrophages, and whole lung tissue showed a similar trend with higher expression of CD115 in old WT mice but not in old TNF KO mice (Figure 2B-D). Although we do not have data on circulating and tissue concentrations of CSF-

1 from these mice, we found that whole lung tissue transcript expression of CSF-1 followed similar trends to CD115 surface expression; however, alveolar macrophages did not show an age-associated increase in TNF (Figure 2E&F).

TNF alters expression of genes associated with myeloid development in monocytes and alveolar macrophages.

To determine how age and TNF influence the expression of genes associated with myeloid development, we sorted bone marrow monocytes and alveolar macrophages from young and old, WT and TNF KO mice and performed RNA sequencing. Preliminary analyses revealed significant differences between Ly6C^{high} and Ly6C^{low} monocytes from young and old WT and TNF KO mice (Figure 3A). Of the few genes that we have identified as differentially expressed between groups, *HDAC1* and *FOXO3* were found to be lower in old WT monocytes and were rescued in old TNF KOs (Figure 3B). Alveolar macrophages from old WT mice had decreased expression of many genes necessary for myeloid development (i.e. *Hdac1, Tet2, Cebpa, Cbfa2t3, Runx1*), which were not decreased in cells from old TNF KO mice (Figure 4). There were also changes to expression of enzymes involved in metabolic regulation of myeloid development and macrophage activation (e.g. *Tet2, Idh1* and *Hif1a*, Figure 5).

Discussion:

We have previously shown a TNF-dependent increase in bone marrow and splenic monopoiesis, thus we wanted to further understand at which stage in monocyte development this occurs. Based on our findings, age-associated TNF may contribute to elevated expression of cKit driving increased survival of bone marrow CMPs and splenic MDPs and cMoPs. The number of $cKit^+$ cells in the bone marrow has been shown to be higher in old mice[459]. Although direct TNF stimulation *in vitro* has been shown to downregulate cKit expression on $CD34^+$ progenitors[460], TNF has also been shown to upregulate the cKit ligand, SCF[461], and it may stimulate other cKit promoting cytokines (e.g. IL-6 , IL-7, Flt-3L[462]) to indirectly increase cKit expression on myeloid progenitors with age. cKit is essential to maintaining hematopoiesis in the bone marrow and spleen, and it is believed to play a role in HSC mobilization to the spleen[463,464]. In fact, splenic hematopoiesis relies on the production of SCF, by endothelial cells and perivascular stromal cells[465]. This suggests that increased expression of cKit could in part be mediating increased splenic hematopoiesis with age.

The increase in cKit expression with age was also accompanied by an increase in Flt-3 expression on splenic MDPs. Activating mutations in Flt-3 are the most abundant ones found in cases of acute myeloid leukemia (AML)[451]. Flt-3 inhibits apoptosis through Akt-dependent inactivation of the transcription factor FOXO3[466].

FOXO3 has been well documented to decrease with age[467] and we found it expressed at lower levels in Ly6C^{high} monocytes of old mice compared to their young counterparts (Fig. 4B). Elevated expression of Flt-3 on MDPs could increase survival and thus contribute to the expansion of myeloid progenitors in old mice that we documented in the previous section. Flt-3 activation additionally inhibits the induction of CCAAT/enhancer-binding protein α (C/EBPα) and PU.1 which are transcription factors necessary for myeloid differentiation[468]. This arrests maturation of granulocytic cells in response to G-CSF stimulation, and in combination with increased proliferation results in the accumulation of immature myeloid cells, which are characteristic of AML[468]. In line with these findings we also found decreased expression C/EBPα in alveolar macrophages (Fig.5). Other studies have also demonstrated that increased Flt-3 signalling mediates MDSC expansion and activation, the latter of which is dependent on STAT3[469]. In contrast to the spleen, bone marrow MDPs showed decreased Flt-3 expression with age. No studies to date have compared the differences in expression of FL in the bone marrow and the spleen or how they change with age, but this would be instrumental in understanding site-specific differences we have found in hematopoiesis. There is some evidence that altered phenotypes of progenitors can in turn influence the hematopoietic niche. In a mouse model of Flt-3 overexpression, there is elevated myeloproliferation and inflammation of the hematopoietic niche, similar to what we find with age[470]. This was thought to be due to the aging of the progenitors themselves, which would be considered cell-intrinsic. Interestingly, when Flt-3

overexpressing bone marrow is transplanted into an irradiated wild type host, non-hematopoietic cells in the bone marrow niche overexpress TNF and the bone marrow environment becomes dysregulated. This suggests that there may be feed forward mechanisms at play where aged myeloid progenitors remodel the hematopoietic niche. Then the inflammatory niche may feedback onto HSCs to alter hematopoiesis and myeloid development, through HSC-extrinsic mechanisms.

In line with increased Flt-3 leading to altered myeloid differentiation, we found significant age- and inflammation-associated changes in the expression of genes associated with myeloid development in monocytes and alveolar macrophages. Decreased FOXO3 activity inhibits apoptosis[466], and we propose the decreased expression we observed in Ly6C^{high} monocytes may promote their survival, contributing to monopoiesis. HDAC1 is increased during myeloid differentiation[101,471] and its decreased expression with age could contribute to the impaired differentiation we have observed. Furthermore alveolar macrophages sorted from young and old, WT and TNF KO mice, displayed clear differences in expression of genes involved in myeloid development. Decreased Tet methylcytosine dioxygenase 2 (TET2) activity is found in many myeloid malignancies[472] and when it is deleted in the hematopoietic compartment, it leads to a phenotype similar to chronic myelomonocytic leukaemia[473]. TET2 is negatively regulated by the metabolite 2-hydroxyglutarate, which is generated by aberrant isocitrate dehyrogenate 1 (IDH1) activity[474]. In alveolar macrophages from old mice

we found an elevated expression of *IDH1*, which could explain the decrease in *TET2* expression. The expression of both genes in old TNF KO alveolar macrophages was similar to that of young mice, indicating life-long exposure to TNF may drive impaired myeloid differentiation (Fig. 6). Future experiments would benefit from performing assays for transposase-accessible chromatin using sequencing (ATACseq) and chromatin immunoprecipitation (ChIP) sequencing which will allow for us to better understand the epigenetic mechanisms by which TNF may be influencing gene expression and myeloid development.

The myeloid transcription factor PU.1 binds upstream of CD115 and initiates its transcription[475]. CD115 transcription is found in early myeloid precursors prior to surface expression, suggesting differentiation decisions are cell-intrinsic and occur prior to signalling through CD115[476]. CSF-1 is necessary to maintain populations of circulating monocytes and tissue macrophages[100]. It is elevated in inflammatory states to increase the proliferation and differentiation of these cells. Thus it is likely that CSF-1-CD115 signalling is not what initiates the monopoiesis and impaired development with age, but likely contributes to the maintenance of progenitors by providing survival and proliferation signals. Our data suggest that CD115 is differentially regulated by age and TNF in the spleen and bone marrow. In bone marrow MDPs and cMoPs, there is no age-associated increase in CD115, but it is more highly expressed in WT compared to TNF KOs, suggesting TNF increases the expression of CD115. In the spleen, CD115 expression on cMoPs is increased with

age independent of TNF. Thus, TNF may drive CSF-1-dependent proliferation in the bone marrow, while in the spleen other age-associated factors are involved.

We found CD115 expression on circulating monocytes increased with age, but not in old TNF KO mice (Fig. 2). Treatment with anti-CSF-1R does not affect monopoiesis or deplete monocytes in mice, but it blocks differentiation from Ly6C^{high} into Ly6C^{low} monocytes[108,477]. Signalling through CD115 is likely important for the transition to more mature monocytes (Ly6C^{low}) and macrophages. CSF-1 has been shown to generate macrophages with improved phagocytosis, cytotoxicity, superoxide production and cytokine production[478]. It decreases expression of TLR-1, -2, -6 and -9, without changing TLR-4; however, it increases the expression of the LPS co-receptor CD14, suggesting it enhances inflammatory responses to LPS, while suppressing the response to CpG DNA[479]. CSF-1 can also induce expression of CD16 on monocytes, causing them to be more inflammatory[204,480,481]. The higher expression of CD115 on monocytes from our old mice may make them more sensitive to CSF-1, priming them to produce pro-inflammatory cytokines and differentiate into hyper-inflammatory macrophages. This is supported by the decreased expression of *Tet2* and *Sirt1*, negative regulators of pro-inflammatory signalling, in alveolar macrophages from old WT mice (Figure 4).

Although CSF-1 activates macrophages to enhance their pro-inflammatory function, it suppresses antigen-specific responses[95]. CSF-1 stimulation of macrophages leads to decreased MHC II expression[482] and an increase in

indoleamine 2,3-dioxygenase(IDO), a known suppressor of T cell proliferation and survival[483].

CSF-1 is necessary for macrophage suppression of T cells responses to allogeneic cells and their response to mitogens[484–486]. Activated T cells can express CSF-1[487], which suggests T cell suppression by macrophages serves as a negative feedback loop. Furthermore, T cell IFNγ and CD40L signalling is involved in regulating macrophage suppression by IDO[483]. The increased expression of CD115 may enhance CSF-1 driven macrophage suppression of antigen presentation and antigen-specific responses, which could contribute to waning responses to infection and vaccination with age. Some of our data suggest that T cells are indeed involved in our age-associated myeloid phenotype as TCRβ KO mice that lack T cells, do not experience age-associated monopoiesis (Supplementary Fig. 4A); however, a marker of macrophage differentiation, F4/80, which is decreased with age[39], is not completely rescued in TCRβ KO's, indicating T cells are not sufficient to drive the aging myeloid phenotype (Supplementary Fig. 4B).

Although we did not measure protein levels of growth factors and cytokines in the bone marrow and spleen microenvironment, we found increased expression of CSF-1 in whole lung tissue from old mice, but not in old TNF KO mice (Fig. 2). CSF-1 expression by stromal osteoblasts increases with age in mice[488,489]. TNF stimulates CSF-1 in stromal cells like osteoblasts and cardiac cells[489,490]. In cardiac cells, TNF activates NFκB to induce CSF-1 production[491]. Although mesenchymal cells are the primary source of CSF-1[108], TNF conditioning of monocytes for 6 hours was shown

to induce CSF-1 expression which was further potentiated by the presence of IFNγ[492]. This could provide yet another feed forward mechanism in our aging model, as monocytes and levels of TNF[39], CSF-1[488,489,493,494] and IFNγ[306,307] have all been shown to increase with age. We hypothesize that TNF stimulates stromal cells and monocytes to express and secrete elevated levels of CSF-1 with age.

A recent study found bone marrow progenitors deficient in TNF were outcompeted by their wild type counterparts in mixed bone-marrow chimera experiments[429]. TNF expression increases upon differentiation from the cMoP to Ly6C^{low} monocytes; however, endogenous TNF was dispensable for monocyte survival as TNF KO monocytes were rescued by exogenous TNF. In mice, both TNF receptors progressively increase during differentiation from the MDP to the Ly6C^{low} monocyte. TNFR expression is necessary in order to transition from a Ly6C^{high} to a Ly6C^{low} monocyte[429], although which one of TNFR1 or TNFR2 is not yet known. These data demonstrate a direct role for TNF in monopoiesis, but TNF may simultaneously be acting indirectly to potentiate monopoiesis. For example, in a mouse model of osteolysis, TNF was needed to induce osteoclast precursors[495], but was reliant on CSF-1 production by TNF-responsive stromal cells. Seeing as TNF can stimulate the release of growth factors and other cytokines from non-hematopoietic tissue, there may be other contributors to our age-associated impairment in myeloid development. In the steady state, we propose that TNF influences myeloid development in 2 ways: 1) directly by driving myeloid progenitor and monocyte survival and Ly6C^{low} monocyte maturation[453], 2) indirectly by stimulating non-

hematopoietic tissues to release of other cytokines and growth factors that instruct monocyte and macrophage polarization, activation, and function(i.e. CSF-1). With age, chronically elevated levels of TNF alter these mechanisms, resulting in elevated monopoiesis and impaired monocyte and macrophage function. A similar model where low-dose LPS was administered daily for 4-6 weeks and replicated the myeloid skewing and HSC exhaustion seen with age. LPS induces TNF along with many of the other pro-inflammatory mediators downstream of TNF[440]. TLR-induced myelopoiesis is mediated by both HSC-autonomous[496] and non-HSC autonomous influences[140,441], and we believe TNF acts in a similar way.

Another interesting avenue to pursue is the differential responses to TNF we witnessed in myeloid progenitors from the spleen and from the bone marrow. The bone marrow hematopoietic niche has been studied in great detail[238], however the splenic niche has not. In chronic myeloid leukemia and myeloproliferative neoplasms, the spleen is a site of impaired myelopoiesis and results in clones that behave differently than those generated in the bone marrow[497,498]. Although there is growing evidence that cells developed in the bone marrow and the spleen are similar but distinct[90,499], we still do not understand how the splenic environment may bias myelopoiesis. Whether this phenomenon is due to intrinsic differences in the progenitors themselves or the tissue environment is not yet clear.

Our *in vivo* TNF neutralization studies have demonstrated the specificity of TNF in driving age-associated increases in monopoiesis[39,453]; however it should be noted that TNF KO and WT mice were not littermate controls. In future studies,

littermate controls will be used to minimize the non-genetic, housing confounder and control for maternal inheritance of the microbiota, which influences immune development and function[500].

CHAPTER 5.3

Monocyte and Macrophage Function

Introduction:

Age-associated changes in immune function increase susceptibility to infections. Infectious disease is responsible for a third of mortality in the elderly and one of the most common infections is bacterial pneumonia caused by *Streptococcus pneumoniae*[501–504]. In our aging mouse model, old mice mobilize a higher number of Ly6C^{high} monocytes following intranasal colonization and have higher levels of pro-inflammatory cytokines (e.g. TNF, IL-6) in the serum and lungs, and yet they have impaired bacterial clearance[39]. We found bone marrow derived and peritoneal macrophages display impaired bacterial killing of *S. pneumoniae*[39,454]. These data suggests that a hyperinflammatory response is detrimental to immune defence to *S. pneumoniae*. In humans, individuals wither higher than age-average levels of TNF and IL-6 are at a 2.5-fold higher risk of community acquired pneumonia[24]. Systemic

infusion of TNF (10 ng/h for 6 days) into young mice increases the bacterial burden following intranasal colonization with *S. pneumoniae* compared to controls[505]. In our model, when mice are aged in the absence of TNF (i.e. TNF KO) they have improved bacterial clearance *in vivo* and their bone marrow derived macrophages maintain their ability to kill bacteria[39,454]. We hypothesize that the elevated levels of TNF with age alter monocyte and macrophage function, contributing to impaired immune defence against *S. pneumoniae*.

Materials and Methods:

Whole blood bacterial binding and uptake assay

S. pneumoniae (strain P1547, serotype 6A) was labelled with TRITC and heat-killed at 65°C for 10 min and was incubated with 100 µL whole blood from young and old, WT and TNF KO mice, at an MOI of 50 (assuming 100,000 cells/mL blood) for 1 hour at either 4°C (binding) or 37°C (binding and internalization). Binding and uptake was arrested by incubation at 4°C and monoclonal antibody cocktail, resuspended in 50 µL of FACS wash, was added and incubated at 4°C for 30 min. Monoclonal antibodies with the following specificities were used for monocyte subset differentiation: CD45 (eFluor 450),Ly-6C (BV510), CD11b (PerCPCy5.5), Ly6G (Alexa Fluor 700), all from eBioscience (San Diego, CA, USA). Samples were then incubated with 1X Fix/Lyse Buffer (eBioscience) for 10 min with frequent inversion and centrifuged at 4°C, washed and resuspended in FACS Wash. Samples

were run on a Fortessa cytometer (BD Biosciences, Franklin Lake, NJ, USA) and analyzed with FlowJo 10 software (Treestar, Ashland, OR, USA). Monocytes were gated as $CD45^+CD11b^+Ly6C^+Ly6G^-$ and neutrophils were gated as $CD45^+CD11b^+Ly6C^-Ly6G^+$. The cells with bound and/or internalized bacteria were $TRITC^+$ and the number of bacteria per cell was quantified by geometric mean fluorescence intensity.

Bone marrow isolation and macrophage culture

Bone marrow was isolated from female WT (C57BL/6) and TNF KO (B6;129S-Tnftm1Gkl/J) mice originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and aged in- house. Singe cell suspensions were created by passing spinal bone marrow through a 40 μm cell strainer. Bone marrow was cultured in L929 conditioned media for 7 days as per standard protocols. Cells were lifted with Accutase® (Sigma-Aldrich), and washed with PBS prior to bacterial killing and a phagosomal assays.

Bacterial Killing Assays

To measure bacterial killing of *S. pneumoniae*, $1x10^6$ bone marrow derived macrophages were incubated with a multiplicity of infection (MOI) of 100 bacteria per macrophage for 30 min at 37°C with gentle inversion to allow for internalization of bacteria[506]. Viable colony-forming units were determined by culturing lysates on tryptic-soy agar plates with 5% defibrinated sheep's blood and 10 μg/mL neomycin.

Amplex Red Assay

Total H_2O_2 production was measured using Amplex® UltraRed as per manufacturer's protocol (ThermoFisher). Bone marrow macrophages were seeded at $1x10^5$ cells per well in a 96-well plate stimulated for 1 hour with assay buffer containing 10 mg/mL zymosan (Sigma-Aldrich). Amplex® UltraRed was added to a final concentration of 10 ng/mL with 1 unit of horseradish peroxidase (Sigma-Aldrich) to each well for 15 min and fluorescence was measured using a GENios Pro microplate reader (Tecan Group Ltd.).

Real-time spectrofluorometric assays of the phagosome

Real-time spectrofluorometric assays[507–510] were employed to investigate phagosomal reactive oxygen species production, acidification, hydrolysis, and phagolysosome fusion in bone marrow derived macrophages. Bone marrow macrophages were seeded at $1x10^5$ cells per well in 96 well black, flat- and clear-bottom tissue culture treated plates (Falcon™). Assays were performed at 37°C in assay buffer (tissue culture grade PBS, 1 mM$CaCl_2$, 2.7mM KCl, 0.5 mM $MgCl_2$, 5mM dextrose and 0.25% gelatin) and fluorescence measurements were taken every 120 seconds. Assays used 3 µm silica particles conjugated to fluorophores, substrates or reporters required for each assay[507–510]. Oxygen species production by NOX2 was quantified by measured increasing fluorescence released by oxidized H2HFF-OxyBURST (Life Technologies) relative to the calibration fluorophore AF594 (Life Technologies)[508]. Phagosomal acidification was calculated by recording the ratio fluorescence emissions of CFSE (Life Technologies) at different excitation wavelengths (490 and 450nm on Fluostar OPTIMA, BMG Labtech) and using a

polynomial regression of a standard curve[511]. Bulk protease activity was measured by labelling BSA coated beads with self-quenching fluorophore DQ green BODIPY (Life Technologies). The substrate-liberated fluorescence was measured relative to the calibration fluorophore Alex Fluor 594 (Life Technologies) on a Flex Station microplate reader (Molecular Devices). Phagolysosomal fusion was quantified by measuring the fluorescence resonance energy transfer (FRET) between donor fluorophore Alexa Fluor 488(Life Technologies) to a fluid-phase acceptor fluorophore Alexa Fluor 594 (Life Technologies) using the Fluostar OPTIMA plate reader. Bone marrow derived macrophages were loaded with acceptor fluor at 50 μg/mL for 18 h then chased for 4 h prior to the assay.

TNF stimulations and neutralization

Macrophages were treated 1 hour prior to bacterial killing and phagolysosomal fusion assays with recombinant mouse TNF (BioLegend) in serum-free RPMI at 1, 10 and 100 ng/mL. Anti-TNF (MP6-XT22, BioLegend) or Rat IgG1k (RTK2071,BioLegend) were used at 5 μg/mL in PBS throughout the bacterial killing assay.

Statistical Analysis

Unless otherwise stated in the figure captions, data were analyzed using a two-way ANOVA with a Fisher's LSD post-test to test the effect of age and TNF. A two-tailed Mann-Whitney test was used to test differences between young and old bone marrow derived macrophages and one-way ANOVA with a Tukey HSD post-test was

used to test the effect of TNF doses. Data were analyzed with Prism (Version 6; GraphPad, San Diego, CA, USA).

Results:

In the previous section we found age-associated TNF influenced myeloid differentiation, which we believe results in changes to monocyte and macrophage function. Monocytes play an important role in their ability to recognize and bind *S. pneumoniae* and induce pro-inflammatory cytokines. We have demonstrated that bone marrow derived macrophages from old mice have impaired bacterial killing and that it is driven by age-associated increases in TNF, as macrophages derived from the bone marrow of old TNF KO mice preserve their ability to kill bacteria. We wanted to determine if deficits in bacterial binding and internalization were evident at the monocyte stage, prior to differentiation into a macrophage. To test this we incubated whole blood with fluorescently labelled *S. pneumoniae* for an hour and quantitated bacteria bound to monocytes and neutrophils by flow cytometry. Monocytes and neutrophils did not show any difference in the number of bacteria they could bind or in the percentage of the population that bound bacteria with age (Fig. 6A, C, E, G); however, fewer monocytes and neutrophils from old mice were able to internalize bacteria when compared to young monocytes (Fig 6D&H). Of the monocytes and neutrophils that did internalize bacteria, they internalized fewer per cell than their younger counterparts (Fig. 6B&F). TNF did not influence bacterial

binding and internalization in monocytes. We can conclude that the age-associated decrease in bacterial internalization by monocytes is driven by age and independent of TNF.

Macrophages from old mice have impaired bacterial killing compared to young mice even after normalizing for lower phagocytosis. This indicates that there is an intracellular killing mechanism that is impaired with age. Bacterial killing by macrophages is mediated in many different ways, but many of these mechanisms occur within the phagolysosome[512]. We hypothesized that phagosome maturation and function was impaired with age. To determine which mechanisms change with age we measured lysosomal acidification, proteolysis, oxidation and the rate of phagolysosomal fusion. There was no difference in either the rate of acidification or the final pH reached (Figure 7C). We found that oxidation and proteolysis were higher in old macrophages than in young (Figure 7A&D). The assays were done with mannosylated and maleylated beads to target uptake by the mannose receptor or by scavenger receptors, respectively. Both beads showed similar results, however the magnitude of difference between young and old macrophages was larger following mannose-mediated uptake.

The most interesting finding was that the rate of phagolysosomal (PL) fusion was lower in old mice (Figure 7E). Since old TNF KO mice preserve their ability to kill bacteria, we hypothesized that they similarly maintain their PL fusion capacity, which they did (Figure 8A). We next tested whether reducing TNF would ameliorate

bacterial killing by old macrophages. We incubated macrophages with anti-TNF antibody for an hour and continued the neutralization for the duration of the killing assay and we found that reducing TNF improved bacterial killing by old macrophages (Figure 8B). This suggested that acute exposure to TNF may be sufficient to impair PL fusion and bacterial killing. To test this we pre-treated young WT macrophages for 1 hour with TNF at 1, 10 or 100ng/mL. What we found was that 1 and 10 ng/mL of TNF significantly slowed PL fusion, but only 1ng/mL impaired bacterial killing (Figure 8C&D).

Discussion:

TNF does not seem to be involved in the decreased bacterial phagocytosis with age in monocytes and neutrophils. We have found decreases in both opsonic (e.g. whole blood assay) and non-opsonic phagocytosis (e.g. macrophage killing assay) with age. This could be due to altered expression of phagocytic receptors, their signalling, cytoskeletal rearrangement, or membrane fluidity. We found no significant difference in binding, suggesting that phagocytic receptor expression is not different with age. A study on alveolar macrophages showed that signalling downstream of phagocytic receptors that mediates cytoskeletal rearrangement is impaired with age[513]. This sort of signalling can also be influenced by lipid membrane composition which changes with age[514].

During homeostatic conditions, macrophages need to degrade and clear internalized debris and apoptotic bodies[510]. To do this they maintain a high level of proteolysis. During infections, when macrophages are activated they switch from relying heavily on proteolysis to using oxidative and nitrosative mechanisms[510]. In DC's, reduced acidification and proteolysis have been shown to enhance antigen presentation[515]. Thus the increased proteolysis, and unchanged acidification, we found in old macrophages may contribute to impaired antigen presentation with age[516]. Macrophages induced to produce high levels of reactive oxygen species are not able to efficiently process and present antigen[511]. Consistent with previous studies[517,518], we found a higher rate of substrate oxidation in old macrophages (Fig. B), which provides another mechanism by which antigen presentation may be impaired with age.

Phagolysosomal fusion is critical for the transformation of the phagosome into a highly bactericidal environment. It delivers various antimicrobial peptides, lipases, hydrolases, proteases, as well as V-ATPases which lead to acidification[512]. Although early phagolysosomal fusion is decreased in old BMDMs, the remaining phagosomal antimicrobial mechanisms (i.e. acidification and proteolysis) were preserved, or even increased in the case of ROS production. We cannot explain why all other mechanisms remain intact while PL fusion does not; however, future studies examining trafficking of proteases, V-ATPases and NADPH oxidase may be informative in this regard. Proteolysis has been found to decrease with age, leading to an accumulation of protein targeted for degradation in the cytosol of aging

cells[517,518]. Our assay specifically measures the activity of cysteine proteases in the phagosome [507], which may explain why we find that proteolytic activity increases rather than decreases. Hydrolase activity is not the rate-limiting step in lysosomal proteolysis; however, improper acidification can inhibit hydrolase activity[515,519], and we show that it remains intact in our system.

The decreased rate of early PL fusion correlated with decreased bacterial killing of *S. pneumoniae* and was dependent on exposure to TNF, but the dose was important. TNF was not required for bacterial killing as young TNF KO macrophages kill similarly to young WT macrophages. Although chronic, lifetime exposure to TNF impairs bacterial killing, acute exposure to TNF is sufficient to slow PL fusion and impair bacterial killing. Young macrophages exposed to 1 and 10 ng/mL of TNF recapitulated the aging phenotype; however, neutralization of TNF only improved killing for old macrophages and not young. These data suggest that there is a threshold past which TNF is inhibitory to PL fusion and bacterial killing. A similar phenomenon is observed with TNF's affect on bacterial killing by neutrophils, where it follows a sigmoidal pattern and is inhibitory at high concentrations[520]. Physiological concentrations of TNF are very low, with serum concentrations at the higher end in patients surviving acute sepsis going up to 5 ng/mL[521] . Experimental studies show maximal induction by 10 ng/mL[86]. The TNFRI has a high affinity for soluble TNF, suggesting that it can detect low levels of TNF[522]. In the future the dose at which TNF inhibits bacterial killing or PL fusion can be titrated by treating young TNF KO macrophages with different doses. Although we do not know the mechanism

by which TNF exerts its effects, we hypothesize it acts through signalling and post-translational protein modifications due to the short time frame in which we saw changes (1 hour), TNF can signal through two receptors and by blocking either of them and adding TNF back into our system we can determine which receptor it signals through, narrowing down our candidate signalling pathways. PL fusion is dependent on many different factors including Akt/PI3K and NFκB activation[512]. How the dose of TNF influences the strength of NFκB activation and the induction of negative feedback responses remain unknown[523–525]; however, PTEN negatively regulates P13K/Akt/NFκB signalling by dephosphorylating PI(3)P[526]. TNF has been shown to induce PTEN at a concentration of 1 ng/mL[527]. It could be that TNF stimulation in that range induces PTEN to inhibit PI(3)P, which is needed for early phagosome maturation and thus may inhibit phagolysosomal fusion.

Our findings suggest that modulating inflammation, specifically TNF, may influence anti-bacterial immunity. Even when the elderly survive a bacterial pneumonia, they are at an increased risk of mortality and it accelerates the acquisition and progression of chronic diseases[528]. This is likely mediated by the elevated and prolonged inflammatory insult caused by the infection. By modulating inflammation and reducing disease severity in the elderly with bacterial pneumonia, there is potential to mitigate chronic disease thereafter.

Figures:

Figure 1. Myeloid progenitors and monocytes differentially express cKit, Flt-3 and CD115 with age. Myeloid progenitors in the bone marrow and splenocytes and monocytes in the blood of young (2mo) and old (20-24mo) WT and TNF KO mice were analyzed by flow cytometry for their expression of cKit, Flt-3, CD115 shown as geometric mean fluorescence intensity on the y-axis of all graphs. CMP-common myeloid progenitor; MDP- macrophage and dendritic cell progenitor; cMoP – committed monocyte progenitor. Statistical analyses were performed using a two-way ANOVA with a Fisher's LSD post-test (*p<0.05, **p<0.01, ***p<0.001; n=5-7 per group)

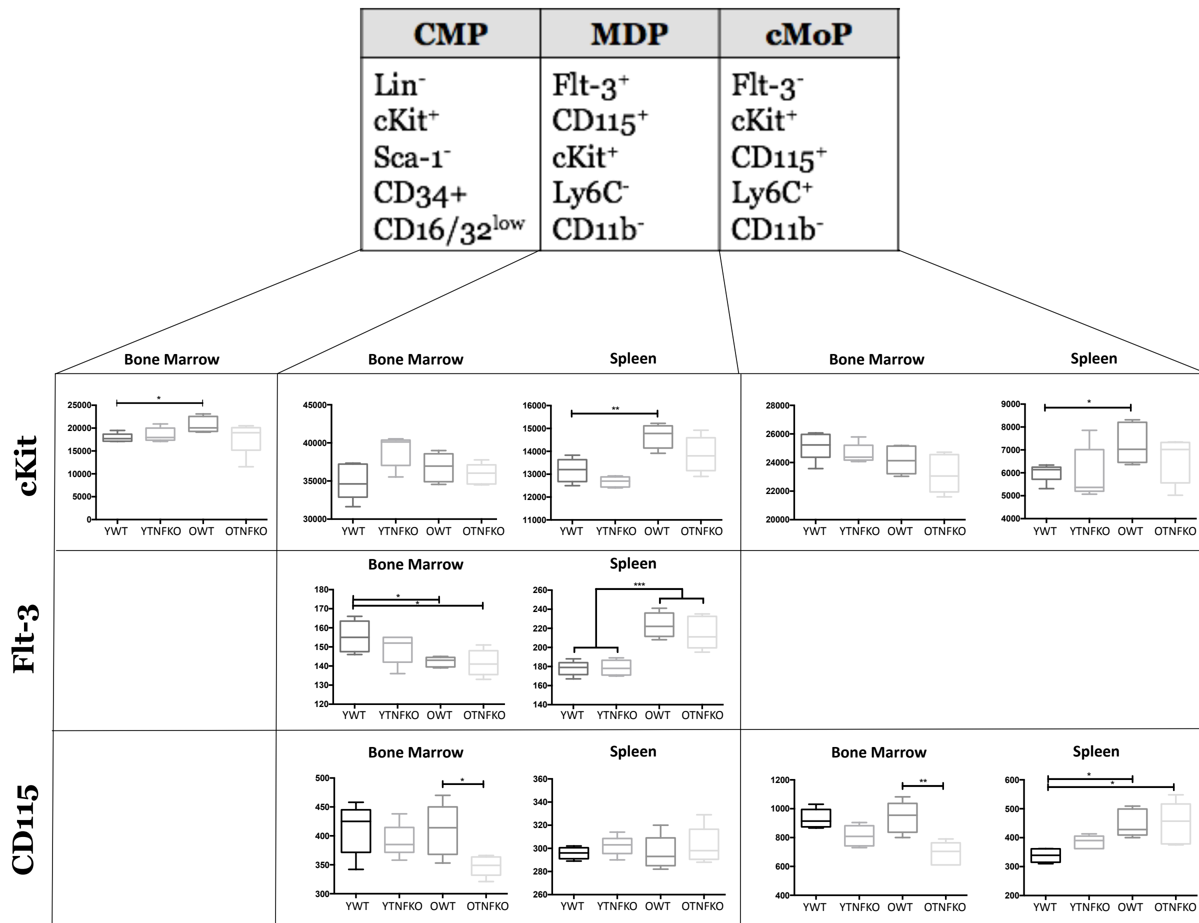


Figure 2. CD115 and CSF-1 are elevated on monocytes and in tissue with age. Surface expression of CD115, as geometric mean fluorescence intensity, on Ly6C^{high} monocytes and Ly6C^{low} monocytes(A) and bone-marrow derived macrophages(B) from young(3mo) and old(20mo), WT and TNF KO mice (n=4-5 per group). C-F) Expression of CD115 and CSF-1 quantified by RNA sequencing of alveolar macrophage (single-cell) and whole lung tissue isolated from young(2mo) and old(22mo), WT and TNF KO mice (n=3 per group). logCPM-log counts per million. Statistical analyses were performed using a two-way ANOVA with a Fisher's LSD post-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

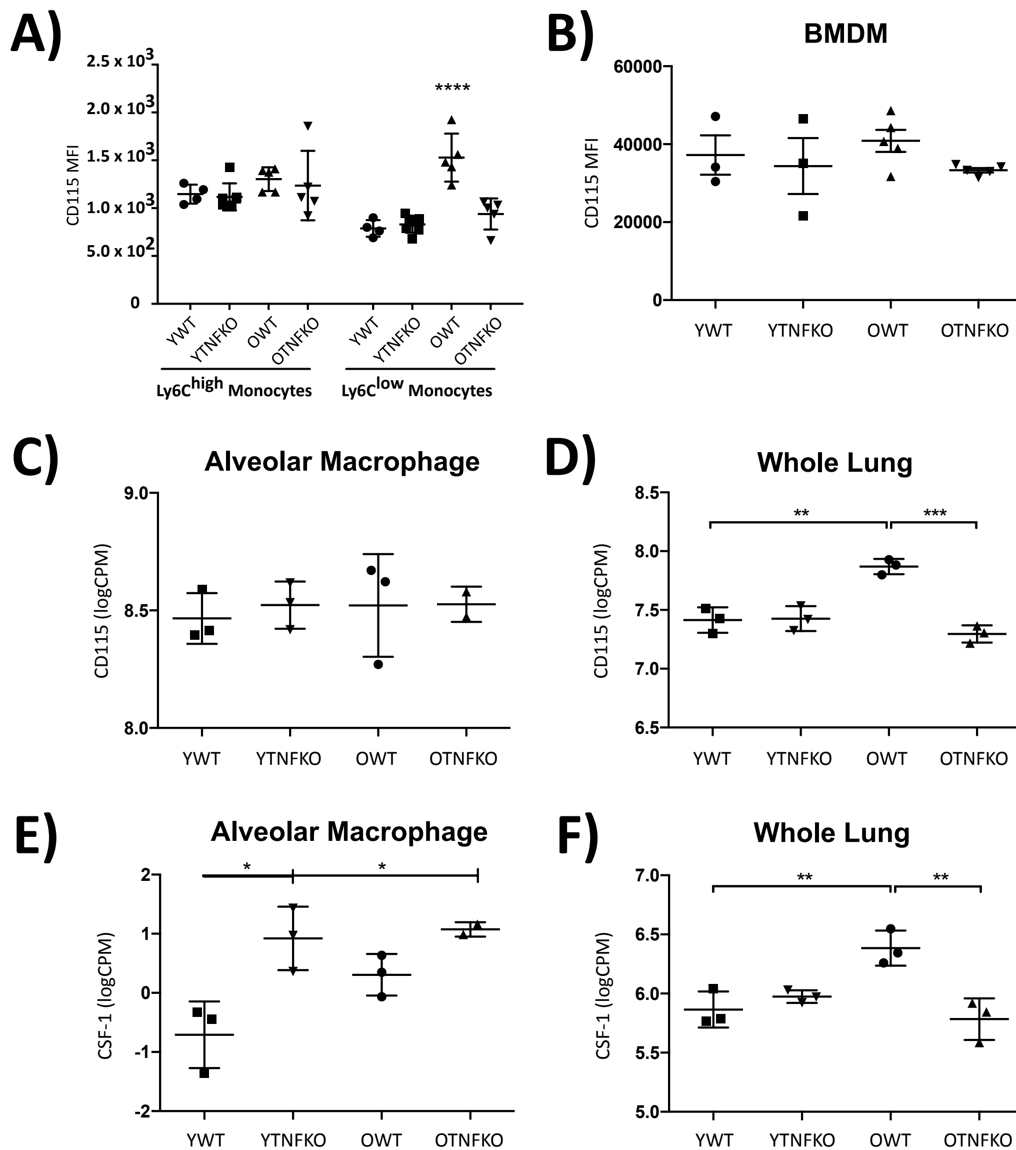


Figure 3. Preliminary transcriptomic analysis of Ly6C^{high} monocytes. A) Principal component analysis performed on differentially expressed genes in Ly6C^{high} monocytes sorted from young(2mo) and old(22mo), WT and TNF KO (n=3 per group) B) Genes differentially expressed with age and not altered in TNF KOs suggest there are age-associated changes mediated by TNF at the transcriptional level. HDAC1- histone deacetylase 1; FOXO3- forkhead box O3. CPM- counts per million. Genes passed a false discovery rate cut off of 5% following a one-way ANOVA. **Figures generated by Dr. Deborah Winter*

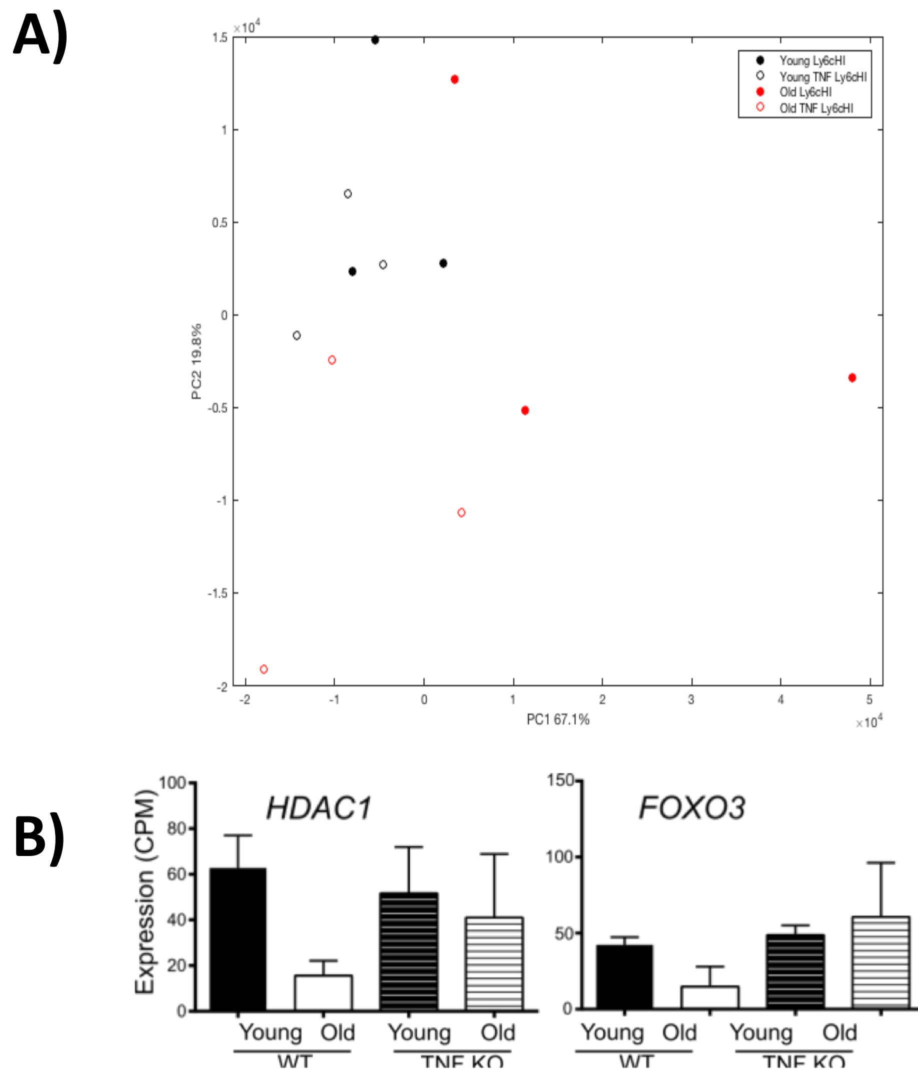


Figure 4. Age-associated TNF influences expression of genes important to myeloid cell differentiation in alveolar macrophages. Genes differentially expressed by single-cell sorted alveolar macrophages from young (2mo) and old(22mo), WT and TNF KO mice (n=3 per group) belonging to the gene ontology process for myeloid cell differentiation are shown here. The block of genes surrounded by the box indicate those that are decreased with age, but not in old TNF KOs. Genes passed a false discovery rate cut off of 5% following a one-way ANOVA.

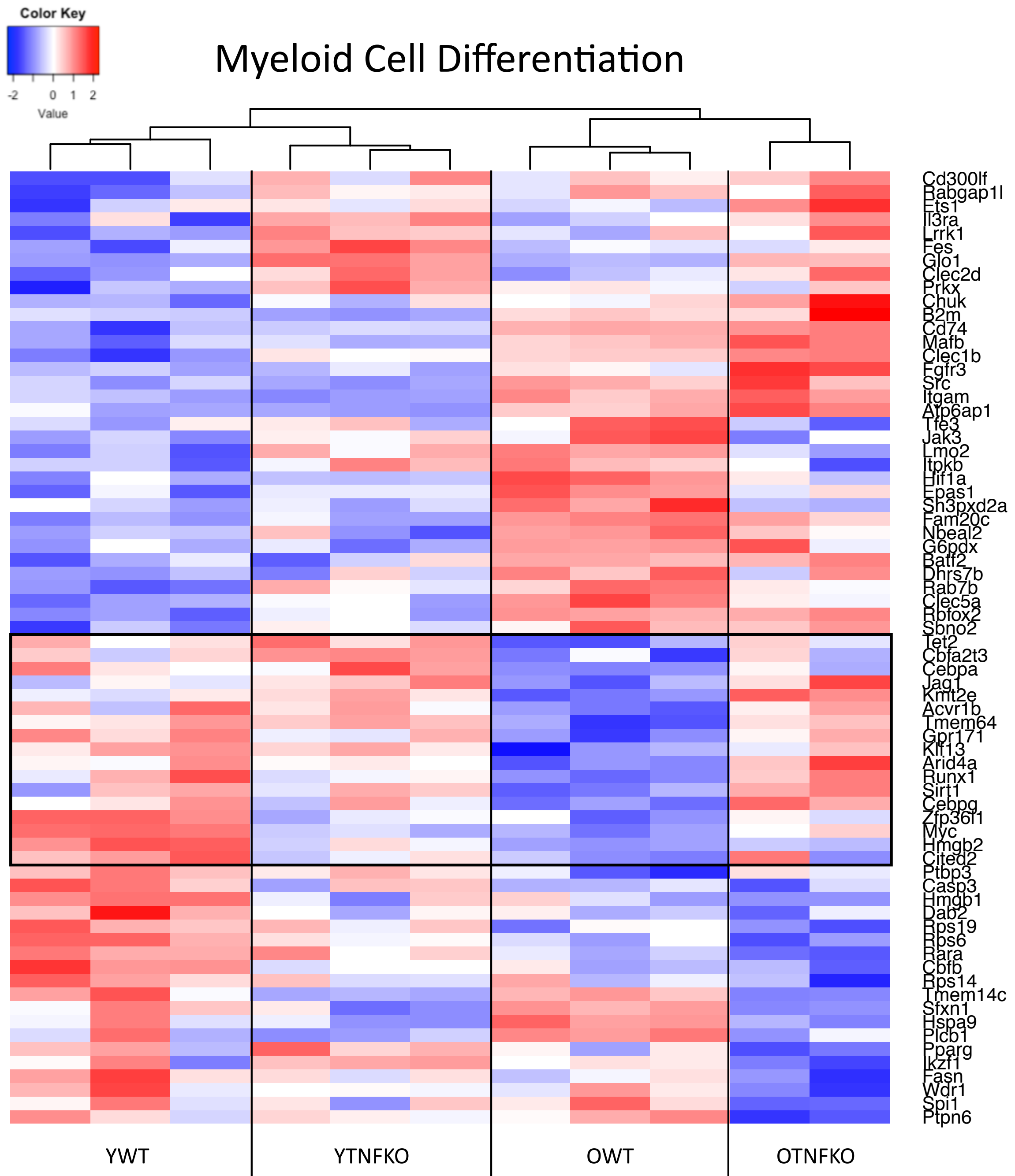


Figure 5. Myeloid development and metabolic genes are differentially expressed with age-associated inflammation in alveolar macrophages. Expression quantified by RNA sequencing of alveolar macrophages isolated from young(2mo) and old(22mo), WT and TNF KO mice (n=3 per group). logCPM- log counts per million; Tet2-methylcytosine dioxygenase 2; Idh1-isocitrate dehydrogenase 1; Hif-1α-hypoxia-inducible factor 1-alpha. Statistical analyses were performed using a two-way ANOVA with a Fisher's LSD post-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

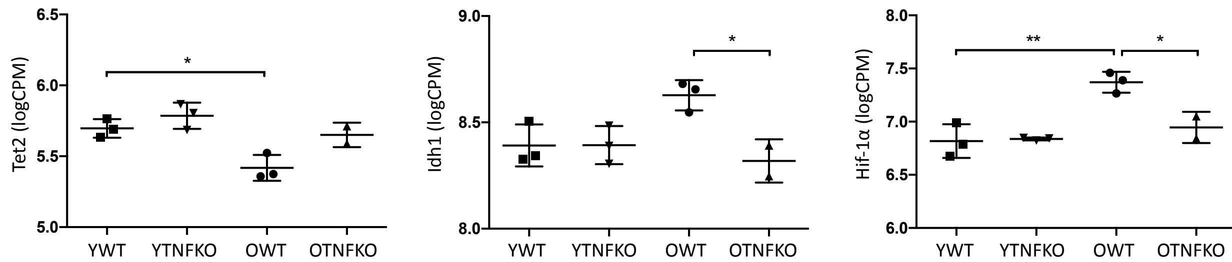


Figure 6. Monocyte and neutrophil phagocytosis decreases with age independent of TNF. Whole blood from young (2mo) and old (16-24mo) WT and TNF KO mice was incubated with TRITC-labeled, heat-killed *S. pneumoniae* P1547 at an MOI of 50 at 4°C(binding: A,C, E, G) and 37°C(binding and internalization: B, D, F, H) for 1h . Samples were stained to differentiate monocytes (Ly6C$^+$; A-D) and neutrophils (Ly6C$^+$ Ly6G$^+$; E-H) by flow cytometry. The proportion of cells positive for TRITC (C, D, G, H) and the quantity of bacteria associated per cell (quantified by geometric mean intensity of TRITC; A,B,E,F) were analyzed. Statistical analyses were performed using two-way ANOVA with a Fisher's LSD post-test (**p<0.01, ****p<0.0001; n=7-9 per group, two independent experiments).

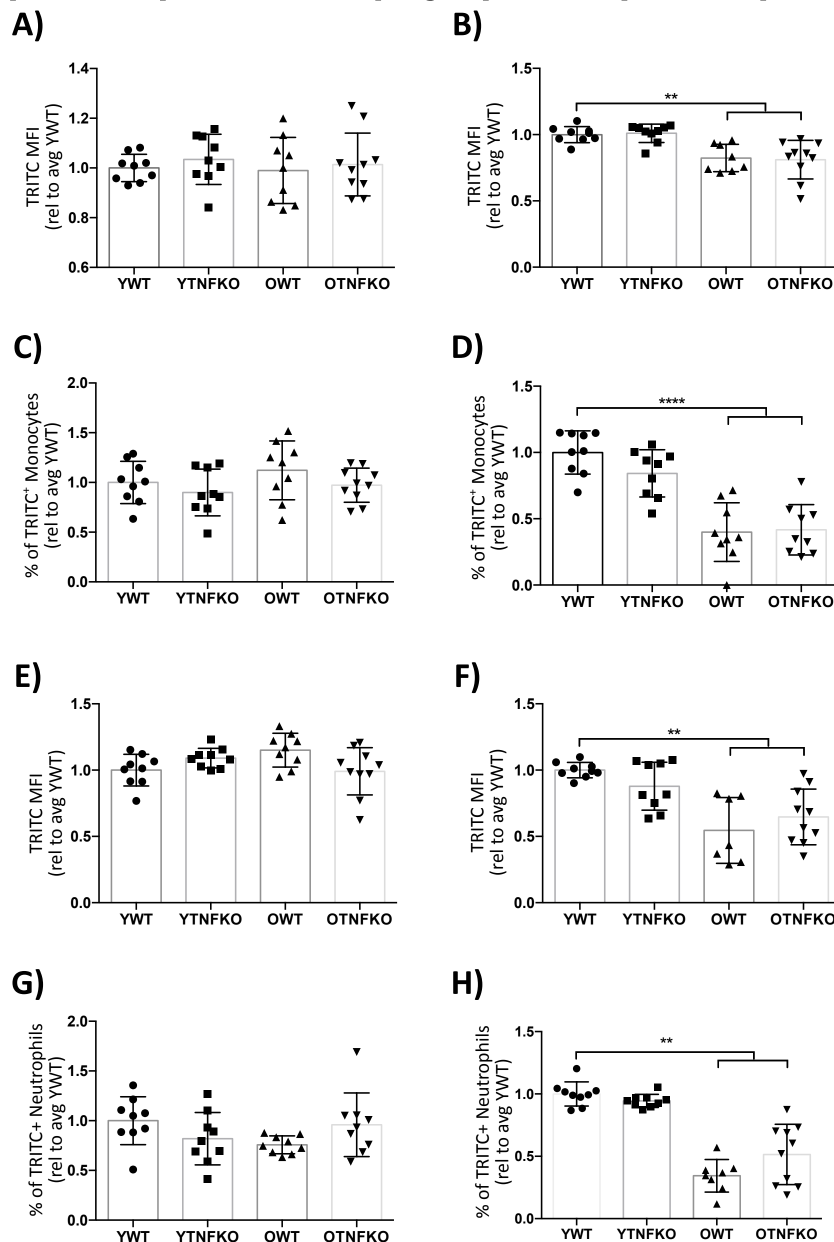


Figure 7. Age-associated changes to phagolysosomal function. Phagosomal oxygen species production(A), global H_2O_2 production(B) and the rate of proteolysis(D, early:40-60mins, late:70-90mins) are elevated in bone marrow derived macrophages from old mice (18mo) mice compared to young (3mo). (C) No difference was seen in phagosomal pH between young and old macrophages. (D) The rate of early phagolysosomal fusion (20-40mins) was lower in old compared to young macrophages. Results are pooled from 1-3 independent experiments with n=4 for young and n=5 for old macrophages. Statistical significance was determined using two-tailed Mann-Whitney test (*p<0.05, **p<0.01).

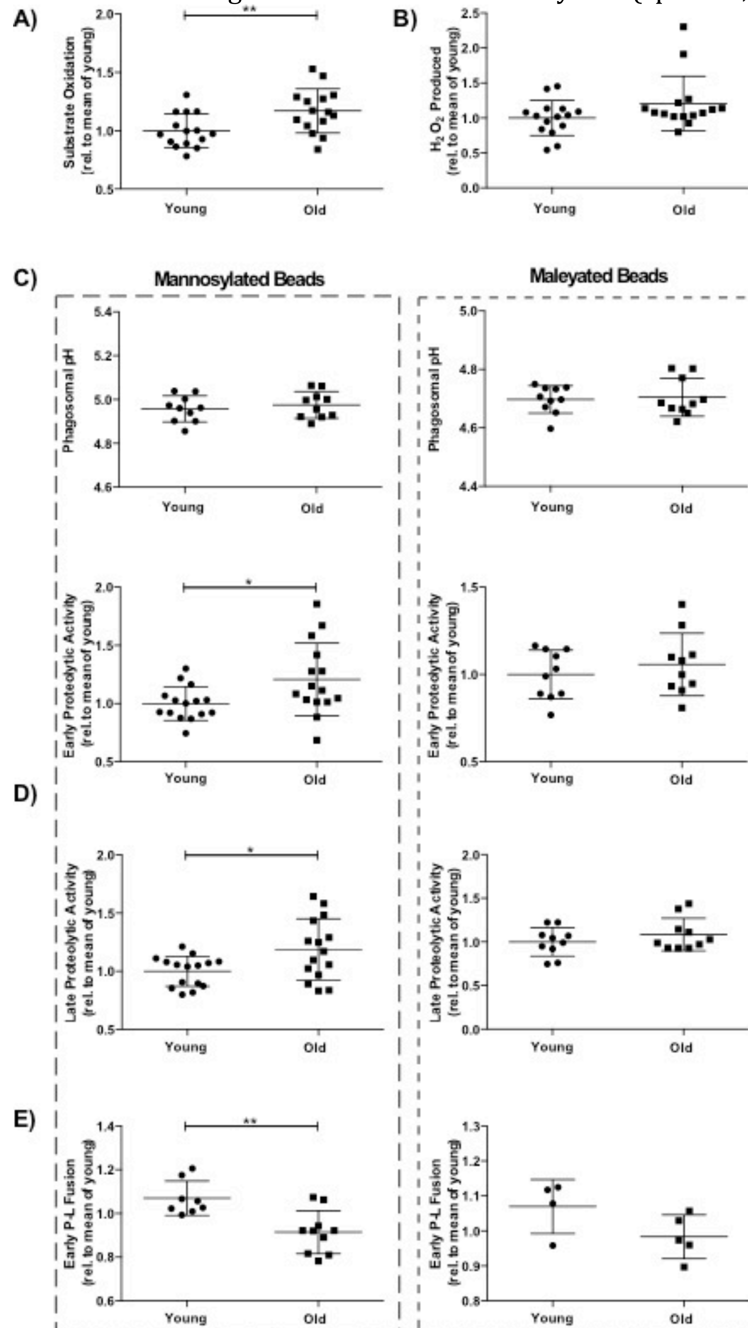
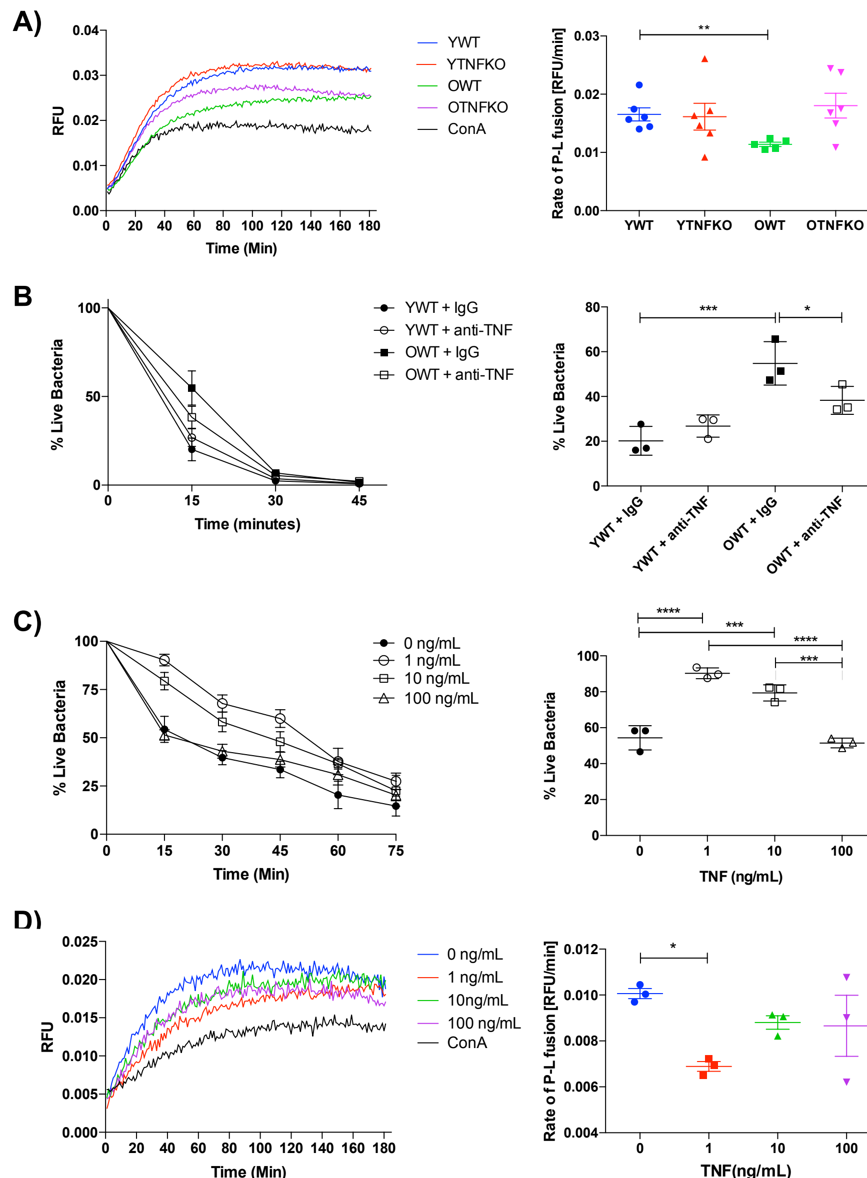
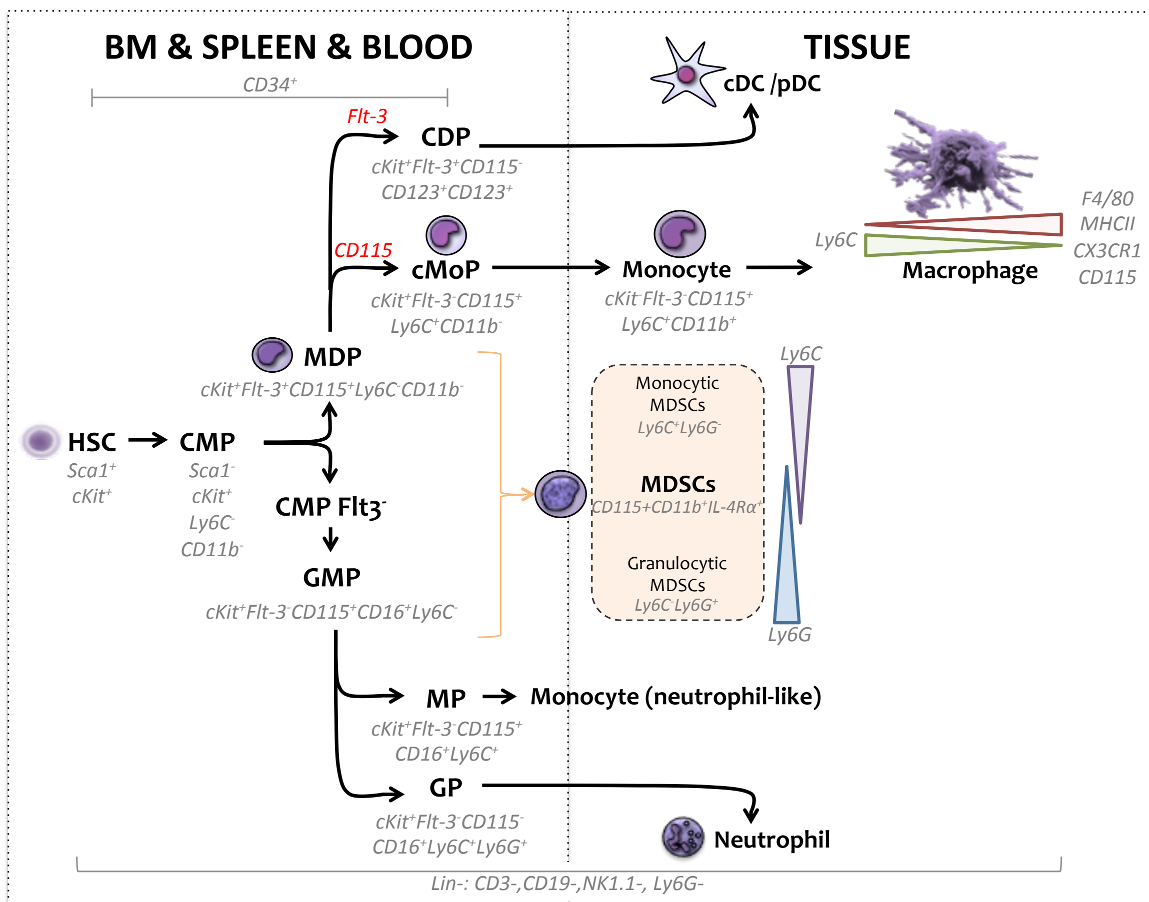


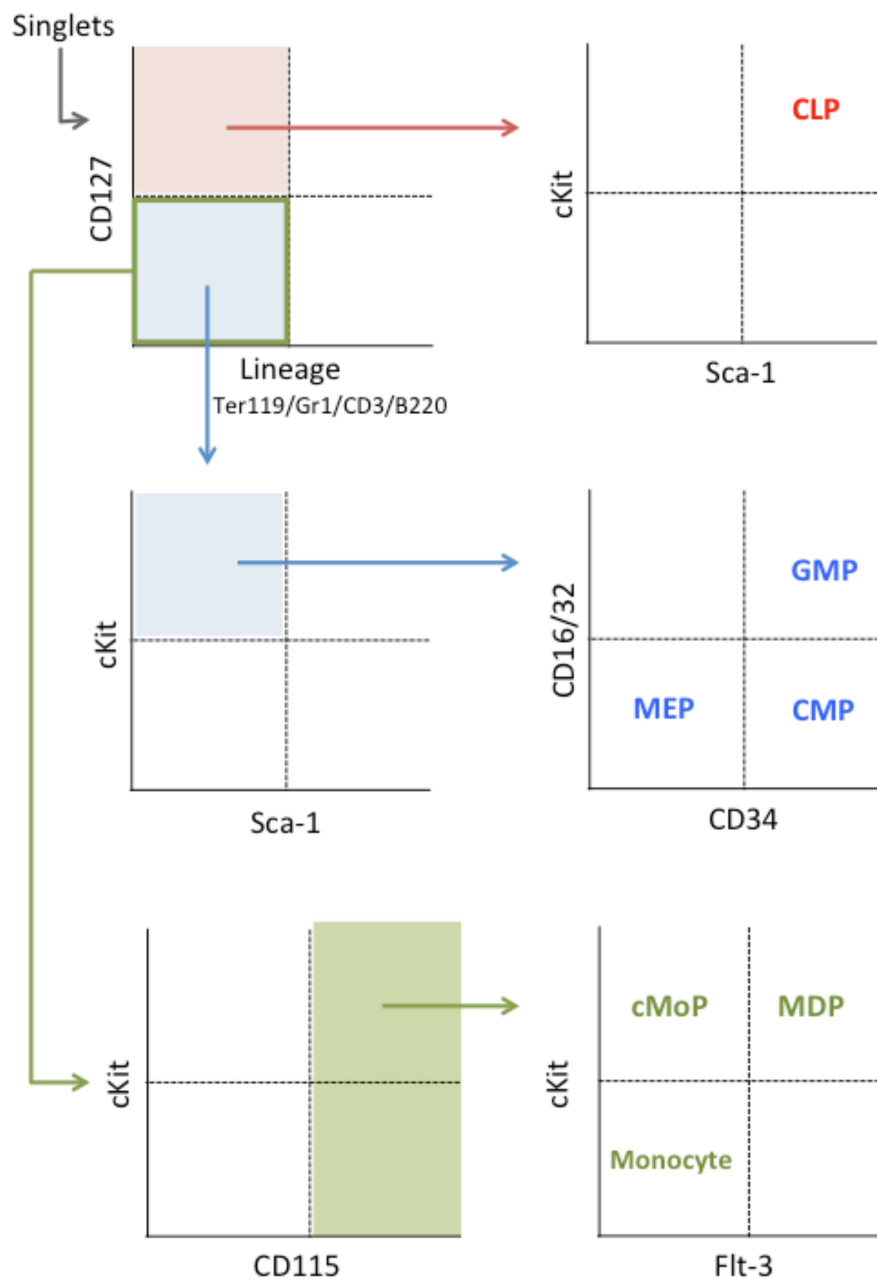
Figure 8. Acute and chronic TNF impairs phagolysosomal fusion by macrophages. A) Representative curves show the degree of phagolysosomal fusion (measured in relative fluorescence units, RFU) in young (3mo) and old(20-24mo), WT and TNF KO bone marrow derived macrophages. The rate of PL fusion was lowest in old WT macrophages, but maintained in old TNF KO. B) Young and old macrophages were incubated with *S. pneumoniae* at an MOI of 100 in the presence of 5ug/mL of anti-TNF or an IgG control. The number of viable *S. pneumoniae* at 15mins was lower in old anti-TNF treated macrophages compared to IgG treated. Young WT macrophages were treated with 0, 1, 10 or 100ng/mL TNF for 1 hour prior to assaying bacterial killing(C) and PL fusion(D). Doses of 1 and 10ng/mL both abrogated PL fusion and bacterial killing. Concanamycin A (conA)- PL fusion inhibitor(100nM). Statistical analyses were performed using a two-way ANOVA (A&B) or a one-way ANOVA (C&D). (* p<0.05, **p<0.01, ****p<0.0001).



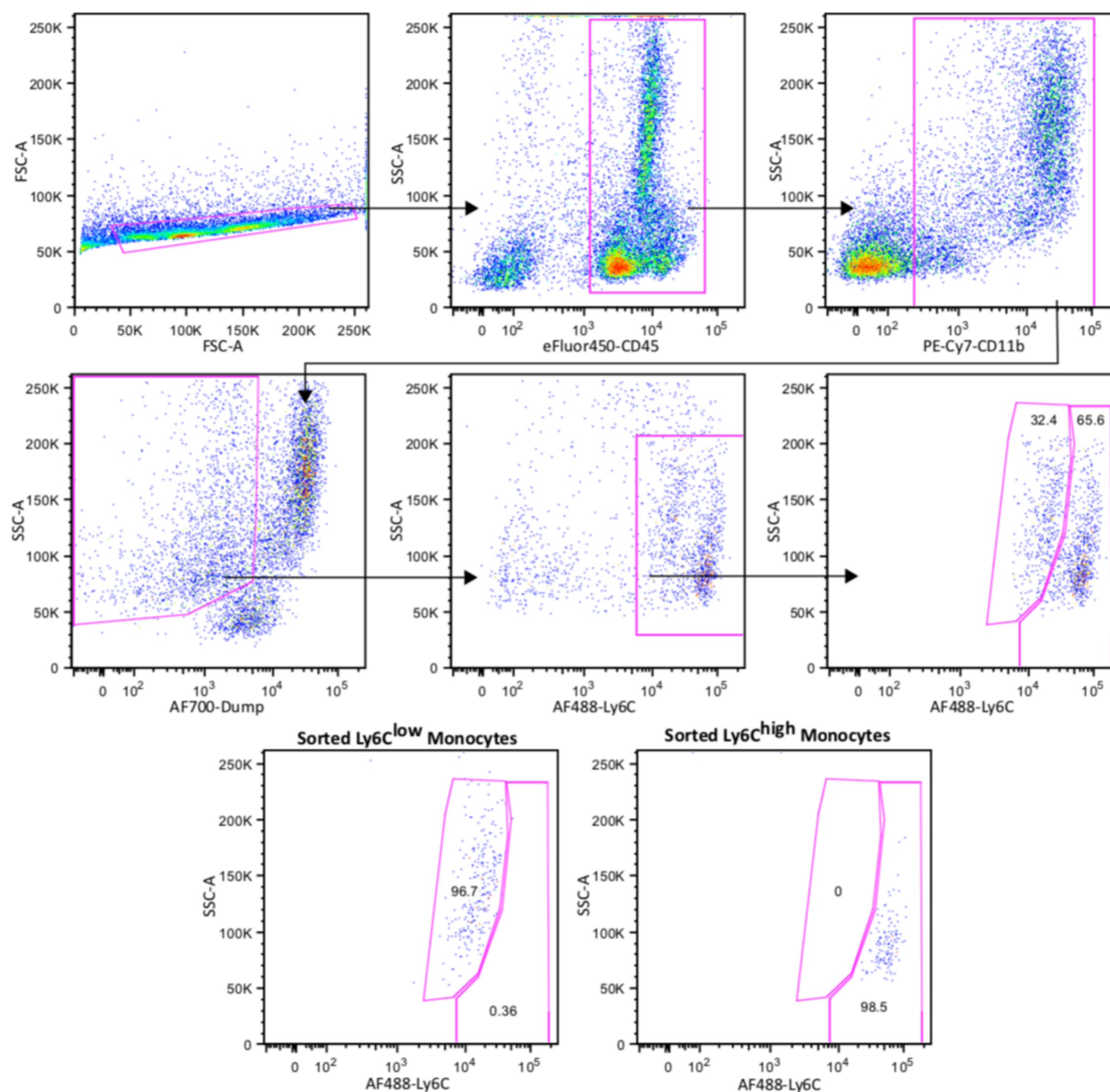
Supplemental Figure 1. Myeloid development progression and surface markers. The progression of myeloid development in mice from the HSC to terminally differentiated dendritic cells, macrophages and neutrophils are summarized in this figure. Hematopoiesis can occur in the bone marrow as well as in extramedullary tissues like the spleen. Surface markers used to discriminate between different myeloid progenitors are shown in grey text. Signalling through Flt-3 on the MDP leads to the differentiation of dendritic cells, while signalling through CD115 leads to the differentiation of monocytes. In the event of acute and chronic inflammation myeloid derived suppressor cells accumulate and exist as monocytic or granulocytic forms based on differential expression of Ly6C and Ly6G. As monocytes mature into macrophages, they lose expression of Ly6C and gain expression of F4/80, MHCII, CX_3CR_1 and CD115.



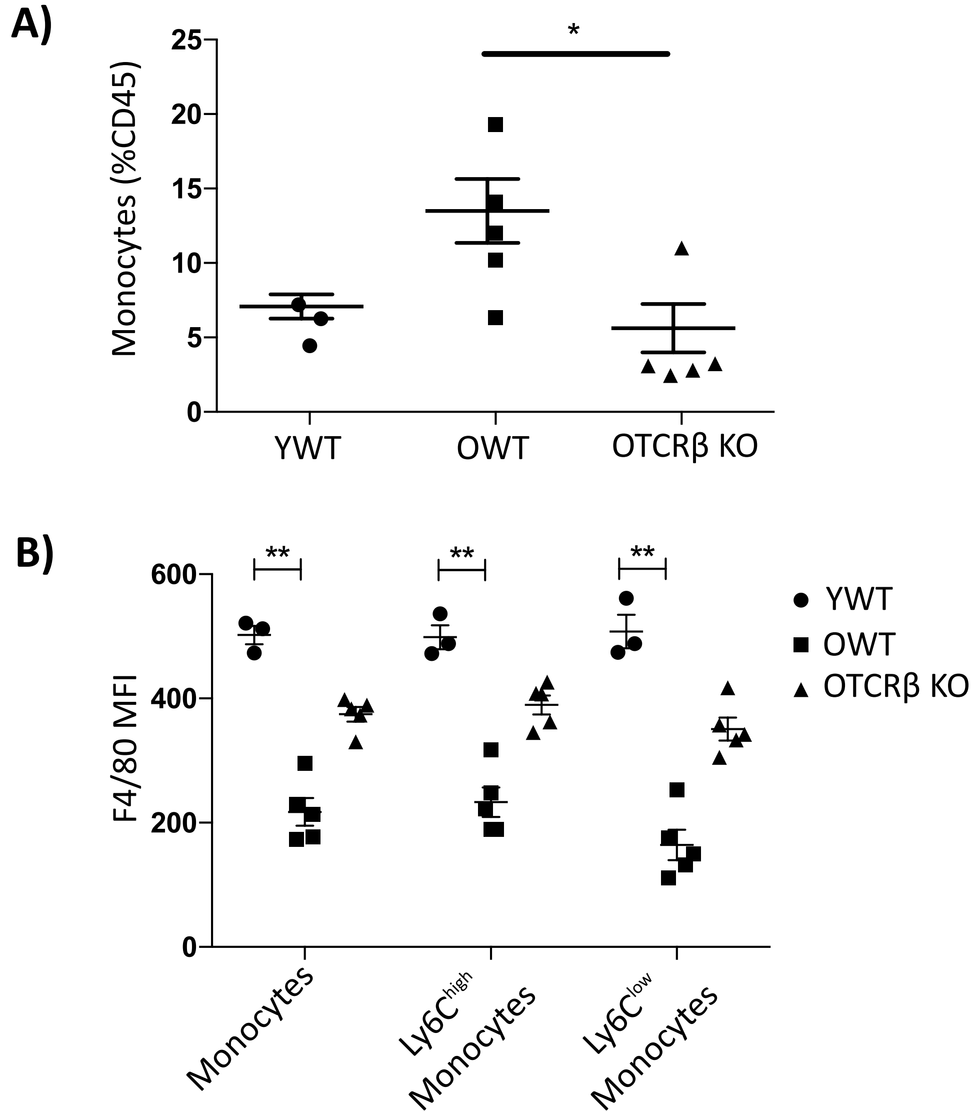
Supplemental Figure 2. Myeloid progenitor gating strategy.



Supplemental Figure 3. Ly6C^high and Ly6C^low monocyte sorting. Gating strategy and purity of sorted populations from murine bone marrow.



Supplementary Figure 4. TCRβ KOs do not display age-associated differences in monocyte numbers and maturity. Monocyte proportions (A) and surface expression of maturity marker F4/80(B) were quantified by flow cytometry from whole blood taken from young (2mo) and old (12mo) WT mice and old(12mo) TCRβ KOs. MFI – geometric mean fluorescence intensity. Statistical analyses were performed using a one-way ANOVA (*p<0.05, **p<0.01).



CHAPTER 6

Discussion

Individuals with higher than age-average levels of inflammatory cytokines (e.g. TNF, IL-6, IL-1β) and biomarkers (e.g. CRP, ESR) are at an increased risk of developing chronic diseases[529]. The biological mechanisms driving these epidemiological associations are not well understood. Monocytes and macrophages contribute to the pathology of many chronic diseases that increase in incidence with age. Inflammation increases the rate of myeloid cell production, which is protective in acute settings, but when it is chronic it compromises myeloid cell differentiation and function and leads to disease. The purpose of this thesis was to investigate how chronic, age-associated inflammation contributes to immunosenescence by influencing monocyte and macrophage development and function. We hypothesized that age-associated inflammation chronically stimulates emergency myelopoiesis. We further posited that the accelerated production of myeloid cells would result in impaired differentiation of monocytes, which would influence their function and persist upon differentiation into a macrophage.

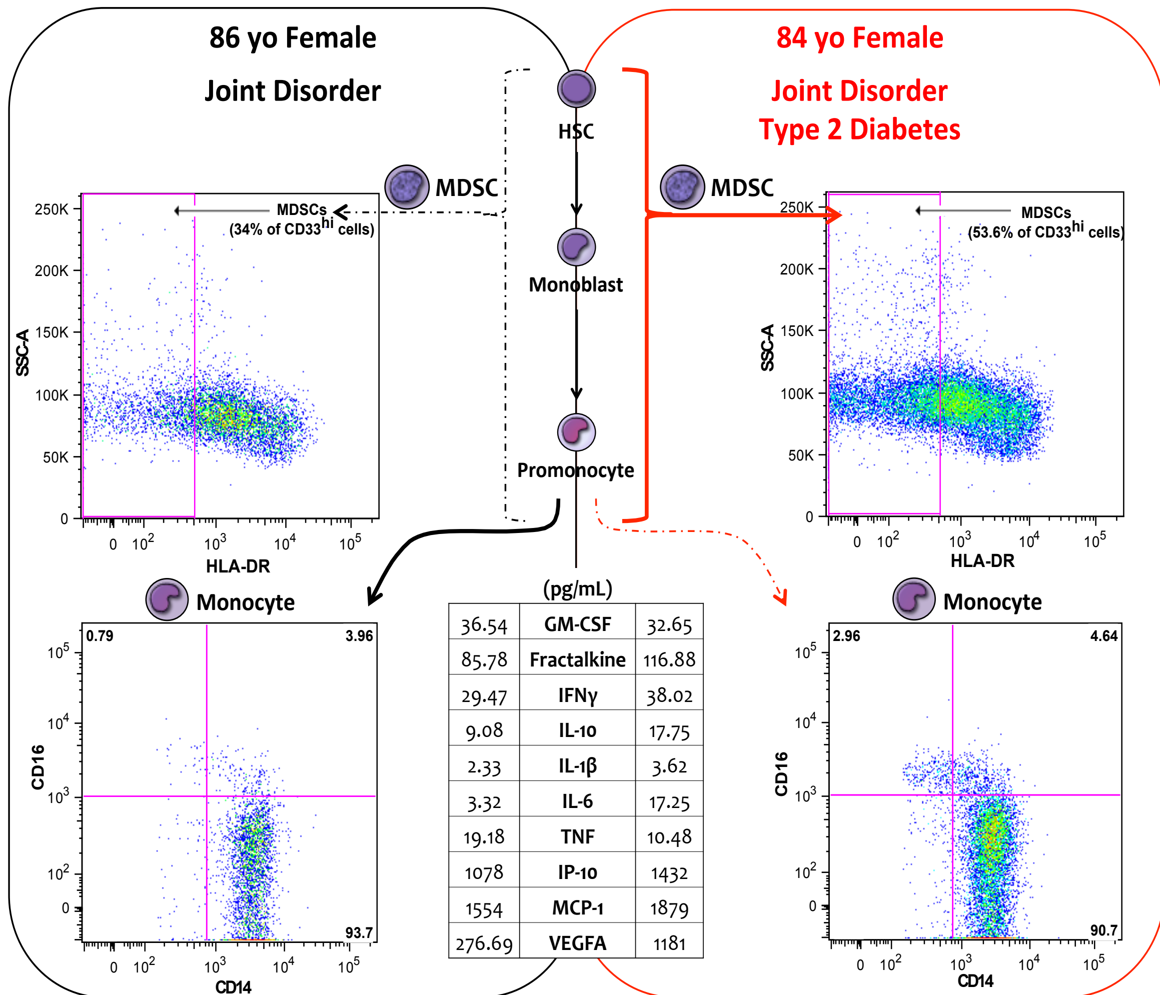
Inflammation and disease shape the myeloid compartment in humans

Ageing is a heterogeneous process that is due to the diversity in individuals' genetics and environmental exposures. It is nearly impossible to study aging in the absence of chronic disease. Although some aging cohort studies address this issue by recruiting "healthy" elderly using the SENIEUR protocol, it is not a perfect solution[530]. The protocol excludes individuals with chronic conditions like atherosclerosis, hypertension, and cardiac insufficiency, as well as those with higher than the age-average clinical measures of metabolic and kidney function, and inflammation. The findings in these studies become difficult to apply to the broader population, thus in Chapter 2 we chose to include individuals with health conditions and limited our exclusion criteria to no recent infections, surgery or major injuries. This allowed us to include health conditions in our analysis and better understand whether inflammation, disease, or age were stronger correlates of myeloid development, monocyte maturity, activation, and trafficking. Our cohort was representative of chronic conditions and their age distribution in the Canadian population. In Chapters 3 and 4 we compared how chronic inflammation due to disease (e.g. RA and OA) changed the monocyte compartment compared to healthy age- and sex-matched controls. In Chapter 3, we were able to determine which of the changes in monocytes were a result of chronic inflammation, as they were reversed following DMARD therapy. Across our three human cohorts we were able to consistently find inflammation significantly remodels circulating monocytes.

In Chapter 2, we found that after controlling for the number of health conditions, the only immune parameters associated with age were MCP-1, IP-10 and the increase in myeloid cells, particularly non-classical monocytes, with a concomitant decrease in lymphoid cells. Non-classical monocytes produce the highest levels of TNF after TLR1/2 and TLR7/8 stimulation[112,190]. In mice, TNF is necessary for monocyte survival and maturation from a $Ly6C^{high}$ to a $Ly6C^{low}$ monocyte[429]. It may be that in humans, the increase in $CD16^{+}$ monocytes with age is a result of their increased survival due to increased production and autocrine signalling of TNF. Non-classical monocytes exhibit a stronger senescent phenotype compared to classical and intermediate monocytes[531], which may be because they have the longest half-lives in circulation[116]. Future studies are needed to investigate how the half-life of non-classical monocyte changes with age and whether this contributes to their expansion with age.

Interestingly, we found no significant association between health conditions and serum cytokines. The health conditions reported in our cohort like cardiovascular disease[532,533], type 2 diabetes[534], and joint disorders[535] are characterized by elevated serum cytokines. This could be due to the fact that every health condition is characterized by a unique pattern of cytokines in addition to our sample size being too small. Unfortunately we did not collect information on disease activity and length of disease duration, which can influence inflammation. We also found that health conditions were significantly associated with an elevated number of MDSCs. These cells are increased in cancer[536,537], trauma[538], sepsis[539], infection[540]

and autoimmunity[259], as well as with age[265,330,541]. Elevated inflammation is common to all of these conditions. MDSCs provide a way by which the immune system can modulate an elevated inflammatory state by producing anti-inflammatory factors (i.e. IL-10, TGFβ, NO, ROS) and inhibiting T cell responses[248]. Although they are not specific to a single disease, they could be indicative of health status. They correlate with prognosis in cancer[542–545] and are elevated in the frail elderly[265]. MDSCs isolated from tumour-bearing mice and transplanted into healthy controls fully differentiate into cells indistinguishable from mature macrophages and dendritic cells[546]. These observations suggest that MDSCs are finely attuned to their microenvironment. In future longitudinal aging studies, it would be interesting to see if these cells increase in circulation prior to clinical presentation of inflammatory disease. Additionally, individuals with health conditions had slightly more $CD16^+$ monocytes than those without, after controlling for age and sex. We also found that mMDSCs were correlated with $CD16^+$ monocytes but were inversely correlated with classical monocytes. We propose that individuals with disease have a block in myeloid differentiation, where myeloid progenitors remain immature myeloid cells (MDSCs) and do not progress to form classical monocytes. Thus in individuals with high mMDSCs, there is a decreased proportion of classical monocytes and an increased proportion of $CD16^+$ monocytes. This is demonstrated in the figure below.



86 yo	(pg/mL)	84 yo
36.54	GM-CSF	32.65
85.78	Fractalkine	116.88
29.47	IFNγ	38.02
9.08	IL-10	17.75
2.33	IL-1β	3.62
3.32	IL-6	17.25
19.18	TNF	10.48
1078	IP-10	1432
1554	MCP-1	1879
276.69	VEGFA	1181

Chronic conditions influence inflammation and myeloid cells. Blood immunophenotyping of two older adult women from our cohort in Chapter 2 demonstrate our model above. Both have a joint disorder; however the 84 year old (yo) additionally has type 2 diabetes. Although similar in age, the woman with two conditions has higher serum cytokines, a higher proportion of MDSCs, a higher proportion of intermediate and non-classical monocytes, and a lower proportion of classical monocytes compared to the woman with one condition.

In Chapters 3 and 4, individuals with RA and OA had decreased monocytes as a proportion of leukocytes when compared to healthy age- and sex- matched controls. There could be two possible explanations for this. The decrease in circulating monocytes could be due to their elevated expression of CCR2 that results in higher recruitment to affected joints, and thus their departure from circulation. To test this we would need paired blood and synovial samples from patients with OA and RA. Circulating monocytes could be labelled with deuterium[116] prior to samples being taken to determine how many of the synovial monocytes and macrophages were derived from circulating monocytes. A second explanation for the lower circulating monocytes could be altered myeloid maturation, similar to what we observed in Chapter 2. Quantifying and characterizing MDSCs in RA and OA would allow us to test this hypothesis. Due to the low abundance of myeloid progenitors in circulation, it is difficult to detect enough of them in blood samples in order to determine where along monocyte differentiation changes in maturity and activation occur.

Based on our data from Chapters 2-4, we propose a model wherein chronic inflammation creates a differentiation block in myeloid progenitors resulting in an increased production of MDSCs. Fewer monocytes are formed, and the ones that enter circulation are bathed in elevated levels of inflammatory cytokines, which results in their activation. We saw evidence of monocyte activation in Chapters 3 and 4, where RA and OA monocytes had elevated expression of CCR2 compared to healthy controls. TNF was correlated with increased CCR2 expression on monocytes in Chapter 2 and 4, which is consistent with our findings in mice and in humans[39].

This association was not found in RA; however, following DMARD treatment in this group, CCR2 decreased and monocyte proportions increased, returning to levels seen in healthy controls. These findings provide evidence for the use of inflammation-modulating treatments to normalize the monocyte compartment, either by reducing their activation and recruitment to sites of inflammation, or by rescuing myeloid development. Neutralizing TNF reduces circulating IL-1, GM-CSF, IL-6 and many other chemokines[405,547]. In future studies, following RA patients as they start anti-TNF biologics will allow us to determine how TNF specifically contributes to myeloid development, and monocyte maturity and activation in humans.

How inflammation increases the risk for chronic disease remains unknown; however, we provide new evidence that this may be due to inflammation-related changes in myeloid development that result in altered monocyte function. We found that MDSCs were elevated in individuals with health conditions and that these cells were correlated with IL-1β and IFNγ when controlling for age and sex. These cytokines were also correlated with increased CD115 expression on monocytes. We also found that CD115 was elevated in monocytes from old mice, but was not in old TNF KO mice. Furthermore, in patients with RA and OA, activated monocytes correlated with clinical features of disease. CCR2 expression and tender joint count both decreased after DMARD treatment in RA patients and CCR2 expression correlated with pain in individuals with OA. In future longitudinal studies of age, we can test our hypothesis by looking for similar parameters of myeloid development,

and monocyte maturity and activation as we did in our studies in addition to inflammation and disease. In theory, the rate of change in inflammation would correlate with changes in myeloid cell parameters, and the rate of change in these variables would be faster in individuals who later develop chronic disease.

We were able to take a more mechanistic approach in mice and test whether age- or age-associated inflammation drove changes in myeloid development. In our aging mouse model, TNF is a key mediator of inflammaging, as mice deficient for it do not experience increased serum and tissue cytokines[39].

Age-associated TNF alters hematopoiesis and myeloid development

The age-associated changes in monocyte numbers and function have been well studied for years, but the mechanisms driving them remain poorly understood. Although some changes may be the result of cell intrinsic aging, our heterochronic bone marrow chimeras confirmed that the aging environment is sufficient to induce altered myeloid differentiation and monopoiesis in young bone marrow[548]. TNF KO mice aged in the absence of age-associated inflammation did not experience this expansion in monocytes that produced increased levels of pro-inflammatory cytokines. How TNF was driving the increase in monopoiesis in old animals was previously unknown. In this thesis we demonstrated that age-associated inflammation driven by TNF results in an increase in the number of proliferating myeloid progenitors both in the bone marrow and in the spleen. Based on the

findings of Chapter 5, TNF could influence myeloid development in 2 ways: 1) directly by driving myeloid progenitor and monocyte survival[453], 2) indirectly by stimulating non-hematopoietic tissues to release other cytokines and growth factors that instruct monocyte and macrophage polarization, activation, and function (i.e. CSF-1). Based on previous findings, endogenous production of TNF is not necessary for myeloid development[429]. Furthermore, the source of TNF driving increased monopoiesis with age is from a non-hematopoietic source, as young TNF KO bone marrow transplanted into an old host results in increased monopoiesis[548]. In this thesis, we found cKit and CD115 expression on bone marrow cMoPs were dependent on TNF, which suggests TNF may act on progenitors by making them more responsive to survival and growth factors essential to myeloid development. Earlier progenitors like the MDP show TNF-dependent regulation of CD115 expression independent of age. Future studies with TNFR KO chimeras will be able to determine if TNF acts directly on progenitors or the microenvironment to alter monopoiesis.

Whether directly or indirectly, we have evidence that TNF alters myeloid progenitors in the bone marrow. This could further be exacerbated by other age-associated factors in the periphery. In contrast to the bone marrow, splenic progenitors had increased expression of cKit, Flt-3 and CD115 with age, but it was not entirely dependent on TNF. Although the bone marrow is exposed to circulating inflammation, the spleen filters the body's blood, increasing its exposure to elevated and chronic inflammatory mediators. It could be that the bone marrow is more

protected from age-associated inflammation than the spleen. We do not know if splenic myeloid progenitors are seeded by the bone marrow or if they are a distinct population seeded during early life that expand with age; however, the microenvironment of the spleen is different from the bone marrow and may interact differently in response to TNF and the process of aging. Many studies of chronic inflammatory states have found that the spleen is a site conducive to the production of MDSCs[549]. It could be that increased exposure to inflammation in the spleen preferentially leads to the development of MDSCs. In fact, we found Flt-3 expression on MDPs elevated in the spleen but not in the bone marrow. Flt-3 activation inhibits the induction of CCAAT/enhancer-binding protein α (C/EBPα) and PU.1, which are necessary for myeloid differentiation[468]. Thus increased expression on splenic MDPs with age might drive the production of mMDSCs rather than monocytes. It would be interesting to test for a similar increase in Flt-3 expression on monocyte precursors of individuals with elevated MDSCs in Chapter 2. Splenic monocytes are known to be mobilized to the heart following myocardial infarct and spinal injury[549]. Determining how much they contribute to circulating monocytes and tissue macrophage populations with age remains to be studied. If myeloid cells being recruited from the spleen more closely resemble MDSCs with age, it may have important health implications.

There are significant changes to tissue macrophage populations with age, likely due to the aging microenvironment and changes to hematopoiesis. With inflammation there is a bias towards the recruitment of monocytes into tissues,

which is why there is an increase in monocyte-derived tissue macrophages in the heart, lungs, spleen and lamina propria[46,69,79]. Recently published work explored the maintenance of tissue-resident alveolar macrophages (TR-AMs) in a fibrosis model[458]. They showed that monocyte-derived alveolar macrophages (Mo-AMs) significantly contributed to fibrosis. These cells persisted following the resolution of fibrosis; however, they adopted a transcriptional signature similar to TR-AMs. Authors suggested that Mo-AMs cells persist in areas where the lung microenvironment is abnormal. With age we know that there are changes to stromal tissue described as a senescence-associated secretory phenotype, which results in increased production of inflammatory cytokines, including TNF[550]; we have also detected increased inflammation in the lungs of old mice[436]. These data suggest senescence of stromal tissue may favour monocyte-derived tissue macrophages over local proliferation of embryonic-derived cells. Senescence of embryonic-derived heart macrophages leads to replenishment by adult-derived tissue macrophages[79]; however in the peritoneum the proliferative capacity of embryonic- and adult-derived macrophages are the same[551]. It may be that adult-derived cells are better adapted to the aging stromal niche, which requires future investigation.

Although Mo-AMs that remain following resolution of lung fibrosis are transcriptionally similar to TR-AMs, it is possible that two cell populations could function and respond differently to stimulation (e.g. injury, infection, inflammation). For example in response to cardiac injury in neonatal mice there is an expansion of embryonic-derived macrophages, which leads to tissue repair [81]. Whereas in adult

mice, there is a significant recruitment of monocyte-derived macrophages that hinder tissue repair and recovery. These functional differences could result from different development and epigenetic regulation of gene expression. As discussed in the introduction, the environment plays an important role in informing monocyte and macrophage phenotype and function. In a study where peritoneal macrophages were transplanted into the lungs of mice, the cells more closely resembled macrophages of the lung; however, the transplanted cells still had 30% of their gene expression that was different from lung macrophages[65]. Whether the 30% of genes contributed to functional differences between peritoneal-derived and lung tissue macrophages was not explored. Thus future research is needed to better understand how much of the macrophage program is hard-wired and irreversible, and how much is modifiable in response to the environment.

This thesis also demonstrated that inflammation-associated changes to adult hematopoiesis, result in impaired development. Transcriptomic analysis of both circulating monocytes and alveolar macrophages revealed decreased expression of genes involved in myeloid lineage commitment and maturation (i.e. *Hdac1, Tet2*). Our aging mouse model lends itself well to testing how much of the age-associated changes in myeloid development are due to age and inflammation by examining cells from young and old WT and TNF KO mice. In the future, we could compare the epigenetic landscape of progenitors and monocytes sorted from the bone marrow, spleen and circulation of these mice. We could additionally inject myeloid progenitors isolated from the bone marrow or the spleen into a host to determine if

they have the same reconstitution, differentiation and functional potential as tissue resident cells, or if they are restricted based on their source.

Inflammation alters monocyte and macrophage function

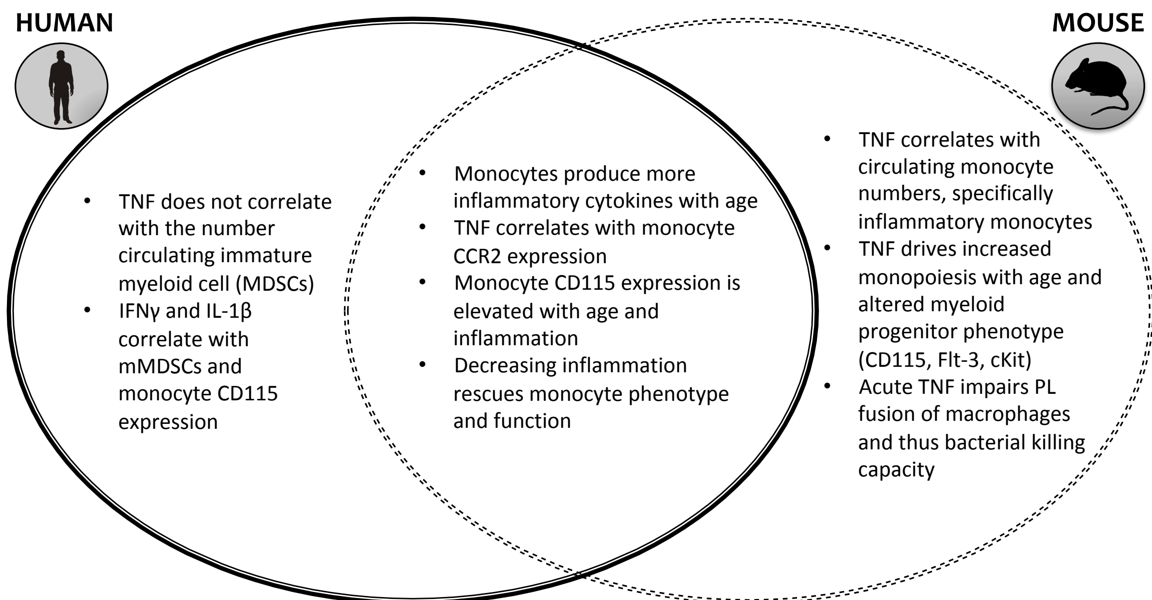
Genetic ablation and *in vivo* neutralization of TNF reduces pro-inflammatory cytokine production by monocytes in old mice[39]. Additionally, macrophages derived from the bone marrow of old TNF KO mice maintain their bacterial killing capacity, compared to old WT. Based on these findings, we hypothesized that exposure to age-associated inflammation would impair monocyte and macrophage function.
We are the first to discover that the age-associated decrease in bacterial killing by macrophages is due to a TNF-dependent decrease in phagolysosomal (PL) fusion. We demonstrated that neutralizing TNF rescued bacterial killing capacity of old macrophages. Additionally, acute exposure to 1 and 10 ng/mL TNF was sufficient to impair PL fusion and bacterial killing, but not at a concentration of 100 ng/mL. These observations suggest that exposure to elevated inflammation can lead to functional impairments in monocytes and macrophages. Future studies should investigate the minimal dose and length of exposure to TNF that is necessary to impair function as well as the mechanism by which it does so. Although 3 weeks of TNF neutralization in old mice is enough to reduce monocyte pro-inflammatory cytokine production [39], we still do not know if acute exposure is sufficient *in vivo*.

In Chapter 4, inflammation was elevated in individuals with OA compared to their healthy controls. We additionally found that in these individuals monocytes had increased expression of trafficking and activation markers (i.e. CCR2, CD16, HLA-DR) and they produced more IL-1β and TNF on a per cell basis in response to LPS stimulation when compared to those of healthy controls. Future work will explore how monocyte and monocyte-derived macrophage function correlates with inflammation. Based on the data generated in this thesis we would expect monocytes in individuals with higher levels of inflammation to produce more pro-inflammatory cytokines following TLR stimulation compared to age-matched individuals with lower levels of inflammation. Furthermore, individuals with higher levels of serum TNF may have decreased bacterial killing and PL fusion. In Chapter 3, DMARD treatment decreased inflammation as well as CCR2 expression and in the future we would predict monocytes from these individuals would perform similarly to age- and sex-matched healthy controls. Testing monocyte function in RA patients being treated with anti-TNF would also allow us to determine if TNF is driving similar changes to monocyte and macrophage function in humans as it does in mice.

Future studies will be needed in order to disentangle if altered differentiation results in functional changes in monocytes and macrophages, or if it is dictated by the inflammatory microenvironment. Although we find acute exposure to TNF drives PL fusion we have not tested how TNF influences phagosomal acidification, proteolysis or oxidation, which are important to antigen presentation and initiating an immune response. Of particular interest is how increased CD115 expression on

monocytes with age influences function. In both humans and mice, we found that age and inflammation contributed to increased expression of CD115. In humans it was correlated with IFNγ and IL-1β, while in mice it was TNF-dependent. How excess CSF-1/CD115 signalling influences monocyte function in the context of age, remains unknown; however, it has been implicated in increasing pro-inflammatory cytokine production, while decreasing antigen-specific T cell responses as discussed in Chapter 5.2.

Summary of findings in mice and humans:



The source of age-associated inflammation

In mice, we know that non-hematopoietic tissue is responsible from impaired monopoiesis and monocyte and macrophage function[39]. We previously reported that increased intestinal permeability and microbial dysbiosis contributes to age-associated inflammation, through increased bacterial products in circulation[436], which can directly stimulate immune cells as well as activate stromal cells to produce pro-inflammatory cytokines. The age-associated increase in inflammation could also be due to senescence of non-hematopoietic cells. Senescence results in a breakdown of many different processes including DNA repair, protein degradation, and cellular metabolism (e.g. dysfunctional mitochondria)[234]. It is highly unlikely that there is one mechanism driving all these changes. Constitutive or repetitive stress placed on these biological systems over time with insufficient recovery may lead to a loss in homeostatic control. Senescent cells themselves are activated and secrete many pro-inflammatory mediators, including TNF, which is described as the senescence-associated secretory phenotype (SASP)[550]. Fat cells acquire a SASP with age and produce pro-inflammatory cytokines as well as angiotensin II, which has been shown to mobilize the splenic monocyte pool[141]. The SASP-related increase in inflammatory cytokines can activate monocytes and macrophages. In addition, monocytes and macrophages can detect the products of senescent cells (e.g. extracellular ATP, fatty acids, urate crystals, free radicals and damaged organelles, etc.). One way they can detect these DAMPs is via NLRP3, which leads to the

production of ROS and inflammatory cytokines (e.g. IL-1β, IL-18), which further feed into the activation of non-hematopoietic cells, monocytes and macrophages. This chronic activation of monocytes and macrophages can lead to the deterioration of homeostasis in tissues across the body, which can eventually result in chronic diseases.

Modulating inflammation to maintain immune function and health

Through this thesis we have determined that age-associated increases in inflammation contribute to elevated myelopoiesis and functional changes in monocytes and macrophages. In mouse models, we have had significant success with neutralizing TNF *in vivo* and *in vitro* to reverse age-associated monopoiesis[39] and recover bacterial killing of macrophages, respectively. The success of this treatment in limiting inflammation lies in its ability to halt the feed forward process of inflammation that occurs with age and in chronic inflammatory diseases (e.g. RA, ankylosing spondylitis, Crohn's disease[552]). Other approaches to inhibit inflammation have also been shown to reverse many facets of the aging process. For example NLRP3 knock out mice are protected from age-associated inflammation, glucose intolerance, thymic involution, neurodegeneration, and osteoporosis[40]. Removing senescent cells increases longevity in mice[553]. Exercise has similarly been associated with increased longevity, due to maintaining metabolic function and decreasing systemic inflammation[554]. Although all these methods may not directly

influence age-associated changes in monocyte and macrophage function, they decrease or limit inflammation of non-hematopoietic tissue, which we know is the source of TNF that drives increased monopoiesis in our old mice[548]. Interestingly, individuals who live past 100 years experience inflammaging but have higher anti-inflammatory responses to counteract age-associated inflammation[262]. Thus the therapeutic implications of this thesis lie in modulating inflammation to rescue non-hematopoietic and hematopoietic tissues.

Limitations

At the time this thesis work was initiated, human monocyte subsets were defined based on their expression of CD14 and CD16; however, more recent analysis of monocyte subsets using single cell RNA sequencing has revealed more heterogeneity in blood monocyte subsets in the steady state[555]. The two major populations of monocytes identified by these methods are consistent with previously described classical and non-classical monocytes, while the intermediate monocyte population was segregated into two populations based on their transcriptomes, which were enriched for genes involved in: 1) cell cycle, differentiation, and trafficking; 2) cytotoxicity. Thus our findings on classical and non-classical monocytes remain valid, however those regarding intermediate monocytes will have to be revisited once the field has defined the functionality of the two newly identified subsets *in vivo* in the steady state and during disease or injury.

We defined human MDSCs based on a subset of previously published surface markers, however it should be noted that no functional assays were performed to test their functional capacity. Most functional studies regarding MDSCs have tested their suppressive capacity *in vitro* with very little done to characterize their suppressive function *in vivo*[248]. This is due to a lack of a well-defined population that can be specifically experimentally manipulated (e.g. *in vivo* depletion). Most studies that eliminate MDSCs *in vivo* either administer pro-differentiation chemicals (e.g. all-trans retionoic acid[546]) or chemotherapeutic drugs that eradicate hematopoietic progenitors (e.g. gemcitabine[556]). Other loss of function approaches used include the use of small molecule inhibitors like phosphodiesterase-5 to downregulate suppressive machinery (e.g. arginase-1 and nitric oxide synthase-2) used not only by MDSCs but other myeloid cells as well[557]. MDSCs are continuously described as a heterogeneous population, however they have been classified using canonical monocyte- and granulocyte-specific surface markers: CD14 and CD15, respectively. This is a biased approach, which does not capture the potential heterogeneity or address the true ontogeny of these cells. In the future the field would benefit from an unbiased single cell RNA sequencing approach to better define MDSCs to address whether they are a distinct population, and their ontogeny (granulocytic vs monocytic). These studies would provide insight as to whether the increase in MDSCs that we have found with age is a beneficial or detrimental adaptation by the immune system.

Concluding Remarks

The studies presented in this thesis have created a link between inflammation and chronic disease by uncovering novel mechanisms by which age-associated inflammation drives changes in monocyte and macrophage development and function. This thesis has additionally opened up many further avenues for investigation in the field of innate immunosenescence and myeloid development. Most importantly, our findings suggest that anti-inflammatory treatments may be sufficient to slow the progression of many chronic diseases.

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