TNF ALTERS MONOCYTE AND MACROPHAGE PHENOTYPE AND FUNCTION IN MODELS OF CHRONIC INFLAMMATORY DISEASE.

By

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ABSTRACT

Aging is accompanied by increasing levels of pro-inflammatory cytokines, such as tumour necrosis factor (TNF), in the serum and tissues and changes in the phenotype and function of leukocytes. This "inflamm-aging" is known to contribute to numerous chronic diseases associated with aging and an increased susceptibility to infection. We hypothesized that age-associated increases in pro-inflammatory cytokines, TNF, contribute to monocyte and macrophage dysfunction, and ultimately, and an increased susceptibility to disease in the elderly and those with chronic inflammation. In this thesis, we conclude that TNF is a key mediator of age-associated inflammation, contributes to changes in inflammatory monocyte development, phenotype and function, and ultimately, impaired anti-pneumococcal immunity. We found that age-associated impairments in monocyte and macrophage function were influenced by the age of the host microenvironment, and not hematopoietic stem cells. Remarkably, exposure of aged immune cells to the microenvironment of a young host could reverse the detrimental effects of age and rescue monocyte and macrophage function. Moreover, in a model of diet-induced obesity, we demonstrated that inflammatory monocyte characteristics, which are regulated by TNF, could serve as a better predictor of insulin resistance in mice and humans than conventional biomarkers. Lastly, we demonstrate that TNF drives age-related defects in muscle function and mobility with age. Together, these studies increase the breadth of our understanding of the cross talk between inflammatory micro-environment and host immunity, and their impact on age-associated diseases. Although there are many more details to be unraveled, these studies indeed have made great progress and could be used to develop novel and much-needed therapeutic strategies to reduce the risk of infectious and chronic disease and improve the quality of life in the aging population.

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LIST OF ABBREVIATIONS

Ab antibody
APC antigen-presenting cell
BMDM bone marrow-derived macrophage
CCR C-C chemokine receptor
CD cluster of differentiation
cfDNA cell-free DNA
CFU colony forming units
CLP common lymphoid progenitor
CMP common myeloid progenitor
CMV cytomegalovirus
CD cluster of differentiation
DC dendritic cell
EBV Epstein-Barr virus
ELISA enzyme-linked immunosorbent assay
GF germ free
HIV Human Immunodeficiency virus
HFD high-fat diet fed
HSC hematopoietic stem cell
HSV herpes simplex virus
IgG immunoglobulin G
i.n. intranasal
i.p. intraperitoneal
i.v. intravenous
IL interleukin
KO knockout
LPS lipopolysaccharide
Ly lymphocyte antigen
MCP monocyte chemotactic protein
MDP muramyl dipeptide
MHC major histocompatibility complex
MSC mesenchymal stromal cell
Nod2 nucleotide-binding oligomerization domain-containing protein 2
NF-κB nuclear factor kappa-B
PBMC peripheral blood mononuclear cell
ELISA enzyme-linked immunosorbent assay
TNF tumor necrosis factor
TLR4 Toll-like receptor 4
WT wildtype

Declaration of Academic Achievement

In accordance with the *Guide for the Preparation of Theses at McMaster University*, the research included is presented as a "sandwich doctoral thesis". The manuscripts presented in Chapters 2, 3 and 4 are three independent and conceptually related scientific articles that, as of June 2016, have been published or submitted for publication. The experiments designed, data collected and conclusions drawn in each manuscript necessitated a collaborative effort involving several colleagues, resulting in multiple authors. As such, my contributions to each article is highlighted below.

Chapter 2: Puchta A* & **Naidoo A***, Verschoor CPV, Loukov D, Thevaranjan N, Mandur TM, Nguyen P, Jordana M, Loeb M, Xing Z, Kobzik L, Larché MJ, and Bowdish DME. TNF Drives Monocyte Dysfunction with Age and Results in Impaired Anti-pneumococcal Immunity. *PLoS Pathogens*. 2016 Jan 15; 12(1):e1005368 -e1005368. *Co-first author

This research was conducted during the period of September 2013 to November 2015. I am the co-first author of this paper. I designed and conducted a significant proportion of the experiments, and analyzed and interpreted the corresponding data. My contributions include the following: 1 of 2 independent experiments for Figure 1A-D, and 2 of 3 independent experiments for Figure 4C-G and 1 of 3 independent experiments Figure 6A-F. I generated the data for Figures 2A (polystyrene depletions of Ly6C^{high} monocytes), 4A-B (heterochronic bone marrow chimeras), 6K (monocyte-mediated *S. pneumoniae* binding and uptake), 7D (inflammatory monocytes in aged TNF knockouts), and Figure 8 (entire panel; monocyte depletion and infection outcome). Further, I performed all the experiments necessary to generate Supplementary Figure 1(D-E), 2 and 3. D. Loukov assessed bacterial burden in old wildtype and TNF knockout mice (Fig.

7C). Dr. C. Verschoor performed and analyzed all the human data (Fig. 3). Dr. A. Puchta produced remaining figures. Dr. L. Kobzik provided thoughtful discussions and contributed to manuscript revisions. Dr. M. Larché, a clinical collaborator, generously provided the adalimumab (*Humira*) used in the TNF neutralization experiments. Dr. Z. Xing generously provided the TNF KO mice, provided insight and critical appraisal of manuscript. Experiments were conceived and designed by Dr. D.M.E. Bowdish, Dr. A. Puchta and myself. Dr. D.M.E. Bowdish contributed to authorship and provided guidance throughout the writing process.

Chapter 3: Naidoo A, Puchta A, and DME Bowdish. The Role of the Aging Microenvironment on Pneumonia Susceptibility in the Elderly. *In Submission.*

This research was conducted during the period of January 2014 to January 2015. I am the first author on this manuscript. I performed all experiments, and analyzed and interpreted the data. Fellow graduate students (D. Loukov, N. Thevaranjan, K. Novakowski) provided experimental assistance. Experiments were conceived and designed by Dr. D.M.E. Bowdish and myself. Dr. D.M.E. Bowdish provided guidance and editorial input throughout the manuscript writing process.

Chapter 4: Naidoo A, Foley KP, Verschoor CP, Loukov D, Novakowski KE, Loeb M, Bowdish DME and Schertzer JD. Monocyte Characteristics Predict Insulin Levels. *In Submission.*

This research was conducted during the period of September 2014 to December 2015. I am the first author on this manuscript. I performed the majority of experiments, with the exception of Supplementary Figure 2, and analyzed and interpreted all the data. Dr. K. Foley assessed the insulin and HOMA-IR values in mouse cohorts (Supp. Fig. 2),

and performed insulin ELISAs on the human plasma samples (Fig. 3G-I). Dr. C. Verschoor performed the human monocyte immunophenotyping required to generate the correlative plots (Fig. 3G-I). Dr. J. Schertzer provided invaluable insight and critical editorial input. Experiments were conceived and designed by Dr. J. Schertzer, Dr. D.M.E. Bowdish, and myself. Dr. D.M.E. Bowdish and Dr. J. Schertzer provided guidance throughout the writing process.

Chapter 5: Identification of the Etiology Behind Inflammaging.

This research was conducted during the period of August 2013 to March 2014. I pioneered the MDP and LPS bioassays, performed the majority experiments, and analyzed and interpreted the data. Dr. C. Verschoor performed the human monocyte immunophenotyping required to generate the correlative plots. Dr. Z. Xing generously provided the TNF KO mice, and Dr. J. Schertzer kindly gave us the HEK293T/mNod2 cell line as a gift. Experiments were conceived and designed by Dr. D.M.E. Bowdish and myself.

Chapter 6: The Role of TNF on Muscle Integrity with Age .

This research was conducted during the period of January 2014 to June 2015. I performed the experiments and analyzed the data, with the assistance Jun Lu from the lab of Dr. P. Bercik, who conducted the open field and novel object behavioural assessments.

Appendix I: **Naidoo A** & Loukov D. (Aug 2015). Immunosenescence: implications for vaccination programs in the elderly. Published in *Vaccine: Development and Therapy.* 5: 17-29.

This article was researched and written over the period of March 2015 –July 2015. D. Loukov and I contributed to review article structure and manuscript preparation equally, which was edited by Dr. D. Bowdish. I independently generated Figure 1, and equally contributed to Figure 2 with D. Loukov.

CENTRAL HYPOTHESIS AND AIMS

Aging is accompanied by increasing levels of pro-inflammatory cytokines, such as tumour necrosis factor (TNF), in the serum and tissues and changes in the phenotype and function of leukocytes. This "inflamm-aging" is known to contribute to a wide spectrum of chronic diseases associated with aging and an increased susceptibility to infection. Above age-average levels of TNF contribute to an increased risk of chronic and infectious disease, and serves as a strong independent risk factor for morbidity and mortality in the elderly. As human longevity increases, age-related increases in pro-inflammatory cytokine levels and their detrimental health outcomes will place a significant burden on healthcare in the near future. Despite need to better understand the mechanistic underpinnings of inflammaging, the field is understudied. As such, the purpose of this thesis is to investigate the etiology of this chronic inflammation and to elucidate its consequences on host immunity.

Little is known about how the innate immune response changes with age and, specifically, whether age-associated changes in monocytes and macrophages contribute to increased disease susceptibility, given that these myeloid cells are involved in the pathology and/or resolution of all age-associated diseases. Previous work in our laboratory demonstrated that old TNF knockout (KO) mice physically aged better than wildtype C57Bl/6 mice. Further, we observed TNF KO macrophages from aged (18-22mo) mice were indistinguishable from those isolated from younger mice. Conversely, macrophages from aged wildtype mice displayed impaired function and produce elevated levels of cytokines compared to young mice. Thus, ***we hypothesized that age-associated***

increases in pro-inflammatory cytokines (e.g. TNF) contribute to monocyte and macrophage dysfunction, and ultimately, an increased susceptibility to disease in the elderly and those with chronic inflammation. We tested this hypothesis using the following aims:

Aim 1. Elucidate whether TNF drives monocyte dysfunction with age and contributes to impaired anti-pneumococcal immunity.

Aim 2. Characterize the role of the aging microenvironment on pneumonia susceptibility in the elderly.

Aim 3. Determine whether the dysregulation of inflammatory monocytes contributes to hyperinsulinemia and insulin resistance during diet-induced obesity.

Aim 4. Identify the etiology behind the chronic inflammatory state observed in the elderly.

Aim 5. Assess the role of TNF on age-related deterioration of muscle integrity and cognition.

Chapter I: INTRODUCTION

IMMUNOSENESCENCE

Aging is a complex process that adversely affects the immune system. The mechanisms that underlie immunosenescence, or changes in the immune system with age, range from intrinsic defects in the pluripotent haematopoietic cells to impairments in peripheral leukocyte migration, maturation and function. These changes culminate in dysregulated immune responses to infection and reduced vaccination responses, which results in increased morbidity and mortality in the elderly.

Hematopoietic Changes with Age

Multipotent hematopoietic stem cells (HSCs) are a heterogeneous population of cells found in the bone marrow of adults which give rise to blood cells[1]. HSCs generate all cells of the hematopoietic system, including myeloid (e.g. monocytes, macrophages, neutrophils, etc.) and lymphoid cells (e.g. NK cells, T- and B-lymphocytes). Age-associated alterations in HSCs underlie many of the changes observed in aged leukocytes[2]. Although the absolute number of HSCs increase with age, the number of independent clones decrease[3–5]. Aging of HSCs is further characterized by a decreased proliferative capacity[6], bias towards myelopoiesis[7–11] (compared to lymphopoiesis[12–14]), decreased polarity (asymmetric distribution of proteins within the cell to determine fate of daughter cells)[15], and increased DNA damage[6]. Age-associated hematopoietic dysfunction contributes to the manifestation of hematological disorders, such as myelodysplastic syndromes and malignancies[6].

HSC aging is an evolutionarily conserved process in mammals[6,16]. The increase in hematopoietic stem cell frequency in aging mice[17,18] and humans[2] is accompanied by a decline in functional activity. Recent studies demonstrate that age-related HSC expansion is not dictated by the age of the bone marrow microenvironment given that upon transplantation into young recipients, HSCs derived from old mice have an increased proliferative capacity compared to HSCs from young mice[5,19]. Aging HSCs favour the development of myeloid cells over lymphoid due to the increased survival of myeloid-competent HSCs and a decrease in lymphoid-competent HSCs. Studies on telomerase-deficient mice have established that telomere dysfunction accelerates myeloid skewing[20]. Common Lymphoid Progenitors (CLPs) from aged mice have a diminished proliferation capacity and impaired responsiveness to IL-7 induced lymphopoiesis[21]. Aging adversely affects the lymphoid differentiation potential of HSCs but does not affect their myeloid differentiation potential or their self-renewal potential[12]. Age-related myeloid bias during bone marrow and extramedullarly hematopoiesis is thought to be due to the increased presence of myeloid growth factors (e.g. GM-CSF) and pro-inflammatory cytokines (e.g. type I interferons and tumour necrosis factor [TNF]) which stimulate myelopoiesis[22]. Age-discordant bone marrow chimeras suggest that both age-related HSC-intrinsic and HSC-extrinsic factors favour myelopoeisis[23]. These age-related changes in HSC lineage potential can directly influence the diseases accompanying hematopoietic aging. For instance, pediatric leukemias primarily involve the lymphoid lineage, while myeloid leukemias are dominant in old adults[24,25]. As the only persistent cell lineage in the bone marrow, HSCs serve as repository for accumulated DNA damage. DNA mutations in

HSCs have been attributed to aberrant function in downstream progenitor cells[19]. In rheumatoid arthritis patients, ineffective DNA repair is a major driving force for accelerated immune aging[26]. DNA damage has been linked to increased inflammation, which is observed with age. Furthermore, HSCs have been shown to respond to pro-inflammatory cytokines, such as interferons (IFNs), and participate in systemic inflammatory responses. Impairment of mechanisms that dampen effects of IFNs on HSCs, such as the effector functions of IFN-inducible immunity-related p47 GTPase Irgm1[27,28], result in enhanced exhaustion, or diminished functionality, suggesting inflammation may contribute to aging of the hematopoietic compartment[2]. The aging hematopoietic system, however, may itself be a source of inflammatory mediators. HSCs isolated from age mice demonstrate an upregulation of genes and signaling pathways (e.g. NFκB) associated with inflammation[3,29]. It is currently unknown whether increased pro-inflammatory gene expression in HSCs occurs in response to the inflammatory microenvironment in aged individuals, or if aging HSCs themselves are responsible for the age-associated inflammatory milieu.

Age-Associated Changes in the Adaptive Immune System

T cell aging in mice and humans is accompanied by progressive loss of thymic function and a shift in the composition of circulating $CD8^+$ T cells population from a naïve to a memory phenotype[30]. Despite declining thymic output due to thymic involution, there is no significant decline in the total number of T cells in the peripheral T-cell pool with age[31] due to expansion of memory and effector T cells in both the $CD4^+$

(helper) and $CD8^+$ (cytotoxic) subsets[32]. $CD8^+CD28^-$ T cell accumulation is one of the most prominent changes associated with human aging[33]. CD28 is expressed on all human T cells at birth, however, by 80 years of age, approximately 10–15% of $CD4^+$ and 50–60% of $CD8^+$ T cells lack CD28 expression[34]. $CD28^-$ T cells are frequently oligoclonally expanded and constrain the adaptive immune system by limiting available space in the T cell niche. $CD28^-$ T cells demonstrate defective antigen-induced proliferation and decreased ability to secrete IL-2[35], reduced antigen receptor diversity, shorter replicative lifespans and increased susceptibility to activation-induced cell death[36], while having enhanced cytotoxicity and regulatory functions[33]. The dramatic increase in the number of memory and effector T cells with age is accompanied by decline in functional responsiveness to new antigens, as evidenced by decreased responsiveness to vaccination compared to younger individuals[37–39]. In vaccination studies, $CD8^+CD28^-$ T cell frequency correlated with defective humoral responses to the influenza vaccine, and were found to hinder CD4 vaccine responses[33,38]. Conversely, *Lelic et al*. showed that elderly individuals produced virus-reactive T-cells that were as stable and long-lived as those generated in young adults, upon infection with a novel pathogen (West Nile Virus)[40]. Instead of using a live virus, this study employed use of synthetic immunodominant peptides for re-stimulation that did not necessitate further processing by antigen-presenting cells (APCs)[40]. Since antigen presentation is compromised with age[41], use of peptide-based booster vaccines may augment vaccine responses in older adults.

Aging adversely affects B-cell output and function. B-cell production in the bone marrow is reduced and this is attributed to a decreased frequency of CLPs[42,43], and

smaller pre-B cell and immature B-cell pools[44]. There exists an age-related reduction in expression of transcriptional regulators (e.g. E2A and E47) essential to the generation of pro-B cells[45–47]. Further, RAG (recombination activating gene) enzymes and lambda-5 genes, which play a key role in the rearrangement and recombination of genes required for B cell receptor (BCR) diversity and are crucial for passage through the pro- and pre-B cell stage, are diminished in developing B cells from aged individuals[48–50]. Together, these findings suggest that both B-lineage commitment and output are compromised with advancing age. Increased levels of TNF in the aging bone marrow have been shown to decrease B cell lymphopoiesis and plasma cell survival[51]. Age-associated changes in B cells have consequences for humoral immunity. Antibodies produced in aged individuals are of lower affinity due to reduced isotype switching and somatic hypermutation, and consequently, both their neutralizing and opsonizing functions are decreased[13]. The duration of humoral immunity is shorter in aged individuals compared to young, and immunoglobulins are less protective due to their low titre and affinity[52–55]. Immunosenescence is also associated with an enhanced production of autoantibodies, which may contribute to autoimmune disease[6]. Age-related defects in B cell function is further attributed to intrinsic defects, including reduced expression of co-stimulatory molecules (e.g. CD86[56]) and defects in B cell receptor (BCR) signalling[57], but may further be compounded by secondary age-related impairments in $CD4^+$ T cell help and soluble factors in the aged microenvironment[13].

AGING, MONOCYTES & MACROPHAGES

The impact of aging on innate immune cells, specifically those of myeloid origin, remains poorly understood. Several studies comparing aged mice and humans to their young counterparts suggest that monocyte and macrophage function is adversely affected by age[58–62]. Given that monocytes and macrophages are involved in the pathology and/or resolution of virtually all age-associated diseases, understanding of the age-associated changes in monocyte and macrophage function is surprisingly limited [63–67]. In Chapter 3, I will elucidate whether impairments in monocytes and macrophages are due to changes in the aging microenvironment (e.g. increases in pro-inflammatory cytokines), or intrinsic age-related defects.

Monocytes

The impact of aging on innate immune cells, specifically those of myeloid origin, remains poorly understood. Murine monocytes can be divided into 2 phenotypically and functionally distinct subsets on basis of expression of CD11b, an integrin family member that regulates adhesion and migration, and Ly6C, a glycerophosphatidylinositol-anchored cell surface protein[68]. $Ly6C^{low}$ monocytes ($CD14^{+}CD16^{++}$ in humans) are key to surveillance of endothelial integrity. This subset is responsible for patrolling the blood vessel lumen, scavenging for oxidized lipids, dead cells and potential pathogens. During infection, they extravasate to sites of inflammation. Though they may briefly be the main producers of TNF at the initiation of the inflammatory response, by 12hrs following recruitment to site of infection, they resemble alternatively activated (M2) macrophages, which serve to promote tissue remodeling and wound healing[68,69]. Conversely, $Ly6C^{high}$

monocytes ($CD14^{++}CD16^{-}$ in humans) functions include rapid extravasation into sites of inflammation and the replenishment of peripheral macrophage and DC compartments[70]. $Ly6C^{high}$ monocytes have been shown to contribute to pathogenesis in several models of chronic inflammation, due to their ability to produce high quantities of pro-inflammatory cytokines[71–76]. During many chronic inflammatory conditions, the number of circulating $Ly6C^{high}$ monocytes correlates with disease progression, and ablation can decrease pathology[75,77–79]. In humans, however, the $CD16^{+}$ positive monocytes ($Ly6C^{low}$ counterparts) constitutively produce more IL-6, IL-1β and TNF basally and upon stimulation, and older people have a significantly larger proportion of $CD16^{+}$ cells than younger people[80]. $Ly6C^{high}$ monocytes egress from the bone marrow in a CCR2-dependant manner during the early inflammatory response[68,69]. Once recruited into inflamed tissues from the blood, this subset can differentiate into TNF- and iNOS-producing dendritic cells (TIP DCs), inflammatory macrophages, or inflammatory DCs, a proportion of which may subsequently migrate to draining lymph nodes[68,69]. $Ly6C^{high}$ monocytes, which differentiate into classically activated (M1) macrophages, secrete high levels of TNF, IL-6 and IL-1β[69]. $Ly6C^{+}$ monocytes that do not differentiate into tissue resident populations return to the bone marrow, upon resolution of inflammation[68]. CXCR4 has been implicated in promoting the return of monocytes from the bloodstream to the bone marrow[81].

Macrophages

Monocytes and macrophages are generally considered to be two related cell types that arise from a continuum of differentiation[82]. Data from recent studies, however, challenge this central dogma of macrophage origins. This paradigm shift began when it was demonstrated in mice that that most adult tissue macrophage populations are maintained in the steady state independently of monocytes[17]. Monocyte input to tissue macrophage populations is limited to inflammatory settings (e.g. infection, tumors) as well as during development and tissue remodeling[70]. Further, tissue-resident macrophages are derived from embryonic precursors that seed tissues prior to birth[70]. Three distinct macrophage progenitors are produced in independent waves during development[70,83–85]. Initially, yolk-sac derived macrophages develop in the extra-embyronic yolk sac from early erythro-myeloid progenitors. This precursor population predominantly gives rise to the brain microglia[86]. The second wave comprises monocytes derived from the fetal liver, which seed the lung and liver to produce Kupffer cells and alveolar macrophages[82,85,87]. These tissue macrophages are self-maintaining populations[82]. The third wave includes hematopoietic stem cells that colonize the bone marrow (BM) and produce the well-known BM-derived monocytes[82] that continuously seed the blood throughout life and contribute to intestinal[88], dermal[89] and cardiac[90,91] macrophage populations.

Macrophages are central effector cells in the innate arm of immunity that are involved in host defense and maintenance of tissue homeostasis. Macrophages play a critical role in the elimination of broad spectrum of pathogens through phagocytosis and production of reactive nitrogen (NOS) and oxygen species (ROS)[92]. Upon recognition of

a pathogen-association molecular patterns (PAMPs) by their pattern recognition receptors (PRRs), macrophages release a host of inflammatory mediators, including cytokines and chemokines that are essential to the initiation and propagation of the inflammatory response[93,94]. Further, macrophages play a key role in the education of adaptive immune cells through presentation of antigen to and activation of $CD4^+$ T cells[95]. Macrophages are important to the maintenance of tissue homeostasis through scavenging of apoptotic cells, clearance of debris, production of growth factors, and promote wound healing through secretion of fibrogenic and angiogenic factors[96,97].

Though macrophages play an integral role in innate immunity, many of their diverse functions (e.g. infection control, initiation of inflammation, tissue homeostasis) become compromised with age. Phagocytosis, which allows macrophages to internalize and degrade pathogens, is reduced with old age[98–100]. In murine models, a decline in opsonophagocytosis, bacterial adherence and killing was observed in peritoneal macrophages[61,101,102]. Further, macrophages isolated from aged mice show a 75% reduced capacity to produce superoxide anions in response to to bacterial ligands[103]. In aged alveolar macrophages, nitric oxide (NO) production in responses to LPS is significantly diminished[104–106]. This is detrimental to the host as macrophage oxygen radical production exerts antimicrobicidal activity against a broad range of pathogens. Aging additionally reduces Toll-like receptor (TLR) expression. In aged peritoneal and splenic macrophages, intracellular expression TLR 3, 7 and 9 and surface expression of TLR 4 and 6 were reduced when compared to macrophages from young counterparts[107]. In humans, decreases in TLR1 surface expression (reduced by 36%) and TLR1/2 signalling

were observed in the elderly compared to young adults[108,109]. Given that pathogen recognition through TLRs is required to link the innate and adaptive arms of immunity, decreased TLR expression could predispose the elderly to bacterial and viral infections. Since macrophages are antigen-presenting cells (APCs), they display antigen on their surface via major histocompatibility complexes (MHC) to initiate effective and specific adaptive immune responses against pathogens. Macrophages from old mice (>18mo), however, express 50% less MHC II compared to macrophages from young mice, following activation with IFNγ[92]. Wound healing is also adversely affected with old age. Macrophages isolated from wounds of old mice demonstrate impaired phagocytic activity, which delays debris removal at site of injury, thereby hindering the wound healing process[100].

Macrophages secrete a wide array of cytokines, growth factors, chemokines and enzymes in response to potential pathogens. However, macrophage responses become dysregulated in the elderly[110]. Macrophages from aged mice and humans have elevated levels of cyclooxygenase-2 (COX-2) and produce increased prostaglandin E(2) (PGE2), a pro-inflammatory mediator[110]. Macrophages isolated from old mice are hyper-inflammatory, secreting elevated levels of interleukin-6 (IL-6) and tumour necrosis factor (TNF), both basally and upon stimulation with LPS. In fact, studies suggest that the progressive increases in inflammation with age, or inflammaging, is largely influenced by an age-associated shift towards hyper-inflammatory macrophages[111–113]. In addition to dysregulated cytokine production, aged macrophages demonstrate aberrant responses to chemotactic stimuli, which influence migration towards the site of inflammation.

Macrophages from old mice have impaired chemotactic responses to complement-derived factors[110]. Impairment in chemotaxis can contribute to delayed pathogen clearance in elderly individuals.

Since macrophages have integral roles in anti-bacterial immunity and maintenance of tissue homeostasis, and many of these roles are adversely affected by age (e.g. tissue repair, infection control, cytokine and chemokine responses), it is surprising that there is limited knowledge on mechanisms underlying age-related changes in macrophage function. A comprehensive understanding of the impact of aging on macrophages is necessary to develop novel therapeutics to improve immune responses in the elderly.

INFLAMMAGING

There is an inevitable increase in pro-inflammatory cytokines in the tissues and circulation with age. Increased levels of soluble and cellular markers of inflammation characterize aging. This *inflamm-aging* is believed to contribute to a wide spectrum of age-associated diseases with inflammation as their shared etiology[63]. Both epidemiological and mechanistic studies strongly support that age-related diseases, including osteoporosis, arthritis, cancer, neurodegenerative and cardiovascular diseases, are worsened by systemic inflammation[114,115]. Elevated levels of circulating pro-inflammatory cytokines, such as TNF, IL-6, IL-1β and CRP (c-reactive protein), serve as strong independent risk factors for morbidity and mortality in older people[116].

Inflammaging contributes to an increased susceptibility to viral, bacterial and fungal infections in the elderly[60,111,117]. Upon recognition of a foreign antigen, activation of the aged immune system leads to dysregulated inflammatory response and an compromised ability to mount efficient innate and adaptive immune responses to newly encountered pathogens or vaccine antigens[118]. How chronic inflammation contributes to an impaired immune response in older adults, however, is incompletely understood[118].

The Etiology Of Inflammaging

Several theories attempt to explain the cause of inflammaging, however, the etiology remains unknown. To date, the major sources of inflammaging have been hypothesized to be: (i) the accumulation of endogenous host-derived cell debris (damage-associated molecular patterns, or DAMPS), (ii) the presence of subclinical, persistent infections (e.g. cytomegalovirus, human immunodeficiency virus, and Epstein-Barr virus), (iii) senescent cells (e.g. memory $CD8^+$ T-cells) and their senescence-associated secretory phenotype (e.g. increased pro-inflammatory cytokine production), and (iv) intestinal microbiota-derived bacterial products and metabolites that induce local and systemic inflammatory events[119]. We will discuss the most prevalent theories below.

One prevalent theory explains that inflammaging is due to low-grade tissue damage that occurs as a result of the ageing process[119]. Once the rate of tissue damage exceeds the rate of repair, a positive feedback loop is established in which pro-inflammatory cytokines secreted by immune cells accumulate and impair immune cell function (*immunosenescence*). The experimental evidence supporting this theory is,

however, weak. A competing theory suggests that inflammaging is a consequence of a cumulative lifetime exposure to an antigen load caused by both apparent and subclinical infections as well as exposure to non-infective, environmental antigens[113]. The resulting inflammatory response, tissue damage and production of reactive oxygen species cause oxidative damage and elicits the release of additional cytokines from immune cells[120]. What results is a vicious cycle that drives immune system remodeling and perpetuates a chronic inflammatory state[113].

Senescent T cells have been implicated in the genesis of inflammaging. Age-related reductions in thymic output results in a decreased T-cell repertoire and increased oligoclonal expansion of memory and effector-memory cells[121]. This imbalance impairs aged hosts in their ability to clear novel pathogens (prolonging infection duration) and increases functionally distinct T cell populations, which have an amplified proinflammatory phenotype[122]. T cells from older adults demonstrate a reduced proliferative capacity and produce more IL-6 and TNF, upon mitogenic and viral stimulation, than their younger counterparts[123,124]. The peripheral memory $CD8^+$ T cell repertoires are dominated by clones with specificity to single antigens, mainly latent viral infections[124–126], as discussed below. Aged $CD8^+$ T cells are extremely potent producers of inflammatory cytokines and have been strongly associated with reduced antiviral immunity and chronic inflammatory disease[125]. Thus, T cell immunosenescence is postulated to be one of the key drivers of inflammaging.

Chronic infections by Herpes Simplex Virus (HSV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) are common in the elderly and provide continuous antigenic

stimulation. These infections are typical acquired asymptomatically during childhood and persist in the host for life and remains latent[127]. EBV infects >90% of the adult population and CMV infects >70% of older addults[128–130]. After infection, the virus establishes lifelong latency within the host and periodically reactivates[32]. Reactivation, though subclinical, is more frequent in the elderly and induces expansion of terminally differentiated effector $CD8^+$ T cells ("megaclones"), limiting space in the T-cell repertoire for other antigen-specific T cells, including those induced by vaccination[124,131]. In longitudinal studies, CMV infection positivity (CMV+) was a strong predictor of morbidity and mortality in the elderly[131] and decreased humoral responses following influenza vaccination[132,133], and combination of CMV+ and high serum IL-6 dramatically increased risk of frailty[63]. Although numerous studies suggest that CMV-specific memory T cells significantly contribute to age-related inflammation, a recent study demonstrated that cytomegalovirus infection did not drive inflammaging (IL-6, TNF and CRP) in healthy older people over a 10-year period, however, the study could not account for other latent infections, suggesting a combined role of other antigenic stimuli in systemic inflammaging[124,134].

In this thesis, we hypothesized that age-associated inflammation is caused by elevated levels of circulating bacterial products, which may due to alterations in intestinal permeability, such as a loss of integrity in the gut epithelia. The basis of such a hypothesis was derived from Elie Metchnikoff, who in the early 1900's, theorized that microbial translocation occurs with age, following an increased penetrability of the gastrointestinal tract[135]. Since his initial observation, a few studies have supported the postulate of

membrane integrity deterioration with age; however, the initial theory remained to be validated. In a study by Ma et al., it was concluded that intestinal permeability increases as individuals age[65]. Further, it was speculated that age-associated deterioration in intestinal barrier functions could permit increased systemic absorption of luminal antigens and contribute to the genesis of antigen-related age-associated diseases[65]. We propose that the microbial products leak into bloodstream via the gastrointestinal tract, first entering the lymphatic system. Whether in the lymphatics or blood, these bacterial products activate immune cells, leading to the production of low levels of pro-inflammatory cytokines, which may contribute to, or even initiate, the aforementioned vicious cycle.

Though the etiology is unknown, inflammaging is likely a consequence of lifelong exposure of the immune system to antigenic stimuli and complex genetic, environmental and age-related mechanisms that expose older individuals, of varying degrees of vulnerability or resilience, to age-associated diseases[119].

CHRONIC INFLAMMATION & DISEASE

Inflamm-aging is believed to contribute to a wide spectrum of chronic diseases linked to advanced age that share an inflammatory etiology[63]. Increases levels of circulating, pro-inflammatory cytokines, such as TNF and IL-6, serve as strong independent risk factors for morbidity and mortality in older people[63,116]. Epidemiological and mechanistic studies strongly support that many age-related diseases, including type II diabetes and neurodegenerative diseases, are initiated or worsened by systemic

inflammation[114,115]. In this thesis, we investigate the how chronic inflammation drives onset of age-associated disease.

METABOLIC DISEASE

Obesity, Inflammation & Insulin Resistance.

The incidence of type II diabetes (T2D) is increasing. This phenomenon has been closely linked to soaring obesity rates, which have reached epidemic proportions in the developed world[136]. Diabetes has 2 primary forms: type 1, known as insulin-dependent diabetes, and type 2 or non–insulin-dependent diabetes, which accounts for 90-95% of all diagnosed cases[137] and will be the focus of our discussion. Among those 65 and older, diagnosed cases of diabetes are projected to reach 26.7 million by 2050, and account for 55% of all diabetes cases. The higher incidence of T2D in the elderly has been attributed to both age-associated increases in pro-inflammatory cytokines (e.g. TNF and IL-6) and age-related increases in adiposity[138]. Diabetes and its complications impose profound economic costs[139]. In 2007 alone, the overall cost of diabetes in the US was approximately $174 billion, with $116 billion in direct medical costs and $58 billion in indirect costs (e.g., work loss, disability, premature death)[140]. Older adults with diabetes have an increased risk of major age-associated co-morbidities (e.g. coronary heart disease, arthritis, stroke, etc.)[137]. Interestingly, diabetes accelerates the aging process and significantly decreases life expectancy (by an average of 8 years)[141,142].

Obesity leads to a state of chronic, low-grade tissue inflammation. Obesity is postulated to be the dominant cause of acquired insulin resistance in individuals with T2D, however the mechanistic underpinnings of how such resistance develops is poorly understood[143,144]. T2D is characterized by resistance to insulin action on glucose uptake in peripheral tissues (e.g. adipose, skeletal muscle), impaired ability of insulin to inhibit glucose production by the liver, and dysregulated insulin secretion[145]. Glucose homeostasis necessitates the fine-tuned orchestration of insulin secretion by pancreatic β cells in response to changes in blood glucose levels and is balanced by secretion of the counter-regulatory hormone, glucagon[146]. In individuals with insulin resistance, glucose accumulates in the circulation rather than being taken up by cells. This state is known as hyperglycemia[147]. Insulin resistance precedes the development of type 2 diabetes (T2D). The systemic state of low-grade chronic inflammation associated with obesity has been implicated in the development of insulin resistance[148]. Consistent with this finding, elevated levels of circulating TNF, IL-6 and MCP-1 (monocyte recruitment chemokine) was found to correlate with hyperglycemia and insulin resistance in T2D[149].

Macrophage-mediated inflammation comprises a key component of insulin resistance[150]. Adipose tissue macrophage (ATMs) accumulation correlates with degree of obesity and magnitude of insulin resistance[151]. M1-like ATMs in obesity are largely derived from circulating monocytes recruited to the adipose tissue in response to tissue-derived chemokine signals. A recent study by Oh *et al.* using fluorescently labeled monocytes demonstrated that increased ATM accumulation only occurs in obese recipient mice, irrespective of monocyte origin (e.g. lean or obese mice)[150]. Thus, it may be

concluded that accumulation of inflammatory tissue macrophages is largely dependent on the adipose tissue microenvironment. Recruitment of adipose-associated macrophages has been demonstrated to contribute to adipose tissue inflammation and insulin resistance[152,153]. However, the initial events of monocyte migration, leading to extravasation and differentiation into tissue macrophages, remain poorly understood [150]. The role of CCR2 and CCL2 in macrophage recruitment to metabolic tissues (e.g. adipose, liver) and their contribution to insulin resistance have been assessed in several studies[154]. Mice with a CCR2 deficiency are protected from insulin resistance and have dramatically reduced macrophage recruitment to adipose tissue[154]. When CCR2 KO monocytes were injected into WT recipients, adipose tissue macrophage (ATM) accumulation is reduced by 40%, and hepatic macrophage accumulation was decreased by 80%[150,153]. As such, it may be concluded that CCR2 is an important contributor to monocyte migration into adipose and liver tissue, in the context of obesity[150]. Though few studies have evaluated the role of the CCR2/MCP-1 chemotactic axis on macrophage recruitment to adipose and the liver, the driving factor underlying monocyte egress remains unknown[150]. In Chapter 4, we will investigate what drives obesity-associated monocyte egress from the bone marrow and accumulation into metabolic tissues, and their implication in the onset of insulin resistance.

TNF & Insulin Resistance.

Chronic adipose tissue inflammation is a central feature of obesity that leads to various health complications, including insulin resistance[151]. Adipocytes constitutively express

TNF and this expression is markedly increased in adipose tissue derived from obese (ob/ob) and aged (>18mo) mice[155]. Adipocyte-derived TNF is thought to function in an autocrine/paracrine manner in adipose tissue and contribute to the development of insulin resistance and glucose metabolism abnormalities, thereby linking obesity to type II diabetes[156]. Increased TNF levels trigger phosphorylation of signaling adaptor protein, insulin receptor substrate 1 (IRS-1), by stress kinases. As a result, IRS-1 signaling is impaired which thereby blocks the intracellular actions of insulin[157]. Emphasizing the role of TNF in disrupting insulin signaling, TNF blockade in obese mice results in improved glucose homeostasis and insulin sensitivity[157]. In another study, neutralization of TNF (using soluble receptors) leads to a significant improvement in insulin resistance in obese mice[158]. Further, TNF-deficient obese mice demonstrated protection from obesity-related reduction in insulin receptor signaling in adipose tissue and muscle[156]. Together, these data suggest that TNF is a key mediator of insulin resistance in obese models by exerting its effects on important sites of insulin action.

AGE-ASSOCIATED COGNITIVE IMPAIRMENT

Aging in accompanied by decline in cognitive capacity. Cognitive decline encompasses a large spectrum of clinical manifestations, with a continuum that ranges from the cognitive changes of normal aging (age-associated cognitive decline (AACD)), through mild cognitive impairment (MCI), and finally, dementia[8,9]. MCI refers to the condition where individuals experience memory loss greater than one would expect for age, as determined by the Mini-Mental State Examination (MMSE), but do not meet the

criteria for clinical dementia[10]. The term dementia encompasses diseases characterized by a significant decline in memory or cognition that impacts an individual's ability to perform day-to-day activities. Dementia affects 15% of the population ≥ 65 yrs, and among those 80 yrs and old, 15-20% need institutional care due to severely diminished cortical functions[12]. Alzheimer's disease (AD) is the most common form of dementia in the elderly, accounting for 60-80% of dementia cases[13]. AD is a progressive, neurodegenerative disorder, associated with cognitive, memory and behavioural impairments[13]. By 2050, AD is expected to affect over 150 million people[14]. In the United States, there exists a 11% risk of developing AD in those > 65 yrs, 32% in those > 75 yrs, and 82% in those >85 yrs[15]. In 2014, over 700,00 deaths in the US, in individuals aged 65 or older, were attributed to Alzheimer's[16]. Since cognitive failure accounts for 40% of admission to institutional care in the elderly and leads to substantial personal, financial and societal costs, further research is required to understand the mechanisms of cognitive aging and the risk factors the contribute to disease progression[9]. In one of the few incidence studies on MCI, a total of 1400 healthy persons (>75 yrs) were monitored for the development MCI over 9 years. Incidence of MCI was found to increase with age, reaching 10 new cases per 100 in individuals 90 yrs and older, and correlated with an increased risk of mortality over the study period[11]. Individuals clinically diagnosed with MCI progressed to dementia at a an accelerated rate compared with healthy age-matched individuals[8].

Currently, there exists a large volume of literature supporting the association between subclinical inflammation and cognitive impairment in older adults[17–19]. Increases

in both systemic and cerebrospinal levels of pro-inflammatory cytokines (e.g. IL-6, TNF) have been linked to the development of mild-to-severe cognitive impairment and dementia[20,21]. The majority of research on age-related cognitive diseases is on AD. While the disease etiology remains unclear, pro-inflammatory cytokines, such as tumor necrosis factor (TNF), may contribute to the pathogenesis of AD[22]. Human studies measuring plasma levels of TNF found significantly higher levels of TNF in centenarians compared to younger control groups, and this high concentration was strongly associated with AD[22]. Further, in patients with Alzheimer's, concentrations of TNF in the cerebrospinal fluid (CSF) were found to be 25-fold higher when compared to controls, with higher levels correlating with increased clinical deterioration[21]. *In vitro* experiments have demonstrated that TNF, in combination with IFNγ, induced production of amyloid-beta (peptides crucially involved in Alzheimer's pathogenesis through contribution of plaque formation in the brain)[23]. Further, amyloid-beta-activated microglia and monocytes secrete high levels of TNF, thought to contribute to neurotoxicity that occurs in AD[20,24]. In a mouse model of brain aging that undergoes early onset cerebral neurodegeneration associated with immunosenescence, known as senescence-accelerated mouse prone 10 (SAMP10), higher brain tissue concentrations of TNF, IL-6, CXCL10 and other pro-inflammatory mediators are observed than in wildtype groups[25]. In another mouse model prone to severe Alzheimer's disease, pharmacological blockade of TNF significantly reduced the cognitive deficit[26]. Further, in a similar study in humans, patients with mild-to-severe AD treated for 6 mo with anti-TNF demonstrated a significant improvement in cognition and memory[22]. Alteration of the tissue microenvironment through use of non-steroidal anti-

inflammatory drugs show promise in the prevention of dementia. However, efficacy may be attained only when given in the appropriate time window during onset of dementia[27]. Therefore, research investigating the pathogenesis of cognitive decline is of great necessity.

Myeloid-derived cells, such as monocytes and microglia, have been implicated in AD pathogenesis[28,29]. Alzheimer's disease (AD) is characterized by widespread death of neurons, and presence of amyloid beta (Aβ) plaques and neurofibrillary tangles[14]. Microglial cells are the resident macrophages of the brain[28]. They share similar functions to peripheral tissue macrophages, inclusive of phagocytosis, antigen-presentation, cytokine production and release of ROS/NOS. They function to enhance neuronal survival through clearance of cellular debris and release of anti-inflammatory mediators (e.g. BDTF, brain-derived trophic factor)[28]. However, in the presence of excess Aβ peptides, microglia become 'activated' and release neurotoxic factors and pro-inflammatory cytokines (e.g. IL-6, TNF, IL-8)[23,30]. Further, in an AD mouse model, a 10-fold increase in bone marrow-derived monocytes and microglia was observed when compared to resident microglia[29]. These data suggest that recruited monocytes may contribute to AD pathogenesis. Consistent with these findings, microglia found in amyloid plaques secrete more CCL2 than non-plaque associated cells[14]. Moreover, several studies suggest that monocyte-mediated neuroinflammation and impairments in Aβ phagocytosis play critical roles in development of AD[14]. Monocytes isolated from AD patients demonstrate impaired Aβ surface uptake, defective transport Aβ into endosomes and lysosomes, and poor clearance[23], in addition to enhanced production of pro-

inflammatory cytokines[24]. Recent evidence suggests that monocyte-related inflammatory events precede the clinical development of AD[29]. Increased levels of serum CCL2 were found in patients with MCI or mild AD, but not severe AD, compared to healthy controls[29]. In a subgroup of MCI patients who progressed to severe AD, circulating CCL2 progressively decreased during the transition[29]. These findings suggest that monocyte recruitment is likely an early event in AD pathogenesis, preceding the clinical onset of disease. The mechanistic underpinnings underlying how blood-derived monocytes contribute to AD onset, however, remains poorly understood.

AGING & PNEUMONIA

In 2010, the estimated total annual excess cost of hospital-treated pneumonia in the elderly exceeded $7 billion in the US alone[182]. The majority of direct expenses were attributed to inpatient costs, which included hospitalization, length of stay, accommodations and physician services[183]. Given the increased risk of *S. pneumoniae* infection with advancing age, costs associated with disease and the growing rates of drug resistance, vaccination is a public health priority[184]. However, while a pneumococcal polysaccharide vaccine (PPV23) has been developed, controversy still exists over the effectiveness of the vaccine in older adults[185,186]. This is largely attributed to the limited randomized control trials that specifically targets patient populations over the age of 65[183]. Additional issues with the vaccine include low rates of adherence (e.g. 60% of adults over 65 have never received the vaccine[187]); specific cohorts demonstrate a poor response to the polysaccharide vaccine (e.g. those low vitamin B12, genetic

immunodeficiencies, cancer or HIV infections[184]), which unfortunately are often the same individuals who are predisposed to pneumococcal infection; and it is uncommon for adults, regardless of age, to respond to all vaccine serotypes.

Susceptibility To Pneumonia In The Elderly

Approximately 30% of mortality in the elderly is attributed to infectious disease, with pneumonia being the most common and costly infection[79,185,188–191]. *Streptococcus pneumoniae* is the most common cause of pneumonia in the elderly[7-9]. Pneumococcal disease is a leading cause of death and hospitalization of the elderly and individuals with chronic inflammatory diseases, and place a significant burden on our healthcare system[192,193]. *S. pneumoniae* is a diplococcus, gram-positive, facultative anaerobic member of the Streptococcus family that elicits a broad spectrum of diseases, including pulmonary pneumonia, meningitis, sepsis, otitis media, pneumococcal bacteremia and sinusitis. The burden of disease lies primarily in older adults. An increase in incidence begins at the age of 55[194], but the highest burden of disease are in individuals over the age of 75 years. In this cohort, the case fatality rate for pneumococcal pneumonia is 40%[194]. While many factors have shown to contribute to this increased susceptibility pneumonia in those of advanced age (e.g. nutritional deficiencies[190], mechanical changes in lung function[191], housing conditions in nursing homes[189], etc.), the major contributing factor is immunosenescence[195].

The Role Monocytes & Macrophages in *S. pneumoniae* Infection

Colonization of the nasopharynx, defined as asymptomatic and transient occupancy of *S. pneumoniae*, is a pre-requisite for pneumonia or invasive pneumococcal disease[62]. The dynamics of cellular recruitment to the nasopharynx in response to *S. pneumoniae* colonization is quite simple, primarily involving macrophages and neutrophils. Recruitment of neutrophils to the nasopharynx were found to be dispensable for *S. pneumoniae* clearance since they exclusively recognize opsonized bacteria and the nasopharynx is a non-opsonic environment[196]. Conversely, macrophages, which are largely derived from recently recruited monocytes, play a predominant role in bacterial clearance due to their non-opsonic, scavenger receptor-mediated recognition of *S. pneumoniae*[197]. As such, if there are changes in clearance capacity, it is likely associated with changes in macrophage quantity or function. During infection, Ly6C^{high} inflammatory monocytes are recruited to the nasopharynx, where they differentiate into classically activated M1 macrophages[198], which reach peak levels at day 7, at which point bacterial numbers begin to decline [199]. In the elderly, inadequate immune control, despite low carriage rates, is postulated to be responsible for the swift transition from asymptomatic colonization of the nasopharynx to symptomatic disease[200,201]. This phenotype can be observed in our aging mouse models. Our aged mice (>18mo, human equivalent of >70yrs) develop pneumonia within 3 days of colonization, whereas young adults (10-12wks, human equivalent of ~25yrs) do not generally demonstrate dissemination of *S. pneumoniae* from the nasopharynx to the lungs[60]. Furthermore, once bacteria disseminates to the lungs, old mice fail to clear bacteria compared to young counterparts[60]. Though age-associated impairments in monocyte and macrophage

function have been postulated to contribute to the transition from colonization of the nasopharynx to dissemination of disease (e.g. pulmonary pneumonia, bacteremia and meningitis), the primary data remains scarce. In this thesis, I aim to elucidate how aging contributes to phenotypic and functional changes in the myeloid lineage, and ultimately, an increased susceptibility to *S. pneumoniae* in the elderly.

Age-Associated Inflammation & Pneumonia Risk

A growing body of epidemiological data strongly suggest that a reciprocal link exists between age-associated inflammation and pneumonia susceptibility and severity[194,202–205]. While a robust inflammatory response is generally believed to be protective against infection, in old age high levels of systemic inflammatory cytokines are associated with increased mortality[203]. Elderly individuals who have higher than age-average levels of circulating, pro-inflammatory cytokines, TNF and IL-6, have an increased risk of acquiring, becoming hospitalized with, or dying from pneumonia than those with lower than average levels[105,107,203,206]. This claim is further supported by a recent study by Yende *et al*[203]. In a 6.5-year longitudinal study in 70- to 79-yr-old healthy, community-dwelling elderly, it was concluded that pre-infection systemic levels of inflammatory cytokines (TNF and IL-6) were associated with a higher risk of pneumonia requiring hospitalization. Furthermore, elevated levels of pro-inflammatory cytokines correlated with disease severity[203]. When mice young mice were infused with TNF, they became significantly more susceptible to experimental infection with *Streptococcus pneumoniae* than those that received saline[206]. Clearly, it is evident that

age-associated inflammation significantly increases susceptibility to pneumonia, however, the mechanistic underpinnings of such a relationship remains to be elucidated.

Can Suppression Of Inflammation Improve Pneumonia Outcome In The Elderly?

Early mortality in patients with community-acquired pneumonia (CAP) is not attributed to failure to eliminate the etiological agents, but rather, a dysregulated host reponse[202,207]. Excessive cytokine responses in patients with severe CAP has been associated with poor prognosis[204,208–211]. Given that CAP is the 6th highest cause of mortality and the 1st among infectious diseases in developed countries [212], strategies to suppress the excessive inflammatory responses during pneumonia to improve prognosis are being considered. Corticosteroids reduce the production of central inflammatory cytokines (e.g. TNF, IL-1β, and IL-6) and subsequent recruitment of inflammatory cells into the site of infection. In a study by *Fernández-Serrano et al.*, researchers administered corticosteroids (e.g. methyl-prednisolone) to hospital-admitted CAP patients prior to antibiotic treatment and observed reduced inflammatory responses and improved clinical outcomes[212]. Another study revealed that patients with CAP who received corticosteroids were discharged from hospital one day earlier, and mortality was reduced by 50%[213]. Further, corticosteroid treatment significantly reduced the need for mechanical intervention and likelihood of complications (e.g. acute respiratory distress)[213]. Additionally, administration of corticosteroids accelerated resolution of clinical symptoms, and thereby reduced the duration of intravenous antibiotic therapy[213,45]. Such phenomena were paralleled in murine studies. Mice infected with severe bacterial

pneumonia that were subsequently treated with corticosteroids and antibiotics recovered more rapidly and had reduced pulmonary inflammation than mice treated with antibiotics alone[214,215]. Immune cells isolated from old mice (>18mo), particularly macrophages, generate a hyper-inflammatory responses to bacteria or bacterial products, when compared to young mice[60]. This dysregulated immune response contributes to an increased disease severity due to enhanced dissemination and greater immunopathology, resulting in a poor disease outcome[212]. Together, these findings suggest that lowering levels of age-associated inflammation may be an effective strategy in improving host defence against *S. pneumoniae* in older adults.

CHAPTER 2: *TNF Drives Monocyte Dysfunction with Age and Results in Impaired Anti-pneumococcal Immunity*

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Declaration of Academic Achievement:

This research was conducted during the period of September 2013 to November 2015. I am the co-first author of this paper. I designed and conducted a significant proportion of the experiments, and analyzed and interpreted the corresponding data. My contributions include the following: 1 of 2 independent experiments for Figure 1A-D, and 2 of 3 independent experiments for Figure 4C-G and 1 of 3 independent experiments Figure 6A-F. I produced the data for Figures 2A (polystyrene depletions of Ly6Chigh monocytes), 4A-B (heterochronic bone marrow chimeras), 6K (monocyte-mediated *S. pneumoniae* binding and uptake), 7D (inflammatory monocytes in aged TNF knockouts), and Figure 8 (entire panel; monocyte depletion and infection outcome). Further, I performed all the experiments necessary to generate Supplementary Figure 1(D-E), 2 and 3. D. Loukov assessed bacterial burden in old wildtype and TNF knockout mice (Fig. 7C). Dr. C. Verschoor performed and analyzed all the human data (Fig. 3). Dr. A. Puchta produced remaining figures. Dr. L. Kobzik provided thoughtful discussions and contributed to manuscript revisions. Dr. M. Larché, a clinical collaborator, generously provided the adalimumab (*Humira*) used in the TNF neutralization experiments. Dr. Z. Xing generously provided the TNF KO mice, provided insight and critical appraisal of manuscript. Experiments were conceived and designed by Dr. D.M.E. Bowdish, Dr. A. Puchta and myself. Dr. D.M.E. Bowdish contributed to authorship and provided guidance throughout the writing process.

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RESEARCH ARTICLE

TNF Drives Monocyte Dysfunction with Age and Results in Impaired Anti-pneumococcal Immunity

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Abstract

Monocyte phenotype and output changes with age, but why this occurs and how it impacts anti-bacterial immunity are not clear. We found that, in both humans and mice, circulating monocyte phenotype and function was altered with age due to increasing levels of TNF in the circulation that occur as part of the aging process. Ly6C$^+$ monocytes from old (18–22 mo) mice and CD14$^+$CD16$^+$ intermediate/inflammatory monocytes from older adults also contributed to this "age-associated inflammation" as they produced more of the inflammatory cytokines IL6 and TNF in the steady state and when stimulated with bacterial products. Using an aged mouse model of pneumococcal colonization we found that chronic exposure to TNF with age altered the maturity of circulating monocytes, as measured by F4/80 expression, and this decrease in monocyte maturation was directly linked to susceptibility to infection. Ly6C$^+$ monocytes from old mice had higher levels of CCR2 expression, which promoted premature egress from the bone marrow when challenged with *Streptococcus pneumoniae*. Although Ly6C$^+$ monocyte recruitment and TNF levels in the blood and nasopharynx were higher in old mice during *S. pneumoniae* colonization, bacterial clearance was impaired. Counterintuitively, elevated TNF and excessive monocyte recruitment in old mice contributed to impaired anti-pneumococcal immunity since bacterial clearance was improved upon pharmacological reduction of TNF or Ly6C$^+$ monocytes, which were the major producers of TNF. Thus, with age TNF impairs inflammatory monocyte development, function and promotes premature egress, which contribute to systemic inflammation and is ultimately detrimental to anti-pneumococcal immunity.

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Author Summary

As we age, levels of inflammatory cytokines in the blood and tissues increase. Although this appears to be an inevitable part of aging, it ultimately contributes to declining health. Epidemiological studies indicate that older adults with higher than age-average levels of inflammatory cytokines are at increased risk of acquiring, becoming hospitalized with and dying of *Streptococcus pneumoniae* pneumonia but how age-associated inflammation increased susceptibility to was not entirely clear. We demonstrate that the increase in the inflammatory cytokine TNF that occurs with age cause monocytes to leave the bone marrow prematurely and these immature monocytes produce more inflammatory cytokines when stimulated with bacterial products, thus further increasing levels of inflammatory cytokines in the blood. Furthermore, although old mice have higher levels of these inflammatory monocytes arriving at the site of *S. pneumoniae*, they are not able to clear the bacteria. By pharmacologically or genetically removing the inflammatory cytokine TNF or reducing the number of inflammatory monocytes we were able to restore antibacterial immunity in aged mice. Thus we demonstrate that monocytes are both influenced by and contributors to age-associated inflammation and that chronic exposure to age-associated inflammation increases susceptibility to *S. pneumoniae* due to altering monocyte maturity and function.

Introduction

Monocyte phenotype and function change with age but whether these changes contribute to susceptibility to infectious disease is unclear. In mice, monocytes can be subdivided based on their expression of the Ly6C antigen into Ly6C^{high} (Ly6C^{high}, CCR2^{high}, CX3CR1^{low}) and Ly6C^{low} (Ly6C^{low}, CCR2^{low}, CX3CR1^{high}) monocytes [1,2]. In humans, the functional equivalents are CD14^{++}CD16$^{-/+}$ and CD14^{+}CD16^{++} monocytes, respectively [1,3]. Ly6C^{high} monocyte output from the bone marrow to the blood increases in a CCR2-dependent manner early during infection [4,5], and they become the dominant monocyte subtype in the blood, preferentially homing to sites of inflammation[6]. Ly6C^{high} monocytes produce high levels of inflammatory cytokines[4,5,7]; thus, they are often called "inflammatory monocytes".

In the elderly, numbers of circulating CD14^{++}CD16^{+} and CD14^{++}CD16^{-} monocytes, are significantly higher[8]. CD14^{++}CD16^{+} monocytes derived from elderly individuals are more senescent (i.e. have shorter telomeres) than other monocyte subsets and produce more pro-inflammatory cytokines (IL6, TNF, IL1β, IL12p70) and have higher levels of some chemokine receptors (e.g. CCR2, CCR5, CCR7, CX3CR1) [9,10]. Due to their ability to produce large amounts of pro-inflammatory cytokines, Ly6C^{high} monocytes contribute to the pathology of several models of chronic inflammation [11,12,13,14,15,16,17]. During chronic inflammatory conditions, the number of circulating Ly6C^{high} monocytes increase progressively[18] and their ablation is an effective strategy for decreasing pathology [16,17,19,20]. Whether Ly6C^{high} monocytes contribute to chronic age-associated inflammation and increased susceptibility to infection is not known and is the focus of this study.

Aging is accompanied by an increase in the levels of pro-inflammatory cytokines such as tumour necrosis factor (TNF) and interleukins 1β (IL1β) and 6 (IL6) in the serum and tissues, a phenomenon that has been termed "inflamm-aging"[reviewed in[21,22]]. This age-associated, systemic state of chronic, low-grade inflammation (defined as "para-inflammation" by Medzhitov[23])is well-documented although its cellular source has yet to be definitively identified. Adipose tissue[24], CD4^{+} T cells or macrophages[25,26] have all been proposed to

contribute. Increases in serum cytokines (particularly IL6 and TNF) are generally thought to be a pathological consequence of aging, as they correlate with risk of classical "diseases of age" such as dementia[27], stroke[28], cardiovascular disease[29] as well as frailty[30,31] and all-cause mortality[32,33]. Conversely, lower than average levels of age-associated inflammation predict good health in age[34]. Furthermore, most chronic inflammatory conditions are characterized by increased numbers of $CD14^{++}CD16^{+}$ and/or $CD14^{++}CD16^{-}$ monocytes [35,36,37,38,39,40,41]. Herein, we investigate the role of monocytes, which are known to be critical mediators of chronic inflammation, contribute to age-associated inflammation.

Inflamm-aging contributes to susceptibility to infectious disease, and particularly pneumonia, which is a major cause of death in the elderly[42]. Susceptibility to pneumonia correlates with increased levels of IL6 and TNF before an infection [43,44,45]. When young mice are infused with TNF, they become as susceptible to experimental infection with *Streptococcus pneumoniae* as old mice[46]. Using a mouse model of pneumococcal colonization, we investigated whether changes in monocyte phenotype adversely affect host defense towards *S. pneumoniae*. We show that with age that there is an in increase in circulating $Ly6C^{+}$ monocytes during the steady state due to increased expression of CCR2. Using heterochronic bone marrow chimeras, we demonstrate that the aging microenvironment, rather than intrinsic changes in myeloid progenitors, drives changes in monocyte phenotype, including decreased expression of F4/80 (a marker of maturity), and increased expression of CCR2 (required for monocyte mobilization). We demonstrate that age-associated increases in TNF are the driving factor behind changes in monocyte phenotype, as TNF deficiency or treatment with anti-TNF antibodies normalizes expression of CCR2 on $Ly6C^{+}$ monocytes. Decreased CCR2 expression results in decreased numbers of monocytes in the circulation and reduced production of TNF and IL6. Finally, we demonstrate that, although TNF levels and the recruitment of $Ly6C^{+}$ monocytes are increased in old mice during nasopharyngeal *S. pneumoniae* colonization, this, counterintuitively, results in diminished bacterial clearance.

To our knowledge, this is the first mechanistic study that investigates the role of $Ly6C^{+}$ monocytes as central mediators of inflamm-aging and demonstrates that TNF is a key contributor to age-associated defects in myeloid phenotype and anti-bacterial function. These data indicate that $Ly6C^{+}$monocyte frequency and increased production of pro-inflammatory cytokines contributes to both age-associated inflammation and declining anti-bacterial immunity.

Results

$Ly6C^{+}$ monocytes increase with age in the blood and bone marrow but are phenotypically and functionally different

It has been reported that with age the proportion of myeloid cells and cytokines in the blood is increased. We quantitated circulating leukocyte populations in old (18–22 mo) mice and found that, consistent with previously published data[47,48], there was a decrease in the percentage of T cells and an increase in the number of myeloid cells when compared with young (10–14 wk) mice (Fig 1A & S1A Fig). Analysis of monocyte subsets indicated that the absolute number of both $Ly6C^{high}$ and $Ly6C^{low}$ monocytes was increased with age (Fig 1A). An increase in $Ly6C^{high}$ monocyte frequency within the blood of old mice was paralleled by a similar increase in the bone marrow (Fig 1B), suggesting that increased myelopoiesis within the bone marrow may precede increased numbers of these cells in the blood. Consistent with this, we also found that the number of M-CSF responsive cells (myeloid precursors and monocytes capable of differentiating into bona fide macrophages *ex vivo*) in the bone marrow was significantly increased with age (S1C Fig).

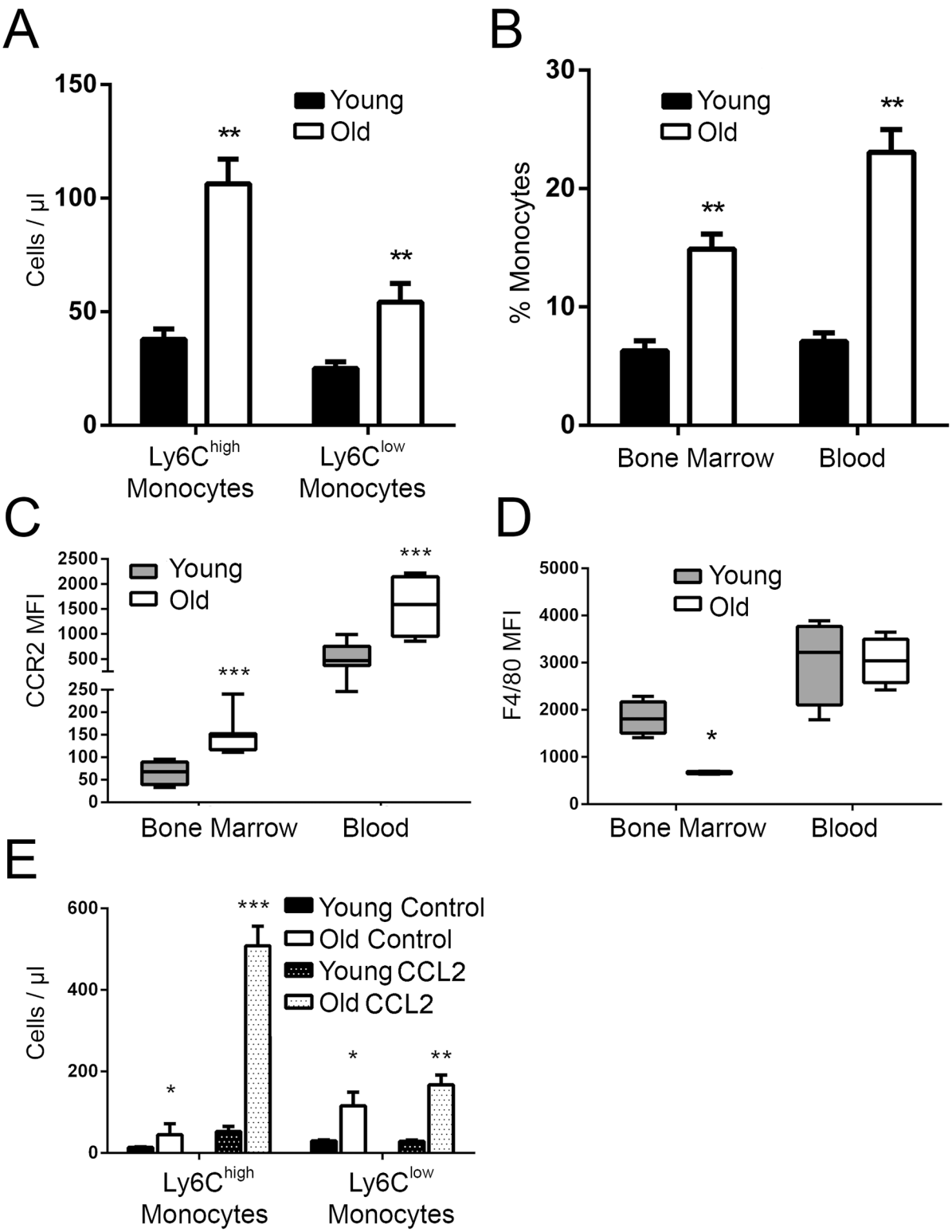

Fig 1. Ly6C^{high} monocytes are increased with age, express more CCR2 and less F4/80. (A) Total numbers of Ly6C^{high} and Ly6C^{low} monocytes were quantitated in the blood of old (18–22 mo) WT C57Bl6/J mice and compared to that from young (10–14 wk) mice. The data represent the mean (± SEM) of 6 mice. (B) Analysis of the Ly6C^{high} monocytes as a percentage of CD45^{+} cells in the blood and bone marrow of young and old mice (± SEM; $n = 6$). (C) CCR2 expression on Ly6C^{high} monocytes in the bone marrow and blood of old mice is higher than young controls as determined by flow cytometry ($n = 6$–8). (D) The mean expression of the macrophage maturity marker, F4/80, on Ly6C^{high} monocytes in the bone marrow and blood of young and old mice ($n = 6$–8). (E)

Cells recruited to the peritoneum were quantitated 4 hours after administration of 100 nM CCL2. The recruitment of Ly6C^{high} and Ly6C^{low} monocytes was greater in old mice (± SEM; $n = 5$). Statistical significance was determined by two-tailed Mann-Whitney-Wilcoxon test or two-way ANOVA with Fisher's LSD post-test where appropriate. * indicates $p < .05$, ** indicates $p < 0.005$, *** indicates $p < 0.0005$ and **** indicates $p < 0.00005$. (A-D) is representative of 4 independent experiments; (E) is representative of 2 independent experiments.

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The C-C chemokine receptor type 2 (CCR2) is expressed at high levels on Ly6C^{high} monocytes and is essential for their entry into the blood in response to the production of CCL2[49]. Since CCR2 is required for monocytes, and especially Ly6C^{high} monocytes, to leave the bone marrow and enter the blood, we hypothesized that enhanced CCR2 expression on Ly6C^{high} monocytes could prompt their premature emigration from the bone marrow and could explain the increased number of Ly6C^{high}monocytes seen with age. CCR2 expression was measured on Ly6C^{high} monocytes in the blood and bone marrow of old mice and found to be dramatically increased (Fig 1C). Consistent with previous research[1], CCR2 expression was more pronounced on Ly6C^{high} monocytes (S1E Fig). As Ly6C^{high} monocytes represent an intermediate stage in monocyte-to-macrophage differentiation, we investigated their maturity using the monocyte/macrophage maturity marker, F4/80. Interestingly, we found that there was an inverse relationship between CCR2 expression and F4/80 expression on Ly6C^{high} monocytes in the blood of old mice. With age, these cells showed significantly decreased levels of F4/80 (Fig 1D), suggesting that their increased CCR2 expression may prompt these cells to enter the circulation in an immature state. When CCR2 expression was measured on myeloid precursors undergoing M-CSF-stimulated differentiation into macrophages, increased CCR2 expression occurred during an intermediate stage of differentiation (day 5) on cells from old mice (S1D Fig).

To determine whether increased CCR2 expression was sufficient to increase Ly6C^{high} monocyte egress, we intraperitoneally injected young and old mice with 100 nM of CCL2 and measured Ly6C^{high} monocyte recruitment after 4 hours. We found that despite administering an equivalent dose of CCL2, Ly6C^{high} monocyte recruitment to the peritoneum was increased ~5-fold in old mice relative to young mice (Fig 1E). A less dramatic increase in Ly6C^{low} monocytes was also observed (Fig 1E), consistent with previous studies.

Monocytes are potent producers of pro-inflammatory cytokines with age

Since we found that there was an expansion of monocytes with age and these cells are known to be potent producers of pro-inflammatory cytokines, we postulated that they might contribute significantly to age-associated inflammation. To determine whether the increased numbers of monocytes with age contributed to age-associated increases in IL6 production, we targeted this cell population using carboxylated polystyrene microparticles (PS-MPs), which have been shown by others to lead to a reduction of primarily Ly6C^{high} monocytes in the blood[50]. We found that when circulating monocytes were decreased in old mice (Fig 2A), this reduced circulating levels of IL6 (Fig 2B). In humans, CD14^{++}CD16^{+}HLA-DR^{+}/intermediate monocytes are the biggest producers of inflammatory cytokines under a variety of stimulation conditions [3]. Intracellular cytokine staining reveals that of the three human monocyte populations (classical, intermediate, non-classical) intermediate monocytes are the major producers of TNF (Fig 3A) and IL6 (Fig 3B) after stimulation with LPS or *S. pneumoniae* and older donors (63–70 yrs) produce more cytokines than younger donors (26–52 yrs). Additionally, CD14^{+} monocytes isolated from PBMCs from older donors produced more TNF (Fig 3C) and IL6(Fig 3D) in response to LPS than did younger donors. As in mice, the numbers of intermediate monocytes may be influenced by levels of age-associated inflammation since the frequency of intermediate monocytes, are positively correlated with plasma TNF (Fig 3E) as has been shown to occur in other chronic inflammatory conditions [51]. A weaker correlation ($p<0.02$) was

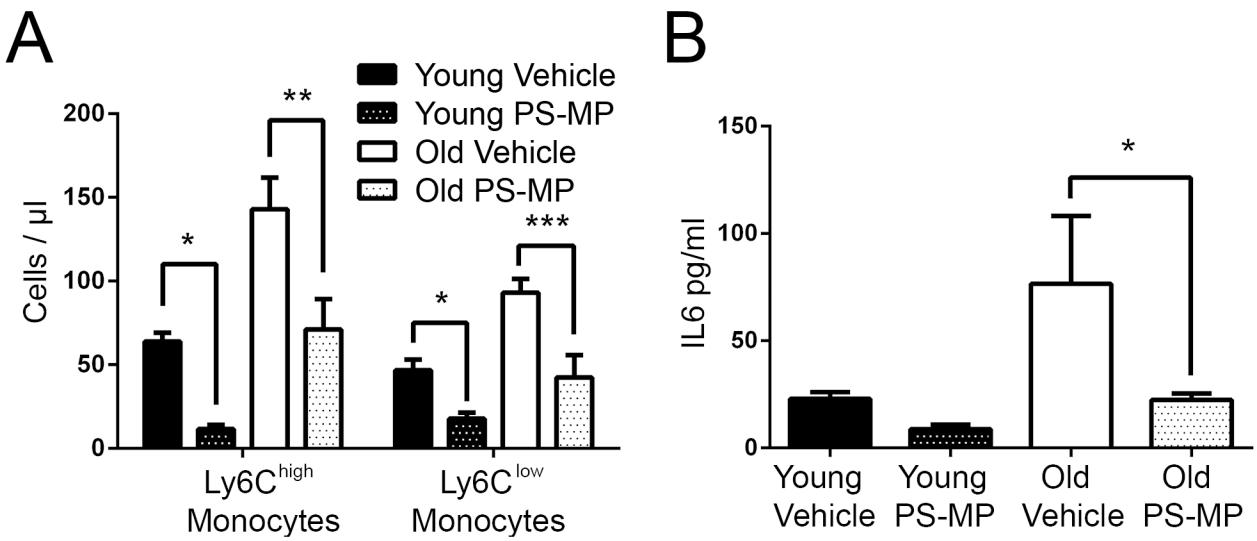

Fig 2. Ly6C^{high} monocytes contribute to elevated levels of serum IL6 and TNF in aged mice. Young and old mice were injected with 500 nm negatively-charged polystyrene microparticles (PS-MPs) previously shown to reduce numbers of circulating Ly6C^{high} monocytes. Circulating monocyte populations (A) and IL6 levels in whole blood (B) were quantitated after 24 hours. Statistical significance was determined by two-tailed Mann-Whitney-Wilcoxon test. * indicates $p < .05$, ** indicates $p < 0.005$, *** indicates $p < 0.0005$ and **** indicates $p < 0.00005$. (A-B) is representative of ± SEM of 5 mice from 2 independent experiments.

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observed between TNF levels and the numerically dominant classical monocytes and no correlation was found between non-classical monocytes and TNF ($p = 0.2$).

The age-associated increase in circulating pro-inflammatory monocytes is regulated by TNF

To determine whether age-related changes in Ly6C^{high} monocyte numbers, phenotype and inflammatory capacity were caused by changes in the aging bone marrow microenvironment or due to intrinsic changes in the myeloid precursors themselves, we created heterochronic bone marrow chimeras. When young bone marrow was transferred to old recipient mice the number of Ly6C^{high} and Ly6C^{low} monocytes was increased to levels comparable to old mice (Fig 1A) or old recipient mice who had received old donor marrow (Fig 4A). In contrast, young recipient mice that had received old donor marrow had Ly6C^{high} and Ly6C^{low} monocyte numbers comparable to young mice (Fig 1A) or to young recipient mice that had received young donor bone marrow (Fig 4A). In addition, the increase in CCR2 expression observed on circulating monocytes from old mice (Fig 1C) was also observed in circulating monocytes from old recipient mice who had received young donor marrow but not on young recipient mice who received old donor marrow(Fig 4B). These data demonstrate that increases of Ly6C^{+} monocytes and increased CCR2 expression occur in a manner entirely dependent on the bone marrow microenvironment.

Since TNF is one of the central cytokines associated with inflamm-aging, we investigated whether TNF was sufficient to drive expansion of the Ly6C^{high} monocytes. We aged TNF knockout (KO) mice (18–22 mo) and quantified Ly6C^{high} monocytes in their blood. We found that, unlike their WT counterparts, old TNF KO mice did not have higher numbers of circulating Ly6C^{high} monocytes (Fig 4C), but did have an increase in bone-marrow Ly6C^{high} monocytes compared to their young counterparts (Fig 4D). Surface expression of CCR2 on Ly6C^{high} monocytes in both the blood (Fig 4E) and the bone marrow (Fig 4F) of old TNF KO mice was comparable to the levels seen in young mice. Similarly there were no changes in Ly6Clow monocytes in aged TNF KO mice (S1D Fig).

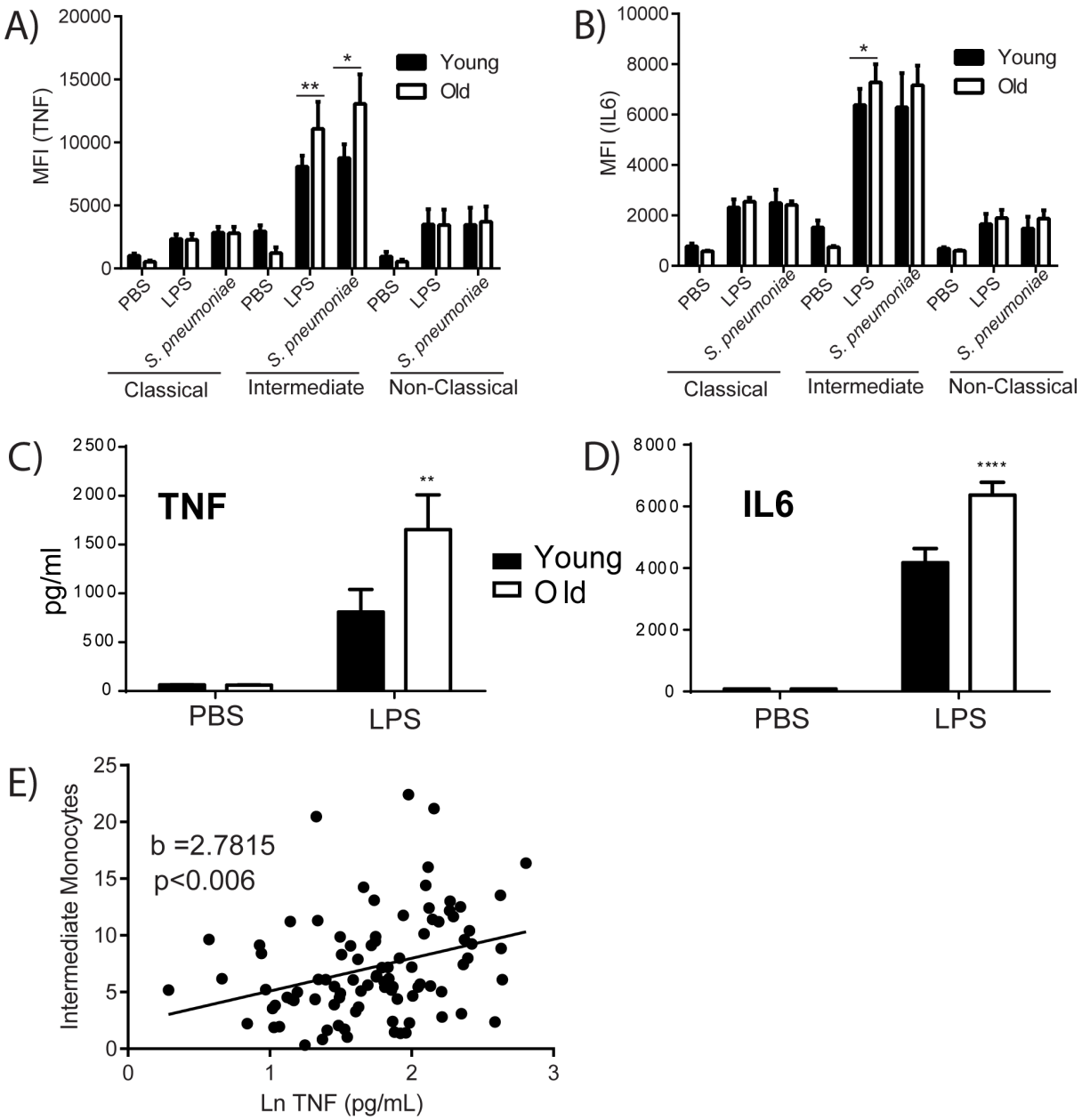

Fig 3. Human CD14^{++}CD16^{+}HLA-DR^{+} (intermediate) monocytes produce more inflammatory cytokines with age. Intracellular production of TNF (A) and IL-6 (B) in classical (CD14^{++}), intermediate (CD14^{++}CD16^{+}) and non-classical (CD14^{+}CD16^{+}) monocytes from young and elderly donors in response to LPS (50 ng/ml) and *S. pneumoniae (5 x10^{6} CFU)*. C) The secretion of TNF and D) IL-6 for isolated CD14+ monocytes in response to LPS for young and older donors. E) The frequency of intermediate monocytes were found to have a significant, positive correlation with the levels of serum TNF (β = 2.78, p<0.006). (A-D) is representative of ± SEM of n = 7 young donors (26–52 yrs) and n = 6 older donors (63–70 yrs) *indicates p<0.05, and ** indicates p< 0.05. Intermediate monocyte (CD14++CD16+HLA-DR+) count (cells per microlitre of whole blood) increases relative to serum levels of TNF in older donors (n = 94, 61-100yrs).

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These data suggest that increased production of Ly6C^{high} monocytes in the bone marrow occur independent of TNF, but that increases in CCR2 expression on these cells in the bone marrow, and their subsequent mobilization to the blood is TNF-dependent. Consistent with our observation that Ly6C^{+}monocytes contribute to elevated levels of circulating cytokines

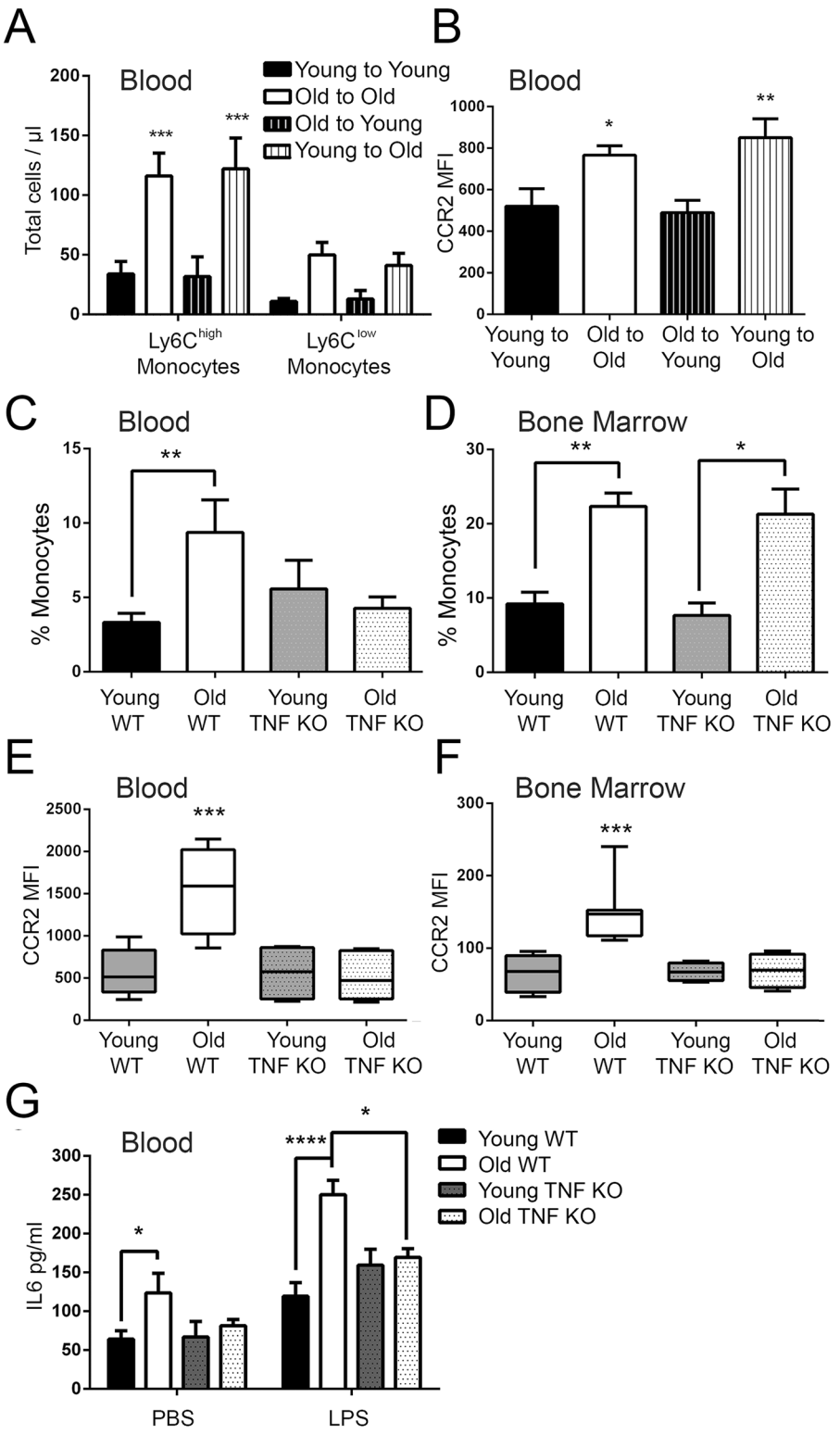

Fig 4. TNF drives increases in circulating Ly6C^{high} monocytes. (A) Total numbers of Ly6C^{high} and Ly6C^{low} monocytes in the blood of heterochronic bone marrow chimeric mice. Old recipient mice which receive young donor marrow have increased numbers of circulating Ly6C^{high} and Ly6C^{low} monocytes which are comparable to old recipient mice that receive old donor marrow. Young recipient mice that receive old donor marrow do not have an increase in Ly6C^{high} and Ly6C^{low} monocytes. The data represent the mean (±

SEM) of 5 mice. (B) CCR2 expression on circulating monocytes is elevated when the recipient mouse is old, indicating that the bone marrow microenvironment drives changes in CCR2 expression (CCR2 MFI± SEM; $n = 5$). (C-D) The percent Ly6C^{high} monocytes as a proportion of CD45^{+} cells in the (C) blood or (D) bone marrow of young and old WT and TNF KO mice was quantitated (± SEM; $n = 4–6$). (E-F) Expression of CCR2 on Ly6C^{high} monocytes in the (E) blood or (F) bone marrow of young and old WT and TNF KO mice ($n = 4–8$) demonstrate that the presence of TNF drives CCR2 expression with age. (G) IL6 production in whole blood from young and old TNF KO mice stimulated with 100 ng/ml of LPS or a vehicle control for 24 hours was quantitated by ELISA (± SEM; $n = 5$). Statistical significance was determined by two-tailed Mann-Whitney-Wilcoxon test, one-way or two-way ANOVA with Fisher's LSD post-test where appropriate. * indicates $p < .05$, ** indicates $p < 0.005$, *** indicates $p < 0.0005$ and **** indicates $p < 0.00005$. (A-B) is representative of 2 independent experiments; (C-G) is representative of 3 independent experiments.

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with age (Fig 2), old WT mice produced more IL6 than young mice following 24 hour stimulation of whole blood with either PBS or LPS (Fig 4G). In comparison, old TNF KO mice, which did not have an increase of Ly6C^{+}monocytes in the blood did not have an age-associated increase in IL6 in whole blood in response to PBS or LPS (Fig 4G).

Blockade of TNF reverses age-associated increases in Ly6C^{high} monocytes and inflammation

We investigated whether it was chronic or acute exposure to TNF that mediated age-related increases in serum IL6 and changes in monocyte phenotype and function. We first sought to determine whether increases in circulating Ly6C^{+} monocytes were inducible after administration of TNF. TNF (5ng/g) was administered intraperitoneally for 3 weeks, a time point chosen because it would allow for multiple cycles of monopoiesis and complete turnover of pre-formed monocytes [52]. Young mice showed a large increase in Ly6C^{high} monocytes in the blood and a less dramatic increase of Ly6C^{low} monocytes (Fig 5A). This was accompanied by a significant increase in serum IL6 in TNF-treated, but not vehicle control mice (Fig 5B). We next asked whether blocking TNF could reduce numbers of Ly6C^{+} monocytes in old animals. Young and old WT mice were administered Adalimumab (HUMIRA), a human monoclonal antibody specific for TNF, or an IgG isotype control at a dose of 50 ng/g for a period of three weeks via intraperitoneal injection. Anti-TNF therapy reduced the levels of plasma TNF from an average of 1.5 pg/ml to below the level of detection (LOD = 0.25pg/ml) in old mice and decreased the number of circulating Ly6C^{high} but not Ly6C^{low} monocytes in the blood to levels similar to young mice (Fig 5C). Anti-TNF therapy also reduced CCR2 expression on Ly6C^{high} monocytes in the blood of old mice to levels that are equivalent to those seen in young mice (Fig 5D) and reduced the percentage of monocytes that stained positive for IL6 or TNF by ICS after LPS stimulation (Fig 5E). Anti-TNF treatment reduces IL6 levels in the circulation of old mice (Fig 5F) and when blood from young and old mice treated with anti-TNF or IgG controls was stimulated with LPS, IL6 levels were lower in old mice treated with anti-TNF compared to those that were treated with IgG(Fig 5G).

Circulating and recruited Ly6C^{high} monocytes are increased with age during *S. pneumoniae* colonization

In order to determine if age-related changes in Ly6C^{high} monocyte numbers or maturity might play a role in defective anti-bacterial immunity with age, we investigated the trafficking of these cells following nasopharyngeal colonization of young and old mice with the bacterial pathogen, *S. pneumoniae*. We selected this pathogen specifically because of the high burden of disease caused by *S. pneumoniae* in elderly individuals and because it has been previously demonstrated that its clearance from the nasopharynx is largely dependent on recruited monocytes/macrophages[53,54]. Following intranasal delivery of *S. pneumoniae*, we found that old

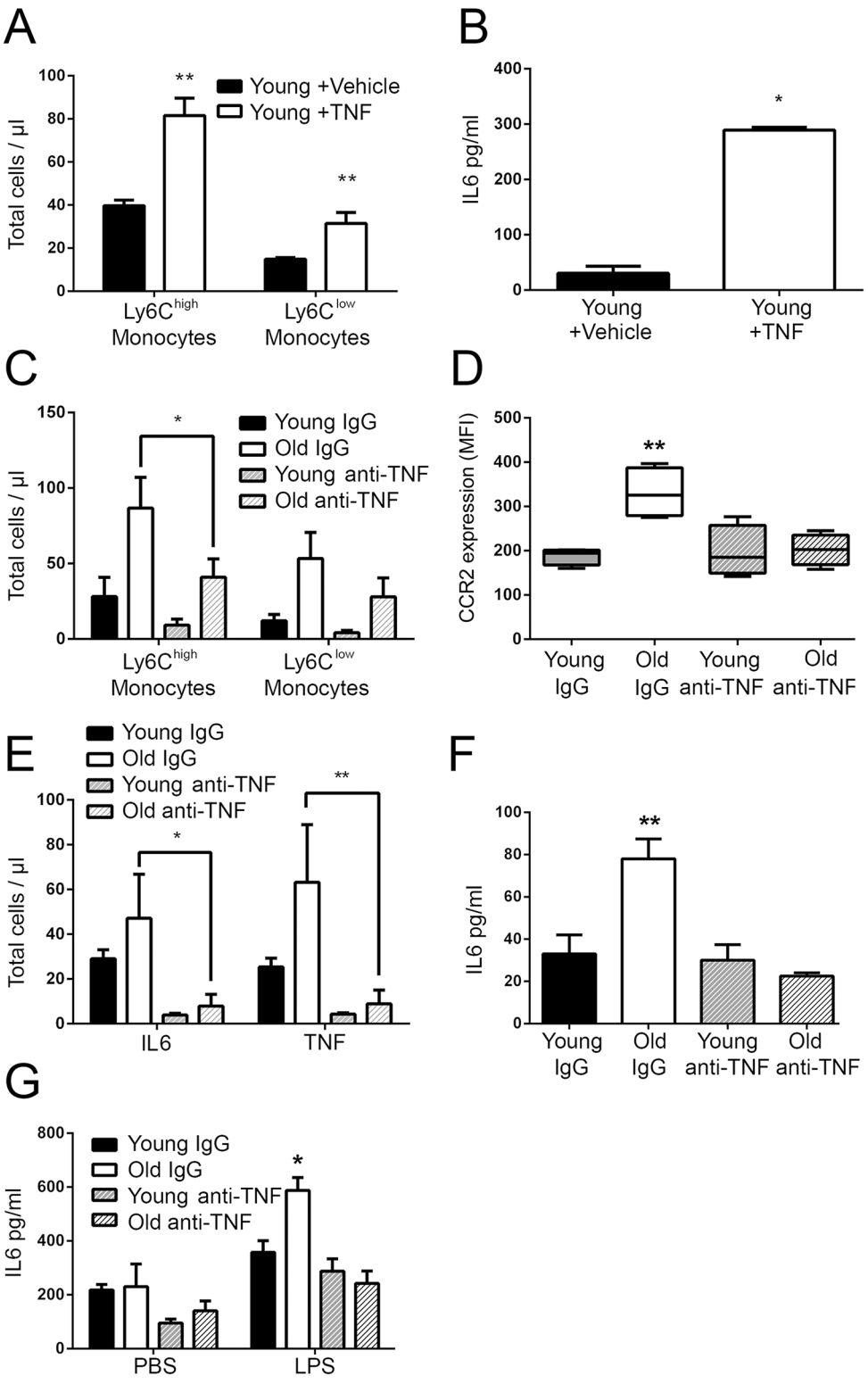

Fig 5. Anti-TNF therapy can reverse the age-associated increase in circulating Ly6C^{high} monocytes. (A-B) Young mice were give 200 ng/ml of TNF intraperitoneally every other day for 3 weeks. Numbers of circulating Ly6C^{high} and Ly6C^{low} monocytes (A) and serum IL6 (B) were quantitated. The data represent the mean (± SEM) of 5 mice. (C) Young and old WT mice were treated for 3 weeks with a neutralizing TNF antibody or IgG control and total numbers of circulating Ly6C^{high} monocytes were quantitated by flow

cytometry. The data represent the mean (± SEM) of 4 mice. (D) The mean CCR2 expression on circulating Ly6C^{high} monocytes in young and old mice treated with either anti-TNF or IgG was quantitated and found to be reduced with anti-TNF treatment (n = 4). (E) Intracellular staining of IL6 and TNF on blood monocytes after a 4 hour stimulation with LPS from young and old WT mice treated with either anti-TNF or IgG demonstrates that the number of monocytes that stain positive for IL6 or TNF are decreased with anti-TNF therapy(± SEM; n = of 4). (F) Serum IL6 is reduced in old mice treated with anti-TNF but not the IgG control. (G) IL6 production in whole blood following stimulation with LPS or a vehicle control after 24 hours from young and old WT mice given either anti-TNF or IgG (± SEM; n = 4). Statistical significance was determined by two-tailed Mann-Whitney-Wilcoxon test, one-way or two-way ANOVA with Fisher's LSD post-test where appropriate. * indicates $p < .05$, ** indicates $p < 0.005$, *** indicates $p < 0.0005$ and **** indicates $p < 0.00005$. (A-G) are representative of 1 experiment with n = 4 mice.

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mice had defects in clearance of the colonization. By Day 21 most of the young mice had cleared the bacteria, while old mice still harbored high bacterial loads (Fig 6A). Old mice were also more susceptible to bacterial invasion to the lungs at day 3 (Fig 6B) and mortality throughout the course of colonization (Fig 6C). Although serum production of CCL2 in old mice was comparable to that of young mice (Fig 6D), old mice had increased Ly6C^{high} but not Ly6C^{low} monocyte numbers in the circulation during colonization (days 3, 7, 14, 21) (Fig 6E).

We next investigated whether the monocytes/macrophages recruited in the context of age had maturity defects (as measured by F4/80 expression). In old mice, circulating Ly6C^{high} monocytes had decreased expression of F4/80 during colonization (Fig 6F), suggesting that the decreased F4/80 expression seen in the bone marrow during the steady state (Fig 1D) perpetuates following their egress during infectious challenge. Despite their inability to control bacterial loads in the nasopharynx, old mice also had a significant increase in the expression of CCL2 in the nasopharynx during colonization (Fig 6G), and had higher numbers of recruited Ly6C^{high} monocytes (Fig 6H) and macrophages (Fig 6I) to the nasopharynx compared to young mice. Although resident macrophages from young and old mice present in the nasopharynx during the steady state expressed equal levels of F4/80, monocytes/macrophages recruited to the nasopharynx during *S. pneumoniae* colonization showed decreased expression F4/80(Fig 6J), similar to that seen in their counterparts in the blood(Fig 6F). In order to determine whether bacterial binding and internalization was different between monocytes derived from young and old mice we compared bacterial binding (measured at 4°C) and internalization/killing (measured at 37°C). Although there was a significant decrease in bacterial binding between young and old mice, this did not appear to affect internalization or bacterial killing (Fig 6K).

Ly6C^{+} monocytes impair clearance of *S. pneumoniae* with age

Although trafficking of blood monocytes was not impaired with age, old mice nonetheless displayed impaired clearance of *S. pneumoniae*. To explain this, we hypothesized that high levels of recruited but developmentally immature Ly6C^{high} monocytes could, in fact, have negative consequences for clearance. Interestingly, TNF, which we showed caused increased numbers of Ly6C^{high} monocytes in the blood (Fig 4A), was increased with age during *S. pneumoniae* colonization in the nasopharynx (Fig 7A) and blood (Fig 7B). We next compared nasopharyngeal bacterial loads in WT and TNF KO mice, to determine whether TNF production affected bacterial clearance. Although TNF had no effect on clearance of colonization in young mice we found that old TNF KOs had significantly fewer CFUs in the nasopharnyx compared to their old WT counterparts at day 3 (Fig 7C). Old TNF KO mice also had decreased recruitment of Ly6C^{high} monocytes in the blood (Fig 7D), confirming that TNF can regulate mobilization of these cells during infection as well as in the steady state.

To determine whether the decreased recruitment of Ly6C^{high} monocytes we observed was responsible for improved bacterial clearance in old TNF KO mice, we preferentially targeted

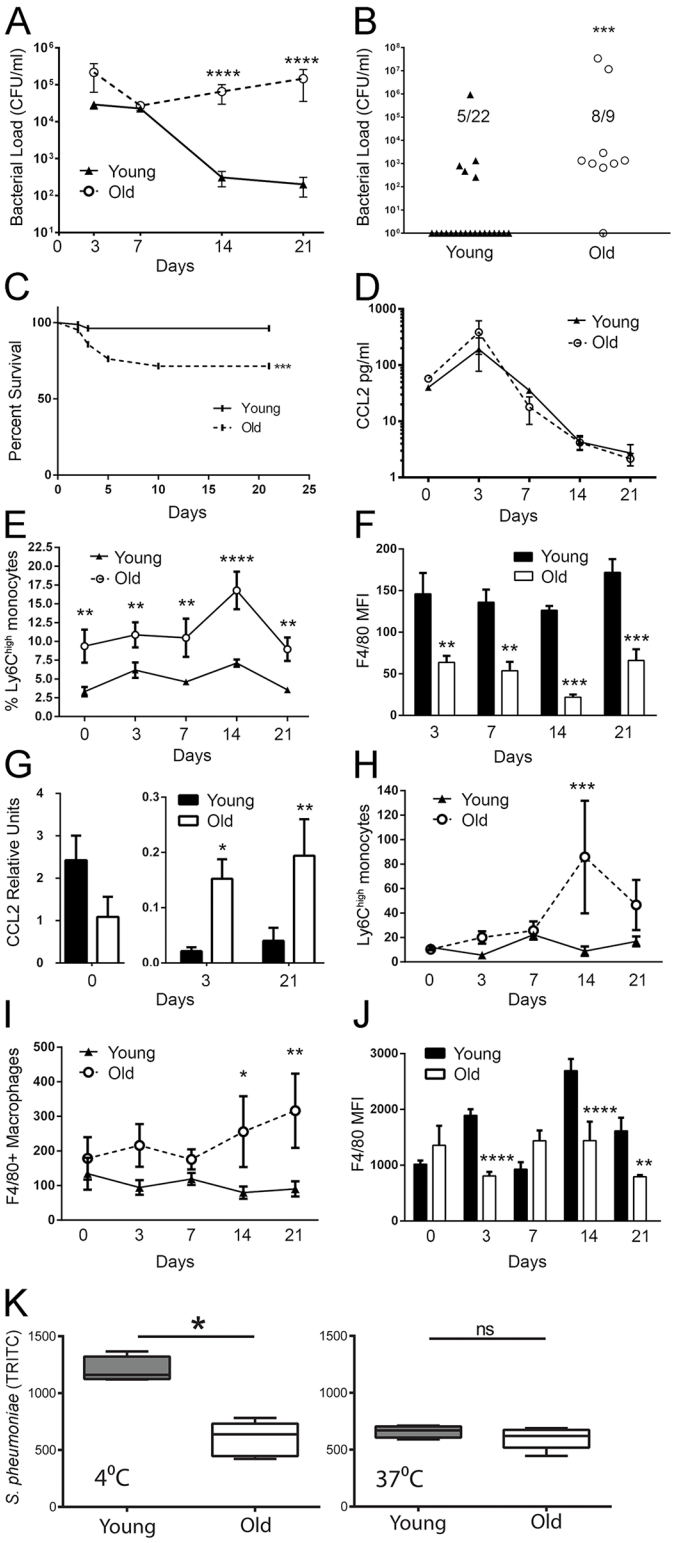

Fig 6. Old mice have increased numbers of circulating and recruited Ly6C^{high} monocytes during the course of *S. pneumoniae* colonization. (A) Colony forming units (CFUs) in nasal lavages from young and old WT mice were quantified on days 3, 7, 14 and 21 following intranasal colonization with *S. pneumoniae* (± SEM; $n = 5–22$). (B) CFUs of *S. pneumoniae* in the lungs at day 3 following intranasal colonization (± SEM; $n = 9–22$). (C) Survival of young and old mice after intranasal *S. pneumoniae* colonization (± SEM; $n = 12$).

(D) Total serum CCL2 in young and old mice following intranasal *S. pneumoniae* colonization was measured by a high sensitivity ELISA. The data represent the mean (± SEM) of 3 mice per time point. (E) Ly6C^{high} monocytes as a percent of CD45^{+} cells in the blood of young and old WT mice during nasopharyngeal *S. pneumoniae* colonization (± SEM; $n = 5–8$) was measured by flow cytometry. (F) Mean expression of F4/80 on Ly6C^{high} monocytes in the blood of old mice during *S. pneumoniae* colonization is decreased as compared to young mice. (G) Levels of CCL2 transcript in the nasopharynx during the course of *S. pneumoniae* colonization were measured by quantitative PCR. (± SEM; $n = 3$). (H-I) Total numbers of (H) Ly6C^{high} monocytes and (I) macrophages detected by flow cytometry in the nasopharnyx of young and old mice during *S. pneumoniae* colonization (± SEM; $n = 3–8$). (J) Mean F4/80 expression on nasopharyngeal macrophages is lower in old mice during *S. pneumoniae* colonization (± SEM; $n = 3–8$). Statistical significance was determined by two-tailed Mann-Whitney-Wilcoxon test, one-way or two-way ANOVA with Fisher's LSD post-test. (K) Circulating blood monocytes from old mice bind fewer TRITC-labelled *S. pneumoniae* (4°C) but there is no difference in internalization of the bacteria (37°C). Survival in (C) was determined by the Mantel-Cox Log-rank test. * indicates $p < .05$, ** indicates $p < 0.005$, *** indicates $p < 0.0005$ and **** indicates $p < 0.00005$. (A-J) is representative of 3 independent experiments.

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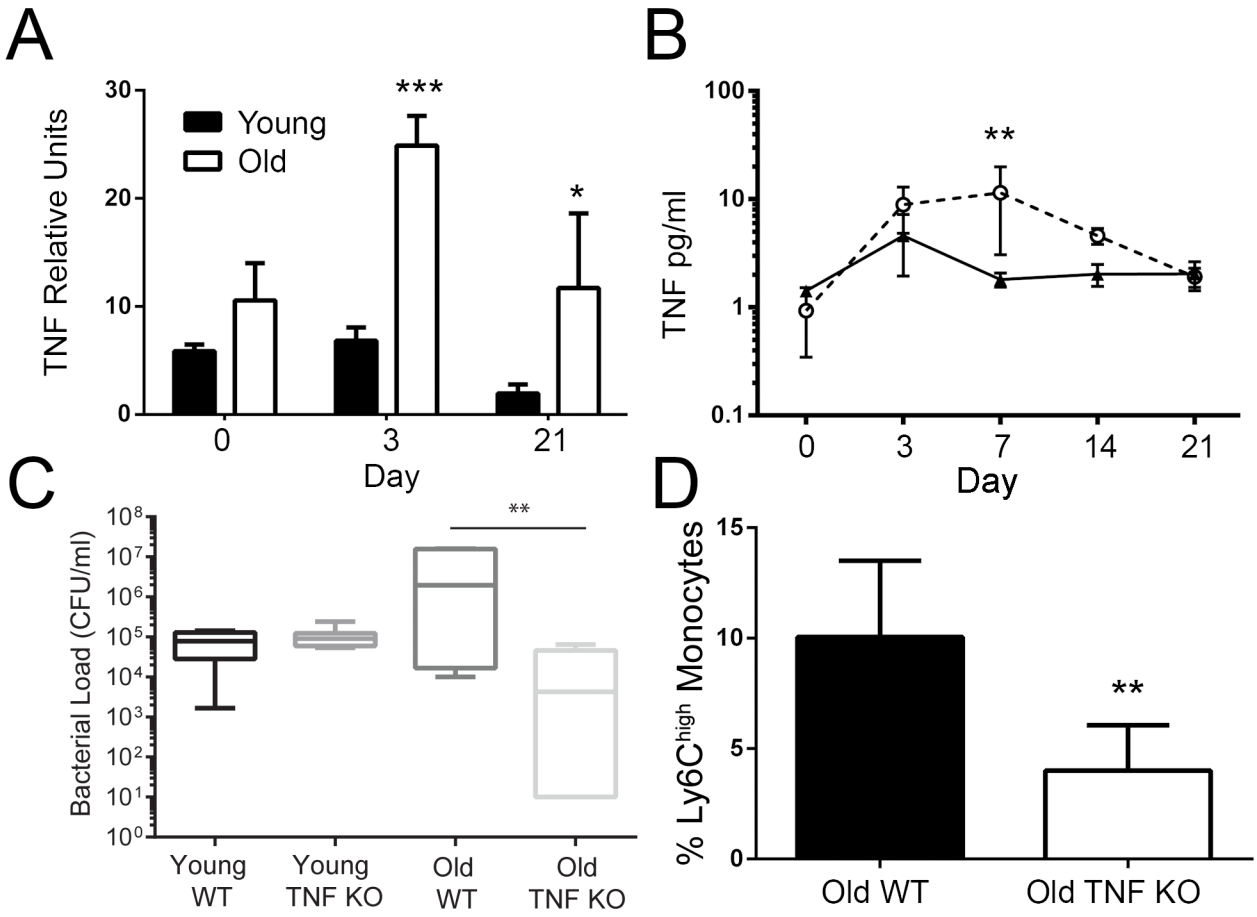

Fig 7. Reducing TNF-regulated recruitment of Ly6C^{high} monocytes during *S. pneumoniae* colonization in old mice reduced nasopharyngeal bacterial loads. (A-B) TNF in the (A) nasopharnyx and (B) serum of young and old mice during *S. pneumoniae* colonization as measured by qPCR and ELISA, respectively (± SEM; $n = 3–5$). (C) CFUs in nasal lavages of old WT and old TNF mice on day 4 after colonization with *S. pneumoniae* (± SEM; $n = 6–8$, one independent experiment of two shown). (D) Ly6C^{high} monocytes as a percent of circulating CD45+ cells in old WT and TNF KO mice on day 4 of *S. pneumoniae* colonization (± SEM; $n = 3–4$, one independent experiment of two shown). Statistical significance was determined by two-tailed Mann-Whitney-Wilcoxon test, one-way ANOVA or two-way ANOVA with Fisher's LSD post-test where appropriate. * indicates $p < .05$, ** indicates $p < 0.005$, *** indicates $p < 0.0005$ and **** indicates $p < 0.00005$.

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this cell population using negatively-charged polystyrene microparticles (PS-MPs) (Fig 8A). We observed that there were also decreases in monocytes in the lungs, but not neutrophils with this treatment (S2 Fig). Old mice were given PS-MPs on day prior to and every 3 days during the course of *S. pneumoniae* colonization and bacterial loads were measured at day 7. PS-MP-treated old mice had increased survival (Fig 8B), less weight loss (Fig 8C)and lower bacterial loads in the nasopharynx (Fig 8D), lungs (Fig 8E) and spleen (Fig 8F) compared to old control mice. Similar results were observed with Gr-1 antibody, which reduces numbers of monocytes and neutrophils. These data confirm that increased trafficking of this cell type during *S. pneumoniae* colonization impairs host defense.

Discussion

Epidemiological data strongly suggests that there is a reciprocal link between pneumonia and age-associated inflammation. Older adults who have higher than age-average levels of the cytokines TNF and IL6 in their circulation have a much higher risk of acquiring pneumonia than their peers who have lower than age-average levels[55]. Although a robust inflammatory response is generally thought to be protective against infection, in the elderly, high levels of circulating inflammatory cytokines during pneumonia are associated with more severe disease and higher mortality[56,57]. Similarly, having a chronic inflammatory disease such as dementia, diabetes, or cardiovascular disease is strongly associated with susceptibility to acquiring pneumonia [58,59,60]. Conversely, having a pneumonia in mid- to late-life can often exacerbate or accelerate sub-clinical or existing chronic inflammatory conditions and can be the harbinger of declining health and decreased quality of life[58,59]. Although descriptions of this reciprocal relationship between chronic, age-associated inflammation and pneumonia, especially that caused by *S. pneumoniae*, are strong, the mechanistic explanations are weak. Herein we demonstrate that monocytes, both contribute to age-associated inflammation and are impaired by chronic exposure to the inflammatory cytokine TNF, and this ultimately impairs their anti-pneumococcal function.

Our data using aged TNF KO mice or anti-TNF therapy indicate that the increased levels of TNF that occur with age impair monocyte development and ultimately anti-bacterial immunity. Although macrophages have previously been shown to promote inflamm-aging[61], our data suggest that this may begin earlier in myelopoesis since monocytes produce more inflammatory cytokines such as TNF and IL6 with age and ablation of monocytes reduces levels of serum cytokines. The increase in circulating monocytes did not occur in old TNF KO mice. Furthermore, by treating young WT mice with a low-dose regime of TNF delivered intraperitoneally, we found that $Ly6C^{+}$ monocytes were increased in the blood in a manner similar to old mice, demonstrating that TNF is sufficient to increase numbers of circulating $Ly6C^{+}$ monocytes. Monocytes appear to be both highly responsive to increased levels of TNF but also seem to be a major source of age-associated TNF.

Our observational studies in humans imply that the numbers of intermediate ($CD14^{+-}$ $^{+}CD16^{-}$) monocytes, which we have previously shown express higher levels of CCR2 with age [62], correlate with increased levels of TNF and contribute to hyper-inflammatory responses to bacterial infection. Studies in patients on anti-TNF therapy for rheumatoid arthritis validate our observations that TNF drives increases in inflammatory monocytes. In these patients anti-TNF therapy decreases the levels of circulating $CD14^{++}CD16^{-}$ monocytes in the blood and synovial fluid as well as decreases CCR2 expression on peripheral blood mononuclear cells and thus is consistent with our data demonstrating that TNF-mediated changes in CCR2 expression are sufficient to alter the numbers of $Ly6C^{high}$ monocytes in the circulation [63,64]. Interestingly, decreases in $CD14^{++}CD16^{-}$ monocytes correlate with a positive prognostic response

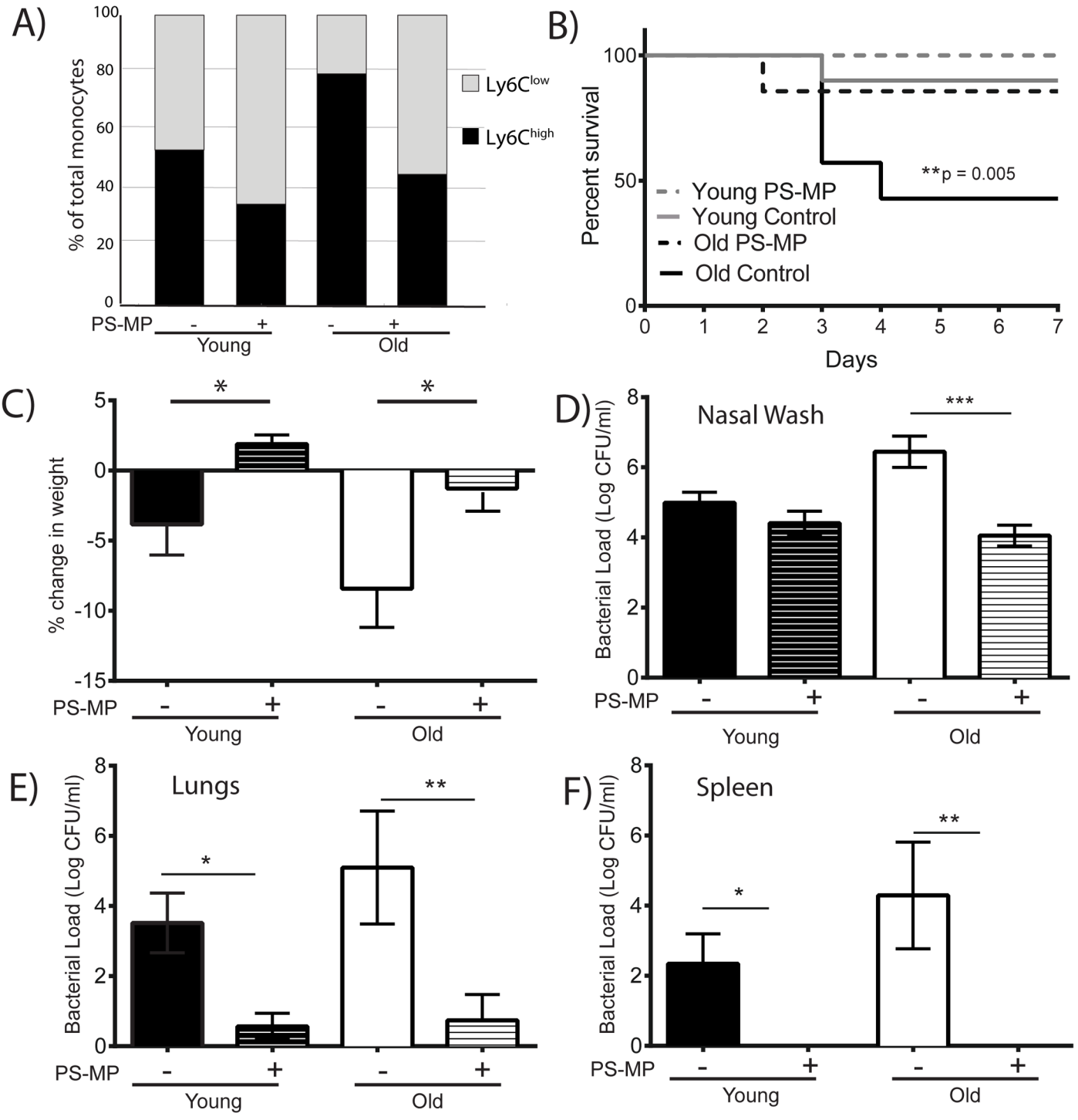

Fig 8. Depletion of inflammatory monocytes improves outcome to S. pneumoniae infection in old mice. Mice (n = 7-10/group) were injected with PS-MP day -1, 0, +1, +3 and +5 during colonization *with S. pneumoniae*. A) The percentage of Ly6C^{high} monocytes was significantly reduced in old mice treated with PS-MP (see S3 Fig). B) Survival was significantly improved in old mice treated with PS-MP (p = 0.005, Mantel-Cox log-rank test). C) Both young and old mice treated with PS-MP lost less weight than their control counterparts (*,p<0.05, one-way ANOVA with uncorrected Fisher's LSD). Levels of *S. pneumoniae* in the D) nasal wash, E) lungs and F) spleen were lower in old mice treated with PS-MP. Fewer young mice had bacteria in their lungs and spleens when they were treated with PS-MP. (*,p<0.05, **,p<0.005 one-way ANOVA with uncorrected Fisher's LSD). CFU count for mice that reached endpoint before day 7 are not included.

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for patients, but whether this is because they contribute directly to disease progression or the inflammatory tone of rheumatoid arthritis is not known [63].

Increases in Ly6C^{high} monocytes are associated with defects in maturity. Interestingly, our chimera data demonstrate that phenotypic changes in monocytes (i.e. CCR2 and F4/80 expression) were not due to intrinsic defects in myeloid precursors but rather the influence of the bone marrow microenvironment, and, since these changes did not occur in TNF KO mice, TNF produced in the context of the microenvironment. Although F4/80 levels were equivalent on blood monocytes during the steady state, they were lower on Ly6C^{high} monocytes/differentiating macrophages recruited during nasopharyngeal *S. pneumoniae* colonization in old mice. These changes had functional significance; despite robust Ly6C^{high} monocyte recruitment and TNF production in old mice, bacterial clearance was significantly impaired. In fact, our data suggest that TNF is detrimental to clearance of *S. pneumoniae* from the nasopharynx with age, as old TNF KO mice had lower bacterial loads compared to their WT counterparts. Although TNF is often thought of as a key anti-bacterial cytokine, mouse studies have demonstrated that TNF is required for control for *S. pneumoniae* bacteremia but not for survival in lung infection[65]. In our study, old TNF KO mice recruited fewer circulating Ly6C^{high} monocytes during *S. pneumoniae* colonization compared to old WT mice and counter-intuitively, this appeared to be protective against infection as when we depleted circulating Ly6C^{high} monocytes using carboxylated polystyrene microparticles colonization, bacterial loads in the nasopharynx decreased. These data are consistent with the clinical observation that rheumatoid arthritis patients (who have high levels of circulating TNF) are at increased risk of pneumonia but that there is no increase in risk of pneumonia for patients on anti-TNF therapy [66]. Whether pneumonia risk is *decreased* with anti-TNF therapy is not known; however, patients on anti-TNF therapy do live slightly longer than their untreated counterparts, despite an increased risk in re-activation of chronic infections[67,68].

These observations have important therapeutic significance, since the belief that host responses to bacteria are impaired with age due to poor innate cell recruitment has been the foundation of two large clinical trials testing the use of cytokines (G-CSF) to mobilize myeloid cells as an adjunct to antibiotics and one clinical trial testing GM-CSF as an adjuvant for pneumococcal vaccination. Although mouse models (tested in young mice) showed promise, these strategies all failed when tested in populations where the median ages were 59, 61 and 68, respectively [reviewed in[69] and[70]]. Our data suggests that use of G-CSF, GM-CSF or other myeloid chemoattractant-based therapies in older adults would enhance recruitment of a population that is fundamentally immature and predisposed towards TNF and IL6 production that provides no functional benefit to the host for clearance and may even exacerbate infection.

In summary, our data suggest that monocytes are both contributors to age-associated inflammation and have altered anti-pneumococcal function as a result of age-associated inflammation. Lowering levels of TNF may be an effective strategy in improving host defence against *S. pneumoniae* in older adults. In fact, it has been shown that immunosuppressive steroid use in combination with antibiotics reduces pneumonia mortality in the elderly[71,72,73,74], although uptake for this therapy has been limited. Although it may be counterintuitive to limit inflammatory responses during a bacterial infection, the clinical observations and our animal model indicates that anti-bacterial strategies need to be tailored to the age of the host.

Materials and Methods

Ethics statement

All experiments were performed in accordance with Institutional Animal Utilization protocols approved by McMaster University's Animal Research Ethics Board (#13-05-13 and #13-05-14) as per the recommendations of the Canadian Council for Animal Care.

Participants or Power of Attorney for participants were approached to determine interest in the study. Informed written consent was obtained from the participant or their legally authorized representative approved by the Hamilton Integrated Research Ethics Board (#09–450).

Animals

Female C57BL/6J mice were purchased from Jackson Laboratories and aged in house. Colonization was performed as previously described[75]. To protect from age-related obesity aging mice are fed with a low protein diet Teklad Irradiated Global 14% protein Maintenance Diet and provided with an exercise wheel, as were young controls. The average weight of a young mouse is this study is 20g+/-1g and the old mice are on average, 27g+/-2.5g. TNF knockout mice (KO) mice (C57BL/6J background) were bred in the barrier unit at the McMaster University Central Animal Facility (Hamilton, ON, Canada) as previously described[76]. All mice were housed in specific pathogen-free conditions. Continual monitoring of the health status of mice was performed.

Human monocytes

Monocyte frequency was measured in whole blood according to staining procedures described in [62]. Briefly, intermediate monocytes were positive for the expression of HLA-DR and CD16, stained brightly for CD14, and were negative for lymphoid and neutrophil markers (CD2, CD3, CD15, CD19, CD56, and NKp46). They are presented as cells per microlitre of whole blood, which was measured using CountBright Absolute Counting Beads (Life Technologies, CA, USA). Serum TNF was measured in elderly donors (61–100 yrs) using the Milliplex High Sensitivity ELISA kit (Millipore, ON, CA).

For intracellular cytokine staining, described in [62], the production of TNF and IL-6 was measured in classical (CD14++), intermediate (CD14++CD16+) and non-classical (CD14 +CD16+) monocytes after a 6 hour incubation period in the presence of 50 ng/ml LPS and 5 x 10^6 CFU of heat-killed *S. pneumoniae*. For cytokine secretion, CD14+ monocytes were isolated from PBMCs of young(26–52 yrs) and older (63–70 yrs)by positive selection procedure (Stemcell, BC, CAN) and stimulated for 22 hours in the presence of 50 ng/ml LPS. TNF and IL-6 were measured by ELISA (eBioscience, CA, USA).

Flow cytometry

Monoclonal antibodies with the following specificities were used in this study: F4/80 (APC), Ly6C (FITC), CD45 (eFluor 450), CD11b (PE-Cy7 or PerCPCy5.5), MHC II (PerCP eFluor 710), CD3 (Alexa Fluor 700), CD4 (Alexa Fluor 605NC), Ly6G (PE), Ter119 (PE), B220 (PE), NK1.1 (PE), CCR2 (PE), IL6 (PE) or TNF (PECy7). Blood and single cell suspensions of lung were stained according to previously published procedures [75]. Total cell counts were determined using CountBright Absolute Counting Beads (Life Technologies). To attain a single-cell suspension of mouse lung tissue, half a lung was collected from each *S. pneumoniae*-colonized mouse and kept on ice. Immediately following, each lung was mechanically dissociated and enzymatically degraded using a Miltenyi Biotec Lung Dissociation Kit (Cat#: 130-095-927) along with the gentleMACS Octo-Dissociator with Heaters (Cat#: 130-096-427). Following dissociation as per protocol, cell suspensions were filtered (70 μM cell filter) and centrifuged at 300 x g for 10 min. Subsequently, single-cell suspensions were re-suspended in phosphate-buffered saline & processed for flow cytometry. A gating strategy for distinguishing $Ly6C^{high}$ and $Ly6C^{low}$ monocytes is presented in S3 Fig.

Cytokine administration

100 nM of recombinant murine CCL2 (endotoxin-free, eBioscience) was diluted in sterile saline and administered intraperitoneally. Recruited cells were isolated via peritoneal lavage and quantitated using flow cytometry. Murine recombinant TNF (eBioscience) diluted in sterile saline was administered intraperitoneally every other day for 3 weeks at a dose of 5 ng per gram of body weight. Adalimumab (HUMIRA, Abbott Laboratories), a humanized anti-TNF antibody, or the human IgG isotype control diluted in sterile saline were administered intraperitoneally at a dose of 50 ng per gram of body weight for a period of 3 weeks.

Ly6C^{high} monocyte depletion

FITC Fluoresbrite 500 nm carboxylated polsytrene microparticles (PS-MPs) were obtained from Polysciences. PS-MPs were injected via tail vein at 4 x 10^9 particles in 200 μl as previously described[50]. Monocyte depletion was confirmed by flow cytometry.

Measurement of cytokine production

Serum TNF and CCL2 was measured using high-sensitivity ELISA as per manufacturer's instructions (Meso Scale Discovery). For quantitative PCR analysis, RNA Lysis Buffer (Qiagen) was used to collect nasopharyngeal RNA via nasal lavage. RNA was extracted using an RNAqueous Micro Kit (Ambion), reverse-transcribed to cDNA using M-MULV reverse transcriptase (New England Biolabs) and qPCR was performed using GoTaq qPCR Master Mix (Promega, WI, USA) and the ABI 7900HT Fast Real-time PCR System (Applied Biosystems, CA, USA) all to manufacturer's instructions. Cycle threshold (Ct) values relative to the internal reference dye were transformed by standard curve, followed by normalization to the housekeeping gene GAPDH. Normalized results are presented as relative to an internal calibrator sample.

Quantitation of monocyte-bound *S. pneumoniae*

100μL samples of peripheral blood, were incubated with TRITC-labeled *S. pneumoniae* (MOI 20) resuspended in 100μL of complete RPMI at 4°C to allow binding, but not uptake. After 30 min of incubation, cells were stained for flow cytometry. Following RBC lysis (1x 1-step Fix/Lyse Solution eBioscience; ref: 00-5333-57) for 10min, cells were washed 2x with PBS to remove excess stain and non-adherent bacteria, and re-suspended in FACS wash (10% fetal bovine solution in PBS). Flow cytometry was performed and the amount of *S. pneumoniae* bound by Ly6C^{high} monocytes was quantitated based on the mean fluorescent intensities of TRITC.

Administration of anti-TNF *in vivo*

Adalimumab (HUMIRA, Abbott Laboratories), a humanized anti-TNF antibody, or the human IgG isotype control diluted in sterile saline were administered to mice. A dose of 50 ng per gram of body weight was given intraperitoneally in a volume of 200 μl every other day, for a period of 3 weeks to young and old WT mice.

Statistics

Unless otherwise mentioned in the figure legend, statistical significance was determined by two-tailed Mann-Whitney-Wilcoxon tests, one-way analysis of variance or two-way analysis of variance with Fischer's LSD post-tests where appropriate.

Supporting Information

S1 Fig. Age is characterized by myeloid skewing in mice. (A) Although total leukocyte numbers were not altered with age, there was a skewing towards cells of myeloid lineage, with increases in the total numbers of monocytes and neutrophils, and a decrease in the total number of T cells in the circulation. (B) The number of bone marrow-derived precursor cells capable of differentiating into macrophages following M-CSF stimulation was increased in old mice relative to young mice. (C) With age, bone marrow-derived precursors differentiating into macrophages ex vivo express heightened CCR2 levels during an intermediate stage of the differentiation process. This is in contrast to precursors from young mice, which do not express peak CCR2 levels until the end of the differentiation process. (D) There were no differences in $Ly6C^{low}$ monocyte levels in the circulation in old TNF KO mice. (E) CCR2 levels were significantly higher on $Ly6C^{high}$ monocytes rather than $Ly6C^{low}$ monocytes. Statistical significance was determined by two-tailed Mann-Whitney-Wilcoxon test, one-way ANOVA or two-way ANOVA with Fisher's LSD post-test where appropriate. * indicates $p < .05$, ** indicates $p < 0.005$, *** indicates $p < 0.0005$ and **** indicates $p < 0.00005$.
(TIF)

S2 Fig. Injection with polystryene microparticles (PS-MP) reduces the percentage of $Ly6C^{high}$ monocytes. Mice (n = 7-10/group) were injected with PS-MP day -1, 0, +1, +4and +6 during colonization with *S. pneumoniae*. Injection of PS-MP reduces in the proportion of Ly6Chigh monocytes in the (A)circulation and (B) lungs of old mice during S. pneumoniae colonization. Consequently the proportion of $Ly6C^{low}$ monocytes in the (C) circulation and (D) lungs increases, while there is no effect on (E) circulating neutrophils. (F) Reduction of circulating myeloid cells using an anti-Gr-1 antibody also reduces the bacterial load in the nasal wash of old mice. (*,$p<0.05$, **,$p<0.005$, ***, $p<0.001$ one-way ANOVA with uncorrected Fisher's LSD). Mice that reached endpoint prior to day 7 were not included in the analysis.
(EPS)

S3 Fig. Flow cytometry gating strategy for $Ly6C^{high/low}$ monocytes. To gate on $Ly6C^{high}$ monocytes (circulating & lung-infiltrating), first A) CD45+ cells (leukocytes) are gated upon. Subsequently, a B) width gate is created to exclude cell aggregates, and C) CD11b+ cells are selected. Using this population, cells can be divided into D) neutrophils and non-neutrophil using SSC and Ly6C surface expression. E) Monocytes are gated upon as $Ly6C^{+}/SSC^{low}$ cells, and those that are F) $Ly6C^{high}$ would be defined as $Ly6C^{high}$ monocytes. Using a dump gate positive for NK1.1, CD19, and CD3, it is apparent that no NK cells, B cells or T cells are found in this population. Isotype controls were used for all experiments.
(TIF)

Author Contributions

Conceived and designed the experiments: DMEB LK AP AN CPV. Performed the experiments: AP AN CPV DL TSM NT PsN. Analyzed the data: AP AN CPV LK PsN DMEB. Contributed reagents/materials/analysis tools: MJL ZX ML MJ. Wrote the paper: AP AN DMEB.

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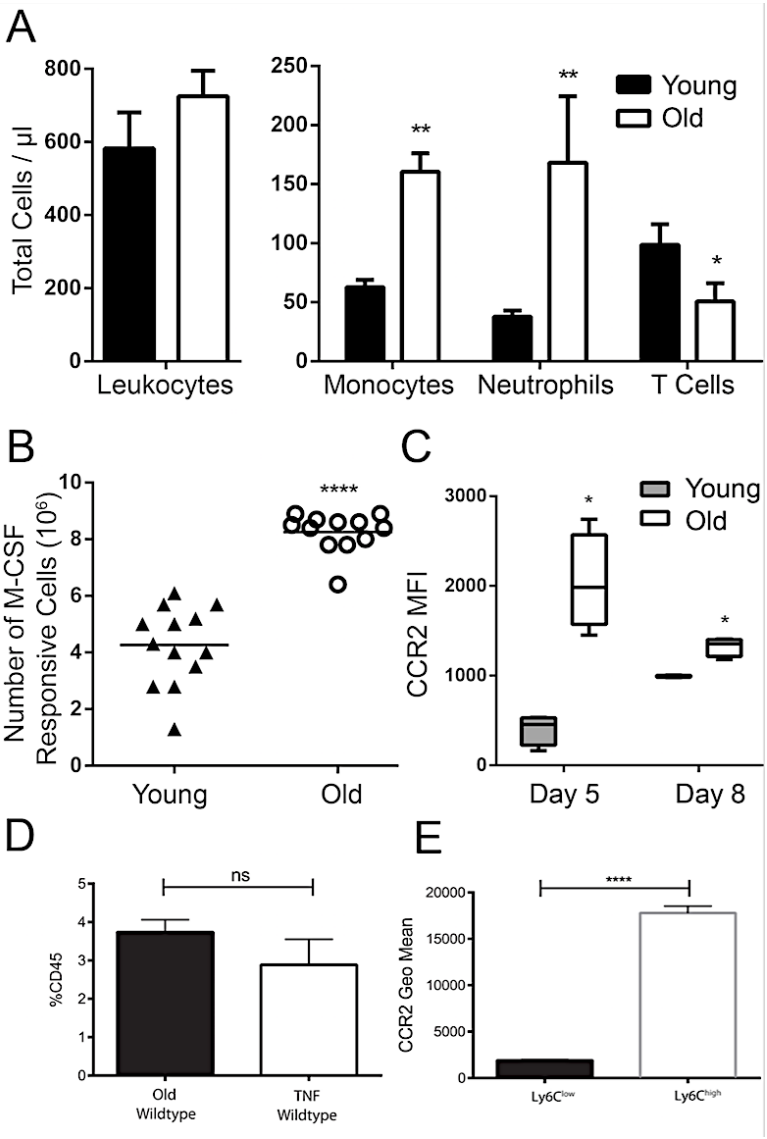
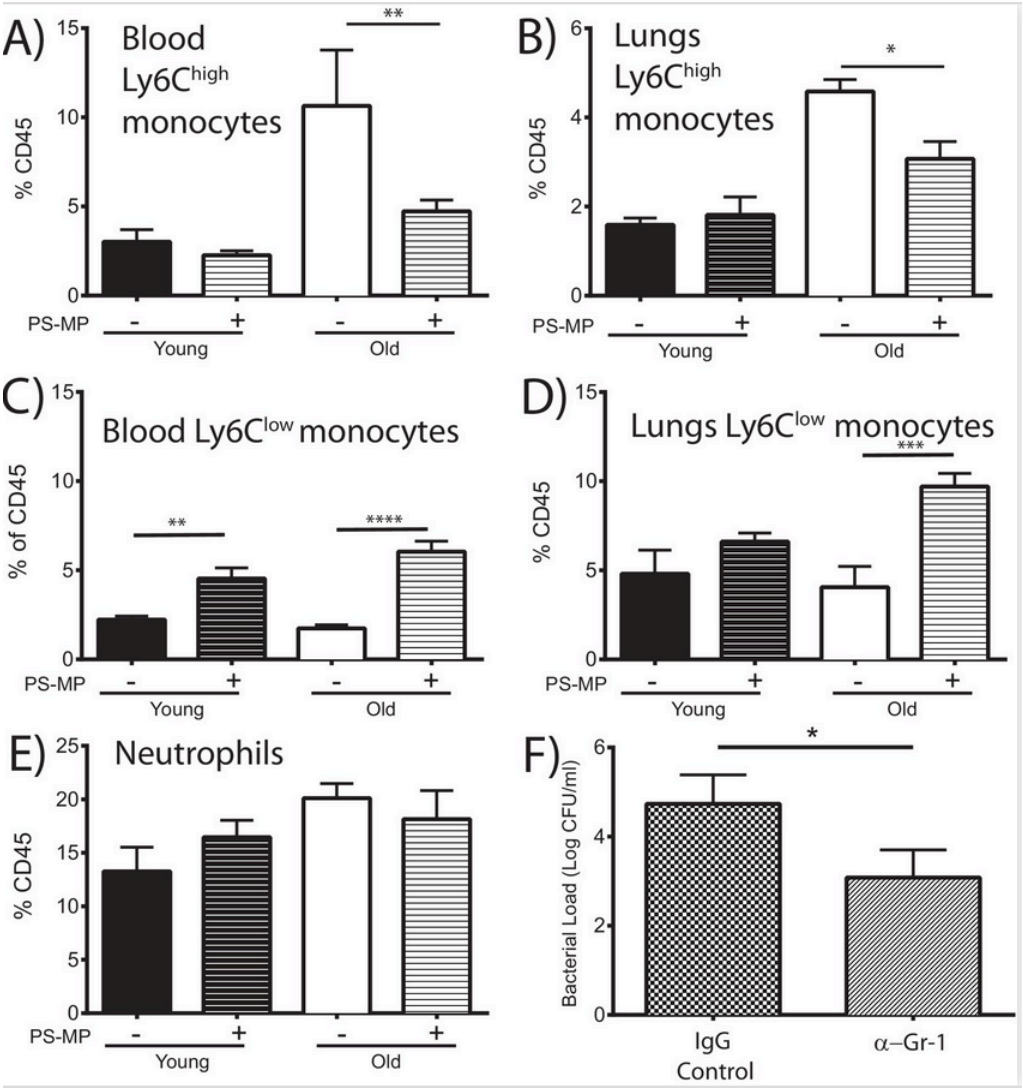
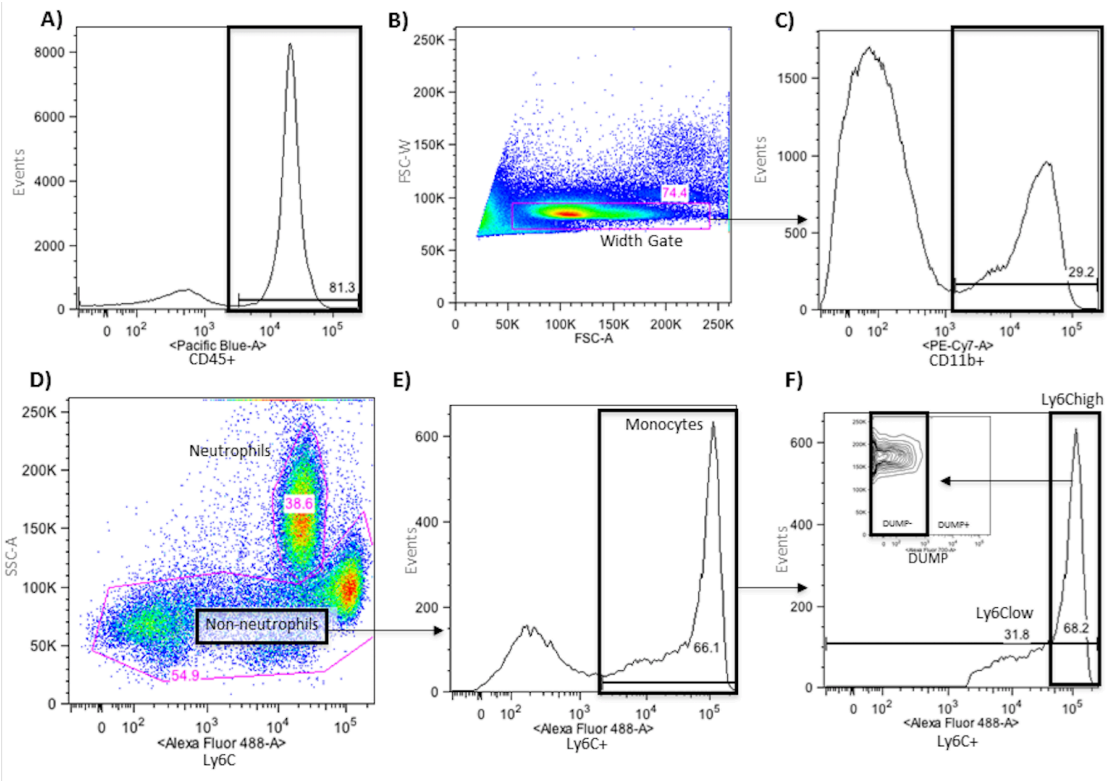
Supplementary Figures



Supplementary Figure 1. ***Aging is characterized by myeloid skewing in mice.*** A) Although total leukocyte numbers were not altered with age, there was a skewing towards cells of myeloid lineage, with increases in the total numbers of monocytes and neutrophils, and a decrease in the total number of T cells in the circulation. (B) The number of bone marrow-derived precursor cells capable of differentiating into macrophages following M-CSF stimulation was increased in old mice relative to young mice. (C) With age, bone marrow-derived precursors differentiating into macrophages ex vivo express heightened CCR2 levels during an intermediate stage of the differentiation process. This is in contrast to precursors from young mice, which do not express peak CCR2 levels until the end of the differentiation process. (D) There were no differences in Ly6C^{low} monocyte levels in the circulation in old TNF KO mice. (E) CCR2 levels were significantly higher on Ly6C^{high} monocytes rather than Ly6C^{low} monocytes. Statistical significance was determined by two-tailed Mann-Whitney-Wilcoxon test, one-way ANOVA or two-way ANOVA with Fisher's LSD post-test where appropriate.



Supplementary Figure 2. ***Injection with polystryene microparticles (PS-MP) reduces the percentage of Ly6Chigh monocytes.*** Mice (n = 7-10/group) were injected with PS-MP day -1, 0, +1, +4 and +6 during colonization with *S. pneumoniae*. Injection of PS-MP reduces in the proportion of Ly6Chigh monocytes in the (A) circulation and (B) lungs of old mice during S. pneumoniae colonization. Consequently the proportion of $Ly6C^{low}$ monocytes in the (C) circulation and (D) lungs increases, while there is no effect on (E) circulating neutrophils. (F) Reduction of circulating myeloid cells using an anti-Gr-1 antibody also reduces the bacterial load in the nasal wash of old mice. (*) $p<0.05$, (**) $p<0.005$, (***) $p<0.001$ one-way ANOVA with uncorrected Fisher's LSD). Mice that reached endpoint prior to day 7 were not included in the analysis.



Supplementary Figure 3. ***Flow cytometry gating strategy for Ly6C$^{high/low}$ monocytes.*** .To gate on Ly6C^{high} monocytes (circulating & lung-infiltrating), first A) CD45+ cells (leukocytes) are gated upon. Subsequently, a B) width gate is created to exclude cell aggregates, and C) CD11b+ cells are selected. Using this population, cells can be divided into D) neutrophils and non-neutrophil using SSC and Ly6C surface expression. E) Monocytes are gated upon as Ly6C^{+}/SSC^{low} cells, and those that are F) Ly6C^{high} would be defined as Ly6C^{high} monocytes. Using a dump gate positive for NK1.1, CD19, and CD3, it is apparent that no NK cells, B cells or T cells are found in this population. Isotype controls were used for all experiments.

CHAPTER 3: The Age of the Microenvironment Influences Pneumonia Susceptibility

Declaration of Academic Achievement:

This research was conducted during the period of January 2014 to June 2015. I am the first author on this manuscript. I performed all experiments, and analyzed and interpreted the data. Fellow graduate students (D. Loukov, N. Thevaranjan, K. Novakowski) provided experimental assistance. Experiments were conceived and designed by Dr. D.M.E. Bowdish and myself. Dr. D.M.E. Bowdish provided guidance and editorial input throughout the manuscript writing process.

The Age of the Microenvironment Influences Pneumonia Susceptibility

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Running Title: The Age Of The Microenvironment, But Not Hematopoietic Stem Cells, Determines Outcome Of Infection

ABSTRACT

As human lifespan increases, a larger proportion of the population will be at an increased risk of infectious disease. Preventing immunosenescence, or the age-related decline in immune function, may reduce the rate of infection in older adults. Myeloid cells such as monocytes and macrophages play a critical role in innate anti-bacterial immunity, however, their phenotype and function changes with aging. Whether these functional impairments in monocytes and macrophages primarily arise from changes in the aging microenvironment, or intrinsic age-associated defects in hematopoietic progenitors, is unknown. Using heterochronic chimeras, we characterized the relative contributions of cell intrinsic and microenvironmental factors on age-associated changes in myeloid cells, and ultimately, their impact on *S. pneumoniae* infection. We demonstrated that changes in Ly6C^{high} monocyte phenotype and macrophage function with age were largely dependent on the host microenvironment. Age-related increases in TNF were found to drive increased monocyte output and premature egress into circulation. When age-discordant chimeras were colonized with *S. pneumoniae*, the age of the microenvironment, not hematopoietic stem cells, determined the outcome of infection. Remarkably, exposure of aged immune cells to the microenvironment of a young host could reverse the effects of age on monocytes and macrophages. Herein, we propose manipulation of the aging microenvironment could rescue monocyte and macrophage phenotype and function in elderly mice and significantly improve anti-bacterial immunity.

INTRODUCTION

Aging is associated with an increased susceptibility to infectious disease, especially pneumonia. In fact, 90% of pneumonia deaths occur in the elderly, with *Streptococcus pneumoniae* being the major causative agent[1–3]. Although the numerous factors contribute to the increased risk of pneumonia in older adults (e.g. housing in nursing homes[4], nutritional deficiencies[5], mechanical changes in lung function[6]), the major contributing factor is the age-associated dysregulation of the immune response, called immunosenescence[7]. Colonization of the nasopharynx is a prerequisite for pneumonia or invasive pneumococcal disease. Monocytes and macrophages play a key role in the recognition, containment and clearance of *S. pneumoniae* in the nasopharynx, due to their non-opsonic, scavenger receptor-mediated recognition of the pathogen[5]. These myeloid-derived cells, however, demonstrate age-associated impairments in anti-pneumococcal activity, resulting in poor infection control.

Monocyte and macrophage phenotype and function is altered in the elderly[8,9]. The proportion of monocytes increases with age due to increased extramedullarly[10] and bone marrow monopoiesis[9], and prematurely egress from the bone marrow, due to age-associated increases in CCR2 expression[9]. In mice, there are 2 distinct monocyte subsets: Ly6C^{low} and Ly6C^{high}. Ly6C^{low} monocytes patrol the blood vessel lumen and promote tissue healing by differentiating into alternatively-activated M2 macrophages[11]. Conversely, Ly6C^{high} monocytes, which are potent producers of pro-inflammatory cytokines[12,13], are recruited to sites of inflammation and differentiate into classically-activated M1 macrophages[14]. Macrophages derived from aged mice demonstrate reduced antigen presentation, diminished phagocytosis

and bacterial killing[5,7,15,16], and these functional changes have been attributed dysregulated or persistent inflammatory responses[17]. We have previously shown that monocytes and macrophages isolated from old mice have enhanced inflammatory responses when stimulated with *S. pneumoniae* and impaired bacterial killing which contribute to dissemination of the bacteria from the nasopharynx to the lung or circulation[9]. Whether these impairments in immune cells are due to changes in the aging microenvironment (e.g. increases in pro-inflammatory cytokines), or intrinsic age-related defects, is controversial[18,19].

Aging is accompanied by alterations in levels of lipids, hormones, pathogen- and danger-associated molecular patterns, metabolic products, and pro-inflammatory cytokines[20,21–24]. These global changes in the aging microenvironment have been postulated to contribute to immune decline. In aged animals, exposure to young plasma through heterochronic parabiosis has shown to improve stem cell function in aged muscular, myocardial and central nervous systems[25–28], however the impact of the aging microenvironment on the immune system is incompletely understood. Pro-inflammatory cytokines (e.g. TNF, IL-6) in serum and tissues increase with age, resulting in a state of low-grade, chronic inflammation, known as *inflammaging*[20]. Chronic exposure to proinflammatory cytokines is believed to culminate in constant activation of immune cells[29]. Mice protected from age-associated inflammation (e.g. TNF KOs) do not demonstrate age-related changes in myeloid cell function[9]. Comparing TNF knockout mice to wildtype mice, we observed that prolonged exposure to TNF with age altered the maturity of circulating, inflammatory monocytes and this phenotype was directly linked to susceptibility to bacterial infection[9].

Conversely, it has been postulated that aging of the hematopoietic stem cells (HSCs) themselves, attributed to accumulated DNA damage over time, may underlie functional impairments in myeloid cells observed with advanced age[18,19,30]. We have observed that bone marrow macrophages derived from old mice maintain age-associated defects once cultured *in vitro* in an age-neutral medium. Despite being cultured in the absence of the aged microenvironment, age-associated impairments (e.g. hyper-inflammatory responses to bacteria, reduced killing capacity) persist, suggesting that defects in macrophage function are stable. However, using anti-TNF to reduce age-associated inflammation, such a phenotype could be rescued[9]; therefore implying that these changes are reversible when the inflammatory microenvironment is altered. Whether age-induced defects in monocytes and macrophages primarily arise from changes in the aging microenvironment[31,32], which are unable to provide appropriate signals to regulate the myeloid progenitors, rather than the progenitors *per se*, has not yet been elucidated.

In this paper, we hypothesized that age-related changes in monocyte and macrophage function, and ultimately, increased susceptibility to *S. pneumoniae,* were due to the inflammatory milieu that arises with age. Using age-discordant chimeras, we determined the relative contributions of cell intrinsic and global microenvironmental factors on age-associated changes in these myeloid cells, and their impact on anti-bacterial immunity. We demonstrate that age-associated changes in Ly6C^{high} monocyte and macrophage development, phenotype and function were largely dependent on the host microenvironment. When heterochronic chimeras were colonized with *S. pneumoniae*, the age of the recipient, and not myeloid progenitor cells, dictated outcome of infection. Strikingly, exposure of aged immune cells to the

microenvironment of a young host counteracted and reversed pre-existing effects of monocyte and macrophage aging.

MATERIALS & METHODS:

Animals

C57BL/6J mice were purchased from Jackson Laboratories and aged in house. Pneumococcal colonization was performed as previously described[12]. To protect from age-related obesity, all mice were fed with a low protein diet Teklad Irradiated Global 14% protein Maintenance Diet and provided with an exercise wheel. The average weight of a young mouse in this study is 20g ±1g and an old mouse is 27g±2.5g. All mice were housed in specific pathogen-free conditions. Continual monitoring of the health status of mice was performed. All experiments were performed in accordance with Institutional Animal Utilization protocols approved by McMaster University's Animal Research Ethics Board (#13-05-13 and #13-05-14) as per the recommendations of the Canadian Council for Animal Care.

Flow cytometry

Monoclonal antibodies with the following specificities were used in this study: F4/80 (APC), Ly6C (FITC), CD45 (eFluor 450), CD11b (PE-Cy7), CD3 (Alexa Fluor 700), CD4), CD19 (AF700), NK1.1 (AF700) and CCR2 (PE). Bone marrow flushed from femurs or peripheral blood (100uL) collected retro-orbitally from mice were analyzed by flow cytometry for Ly6C^{high} monocytes, as previously published[9]. Absolute cell counts were determined using CountBright Absolute Counting Beads (Life Technologies).

Establishment of Chimeras

Cohorts of 10 old (18 mo, C57Bl/6 (CD45.2); The Jackson Laboratory) and 10 young (6–8wk, B6.SJL-Ptprca Pepcb/BoyJ C57Bl/6 (CD45.1); 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 days (e.g., sulfamethoxazole/trimethoprim; Septra, Pfizer, New York, NY, USA) prior to receiving 2 doses of 550 rads of gamma irradiation (1100 rads total). Within 2 h of the second irradiation, mice were injected intravenously with $10x10^6$ 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 assessing the ratios of CD45.1 to CD45.2 cells using flow cytometry and was >95% in all recipients' 3mo following transplant (**Fig. S1**). See **Fig. S2** for experimental outlines for heterochronic, TNF knockout and TNFR knockout bone marrow chimeras.

Immunohistochemistry

Histologic analysis was performed on lungs from chimeric mice. Upon collection, lungs were fixed in 10% neutral buffered formalin at room temperature for a minimum of 24 hrs prior to embedding in paraffin. Tissue blocks were cut into 3 mm sections and stained with H&E at the Core Histology Facility, McMaster Immunology Research Centre. Images were acquired with a DM LB2 microscope at a magnification of 20x (Leica, Bannockburn, IL, USA). A trained pathologist performed histological assessment of lung pathology.

Macrophage Culture and Stimulation

Bone marrow-derived macrophages were obtained by flushing femurs and tibias of mice with cold PBS. Flushed bone marrow was washed and resuspended in 25 mL RPMI 1640

supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100mg/ml), 10 mM L-glutamine (R10 media) and 15% L929-cell conditioned medium (contains M-CSF). Macrophage progenitors are cultured for 7 days, with fresh media changes at day 3 and day 6. To isolate peritoneal macrophages, the peritoneal cavity was lavaged with 10 mL of ice, cold PBS. Cells were then washed once with RPMI-1640 and resuspended in R10 media and plated in 10 mL on 10 cm tissue culture dishes. Cells were allowed to adhere for 2 h and then non-adherent cells were removed by washing with warm media. Adherent cells were incubated at 37° C overnight.

All differentiated macrophages were cultured and maintained in a humidified environment at 37° C with 5% CO_2 in R10 media. For assays involving stimulation of cultured cells, 100 ng/mL of LPS or a vehicle control was added in RPMI-1640 containing 1% FCS and L-glutamine was added to $1x10^6$ cells in 6-well plates for 16 hrs. Supernatants were collected and stored at -80° C for quantitation of cytokines by ELISA.

Macrophage Killing Assays

Macrophages (bone marrow-derived or peritoneal) were suspended in 1 ml HBSS at a concentration of $1x10^6$ cells/ml. Live *S. pneumoniae* (strain P1547, serotype 6A) in the log phase of growth (OD: 0.5) was added at an MOI of 10, and solution was mixed on a nutating mixer at 37° C for 30 min. The cells were then separated from unbound bacteria by centrifuging at 1500 rpm for 5 min, and resuspended in 1 mL of HBSS. Macrophages were incubated with bound bacteria for 30 min. To measure macrophage killing of *S.pneumoniae*, aliquots were collected at 30 min and macrophages were lysed in sterile H_2O. Serial dilutions were performed in H_2O and plated on sheep's blood agar supplemented with 10 mg/ml neomycin. Colonies were counted

the next day to quantitate viable bacteria.

Cytokine ELISA

ELISAs for IL-6 were performed as per the manufacturer's directions (eBioscience). Macrophage supernatants were diluted 1:2. To assess plasma levels of IL-6 and TNF, high-sensitivity Milliplex kits (EMD Millipore, Billerica, MA, USA) were used, as per the manufacturer's instructions.

Statistical Analysis

Data was analyzed with GraphPad Prism 6 software to determine statistical significance as indicated in figure legends. Statistical significance was assessed using one-way ANOVA using Fisher's LSD post hoc test or nonparametric Mann-Whitney t-tests. Data presented as bar graphs are presented as means ± SEM, unless otherwise noted. A p value <0.05 was considered statistically significant.

RESULTS

Inflammatory, Ly6C^{high} monocytes are increased in the blood and bone marrow of aged hosts but are phenotypically different.

Myeloid skewing occurs with age. We have previously shown an age-associated increase in the proportion of monocytes, particularly of the Ly6C^{high} subset[9]. Furthermore, we have shown that their production of high levels of pro-inflammatory cytokines contributes both to age-associated inflammation and declining anti-bacterial immunity[9]. To determine whether the aging microenvironment alters inflammatory monocyte phenotype, we assessed Ly6C^{high} monocytes frequency and CCR2 expression in the steady state using heterochronic bone marrow chimeras (**Fig. S1**). As observed in **Fig. 1A**, the microenvironment dictated the percentage of Ly6C^{high} monocytes in the circulation, regardless of whether myeloid precursors were derived from a young (3mo) or old (>18mo) bone marrow donor. Furthermore, CCR2 expression increased in old recipient mice (**Fig. 1B**). CCR2 expression on *young* monocytes in an old microenvironment was equivalent to old controls, while CCR2 levels on *old* monocytes in the young microenvironment was equivalent to young controls. Because increases in Ly6C^{high} monocyte frequency and CCR2 expression occurred in both blood and bone marrow (**Fig. 1C-D**), the influence of the microenvironment likely begins in the bone marrow. Intracellular levels of TNF and IL-6 were significantly greater in Ly6C^{high} monocytes isolated from aged microenvironments (**Fig. 1E**). Together, these data demonstrate that the quantity and phenotype of inflammatory monocytes are dependent on the age of host, but not the age of the donor bone marrow.

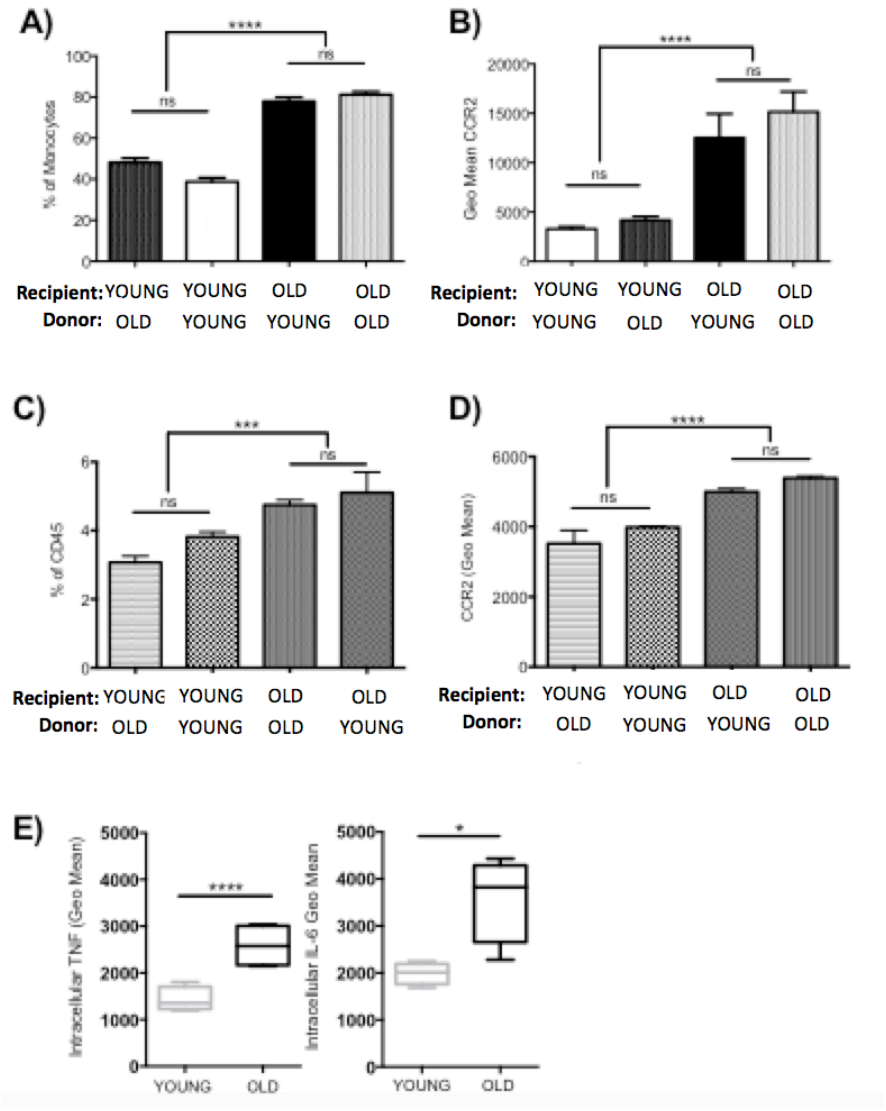


Figure 1. ***Inflammatory Ly6C^{high} monocytes are increased in the blood and bone marrow of aged hosts but are phenotypically different.*** Peripheral blood was collected from chimeric cohorts (n=4-5 per group) and the phenotype of inflammatory Ly6C^{high} monocytes were assessed by flow cytometry. The **A)** percentage of Ly6C^{high} monocytes and **B**) their expression of CCR2, which is required for egress from the bone marrow, was found to be increased in aging microenvironments. These changes in inflammatory monocyte quantity and phenotype were paralleled in the bone marrow **(C-D)**. Ly6C^{high} monocytes isolated from old microenvironments produced increased **E)** intracellular TNF and IL-6. Data representative of 2 individual experiments, where n=4-5 mice per group ± SEM. Statistical significance determined by two-tailed Mann-Whitney-Wilcoxon tests or one-way ANOVA with Fisher's LSD post-test where appropriate, p≤0.05(*), p≤0.01(**), $p \leq 0.001$(***), p≤0.0001(****).

Age-associated increases in TNF contribute to changes in monocyte phenotype and function. TNF is a key cytokine associated with inflammaging and is elevated in circulation with age (**Fig. S1**). Thus, we investigated whether the increased presence of TNF was sufficient to drive age-associated changes in the frequency of inflammatory monocytes. We measured Ly6C^{high} monocytes in the blood of old TNF knockout (KO) mice (18–22 mo). Strikingly, it was observed that old TNF KO mice had levels of inflammatory monocytes comparable to young wildtype mice (**Fig. 2A**). Further, inflammatory monocytes derived from old TNF knockout mice did not have increased CCR2 expression (**Fig. 2B**). When monocytes isolated from young mice (3mo) were stimulated with recombinant TNF (50 ng/mL), they proliferated at a comparable rate to unstimulated monocytes derived from old mice (**Fig. 2D**). Not only does our data support that TNF drives the egress of Ly6C^{high} monocytes into the blood, but it also demonstrates that TNF induces monocyte proliferation. Parallel to the steady state, when young and old wildtype and TNF knockout mice were colonized with *S. pneumoniae* for 5 days, a decrease in circulating Ly6C^{high} monocytes was observed in the aged TNF KOs compared to their old counterparts (**Fig. 2C**).

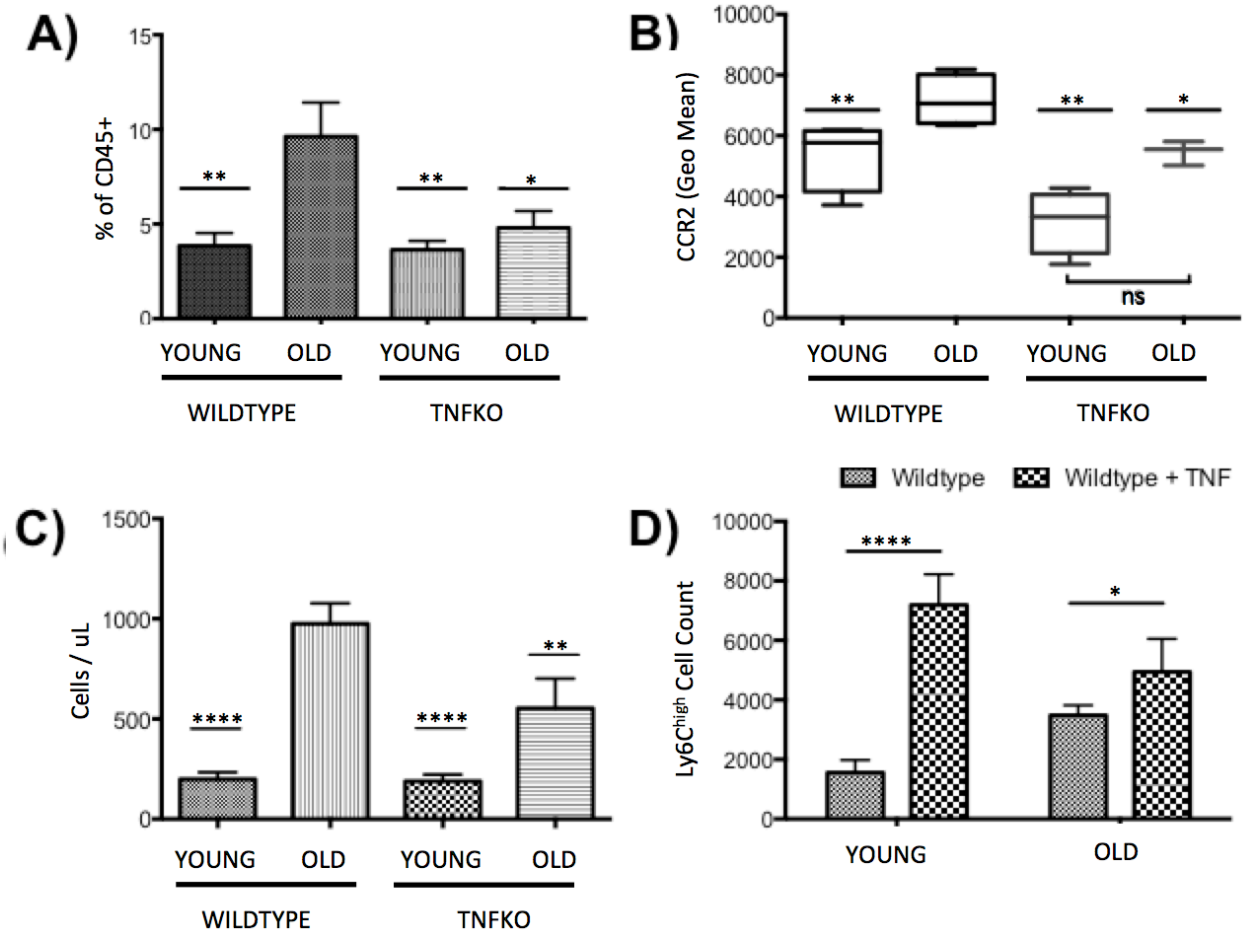


Figure 2. ***Age-associated increases in TNF contribute to changes in inflammatory monocyte phenotype and function.*** **A)** Ly6C^{high} monocytes in the blood of young (3mo) and old (>18mo) wiltdtype and TNF KO mice was quantitated as a proportion of CD45+ cells (± SEM; n = 3–6). **B)** CCR2 expression on circulating Ly6C^{high} monocytes in young (10-12 wks) and aged (>18 mo) wildtype and TNF KO mice (± SEM; n=3–6) support that the TNF drives CCR2 expression with age. When aged TNF KO mice were colonized *with S.pneumoniae* for 5 days, **C)** reduced levels of Ly6C^{high} monocytes were observed when compared to old wildtype mice (± SEM; n = 3–6). **D)** Monocytes from young mice stimulated with TNF (10ng/mL) demonstrated proliferated to a level comparable to that of old mice. Statistical significance determined by one-way ANOVA with Fisher's LSD post-test where appropriate, p≤0.05(*), p≤0.01(**), $p \leq 0.001$(***), $p \leq 0.0001$(****).

Macrophage function is intimately tuned to the host microenvironment.

Macrophages derived from aged mice (>18mo) demonstrate a diminished capacity to phagocytose and eliminate *S. pneumoniae*, and produce a hyper-inflammatory response to bacteria or bacterial products, compared to young mice[9,16,33,34]. To determine whether these impairments are due to changes in the aging microenvironment (e.g. increases in pro-inflammatory cytokines), or intrinsic age-related defects, we isolated macrophages from the peritoneal cavity and bone marrow from our heterochronic chimeras. As observed in **Fig 3A**, when resident peritoneal macrophages (originally derived from *young* bone marrow) were isolated from the old recipients, their ability to kill *S. pneumoniae* was equivalent to old age-concordant chimeras. When peritoneal macrophages (originally from *old* bone marrow precursors) were isolated from young recipients, they regained their ability to efficiently phagocytose and destroy bacteria. This functional plasticity in response microenvironmental signals also occurred in bone marrow-derived macrophages (**Fig. 3B**). When macrophages derived from *old* bone marrow are placed in young recipients, their ability to kill bacteria increased and they produced cytokines at levels equivalent to young **(Fig. 3C)**. Thus, changes in host microenvironment, and not intrinsic changes at the level of the hematopoietic stem cell, cause dysregulated anti-bacterial immunity with age.

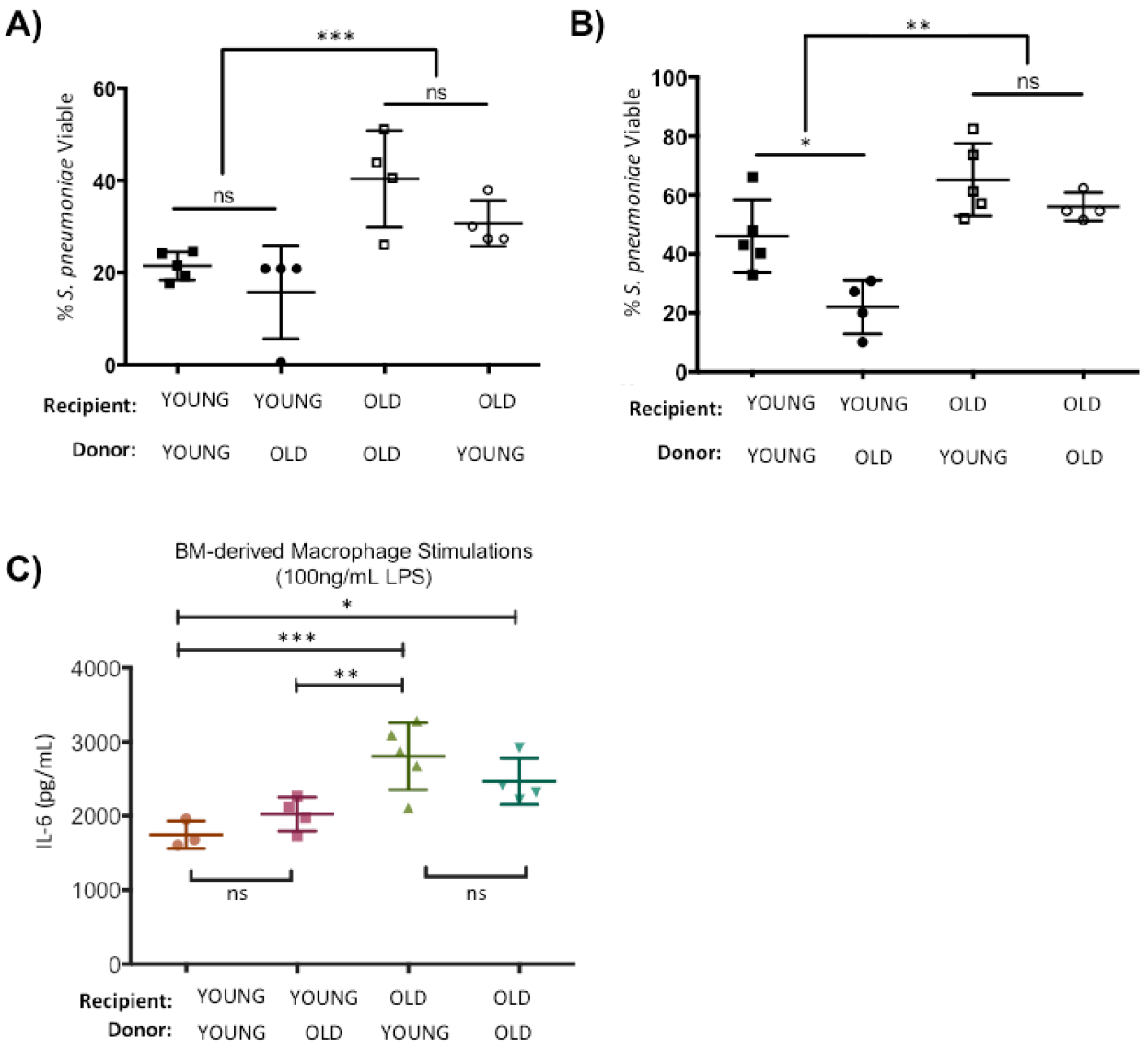


Figure 3. ***Macrophage functionality is intimately attuned to the host microenvironment.***

A) Peritoneal and **B)** bone marrow-derived macrophages isolated from chimeras were incubated with *S.pneumoniae* (P1547, serotype 6A). After 30min, cells were lysed and viable bacteria were plated to determine killing capacity (n=5 per group). Further, **C)** bone marrow-derived macrophages were stimulated with 100ng/mL LPS for 16 hours and IL-6 levels were determined by ELISA to assess inflammatory responses to bacterial products. Statistical significance determined by one-way ANOVA with Fisher's LSD post-test where appropriate, $p \leq 0.05$(*), $p \leq 0.01$(**), $p \leq 0.001$(***), with n=4-5 mice per group ± SEM.

The age of the microenvironment, but not hematopoietic stem cells, influences the outcome of infection.

To investigate the importance of the aging microenvironment *in vivo*, heterochronic chimeras were intranasally colonized with $1x10^5$ CFUs of *S. pneumoniae* (P1547, serotype 6A) (**Fig. 4A**). As observed in **Fig. 4B**, *old* recipient mice fared poorly in response to infection. Old recipient mice experienced a significant loss in body mass, compared to young counterparts. Old recipients had higher levels of serum IL-6 than young recipient mice (**Fig. 4C**). At day 5, mice were sacrificed and lungs and spleens were collected and CFUs were quantitated to assess bacterial dissemination. Little to no dissemination was observed in *young* recipient mice (**Fig 4D**). Old recipient mice had a significantly increased bacterial burden in lungs and spleen (**Fig. 4E**). Thus, the aging microenvironment, not the age of the myeloid progenitors, determines the outcome of *S. pneumoniae* infection.

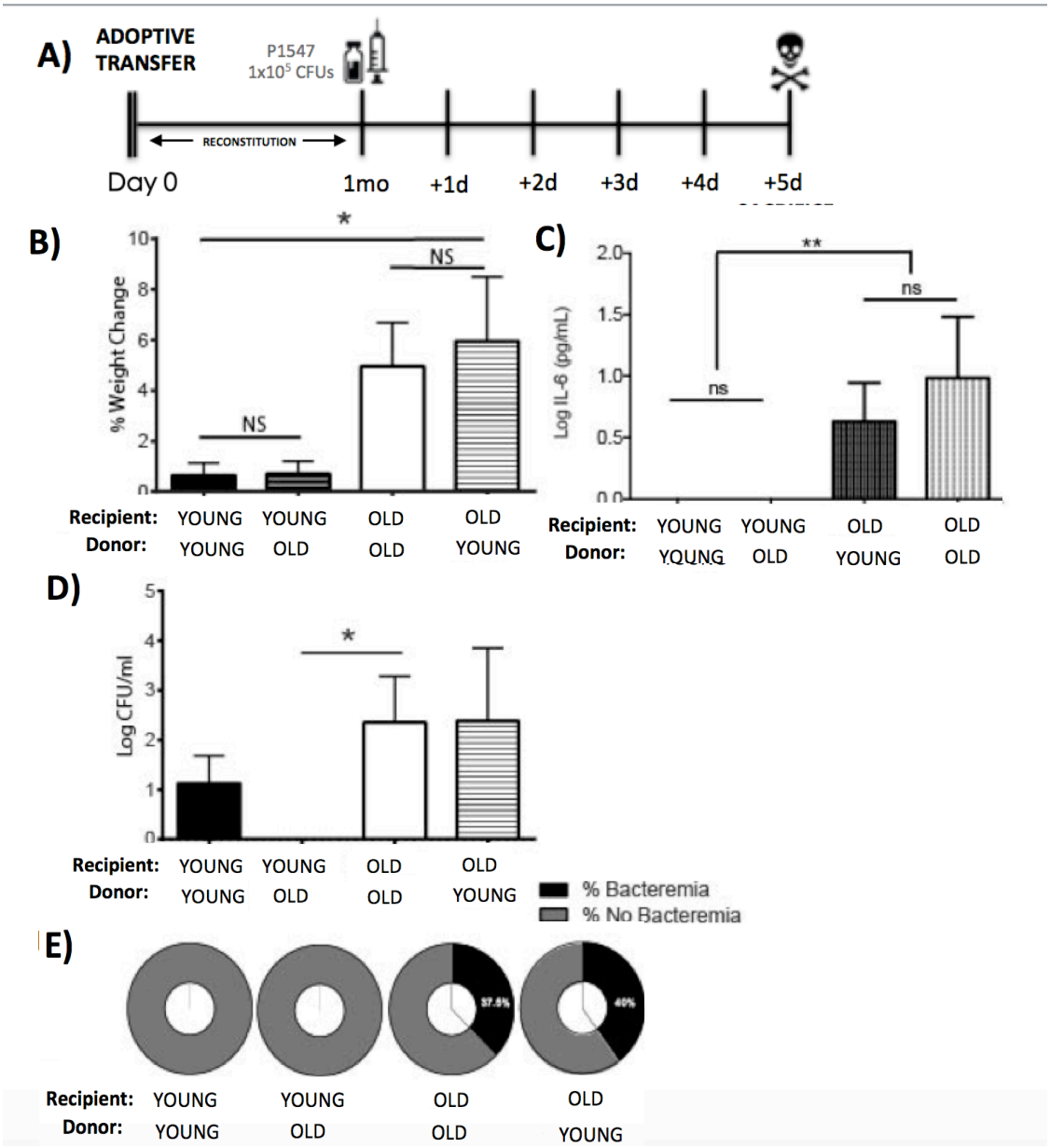


Figure 4. ***Outcome of S. pneumoniae infection is improved when donor marrow is adoptively transferred to young recipients.*** Following irradiation and adoptive transfers with age-discordant or –concordant bone marrow (BM), mice were allowed 1mo for reconstitution (>95%) with donor bone marrow. Following reconstitution, heterochronic chimeras were **A)** colonized intranasally with $1x10^5$ CFUs of *S. pneumoniae* (P1547, serotype 6A). Changes in **B)** mouse weight between pre-colonization and at endpoint were determined (± SEM; n=5-8 per group). **C)** Old recipients had increased levels of circulating IL-6. At day 5, mice were sacrificed and **D)** lungs were collected, homogenized and plated on blood agar plates. Mice with aged microenvironments had a significantly

increased bacterial burden in lungs, indicative of pneumonia severity (± SEM; n=5-8 per group). **E)** *S.pneumoniae* presence in the spleen as a percentage of total colonized mice (per group) was assessed (n=5-8). Statistical significance was determined by one-way ANOVA with Fisher's LSD post-tests where appropriate; $p<0.05$(*).

Inflammatory Monocyte Maturity is Negatively Correlated with Clearance of *S. pneumoniae*.

We have previously shown that increased numbers of inflammatory monocytes in old mice correlated with impaired host defense[9]. Thus, we investigated whether the changes in Ly6C^{high} monocytes, which occurred due to changes in the aging microenvironment, influenced the outcome of *S. pneumoniae* infection. As illustrated in **Fig. 5A**, the proportion of Ly6C^{high} monocytes is dictated by the age of the recipient, regardless of whether the myeloid progenitors were from a young or old donor. Circulating inflammatory monocytes were significantly increased in *old* recipients, during infection. Since Ly6C^{high} monocytes represent an intermediate stage in monocyte-to-macrophage differentiation, we investigated their maturity using the monocyte/macrophage maturity marker, F4/80. As illustrated in **Fig. 5B**, Ly6C^{high} monocytes isolated from *old* recipients were found to be considerably less mature than those from young recipients. Interestingly, a strong inverse relationship ($r = -0.6$) was observed between Ly6C^{high} monocyte maturity and bacterial burden in the nasopharynx (**Fig. 5C**). To validate whether these phenotypic changes in inflammatory monocytes could impair bacterial clearance, we selectively targeted Ly6C^{high} monocytes using negatively charged polystyrene microparticles (PS-MPs). Old mice were given PS-MPs 1 day prior to and every 3 days during the course of *S. pneumoniae* colonization and survival was measured at day 7. As demonstrated in **Fig. 5D**, old mice partially depleted of Ly6C^{high} monocytes had a significantly improved survival (>85%), as compared saline controls (<45%). Collectively, these data for the first time

demonstrate that the aged microenvironment influences both monocyte quantity and maturity and ultimately, disease outcome to pneumococcal infection.

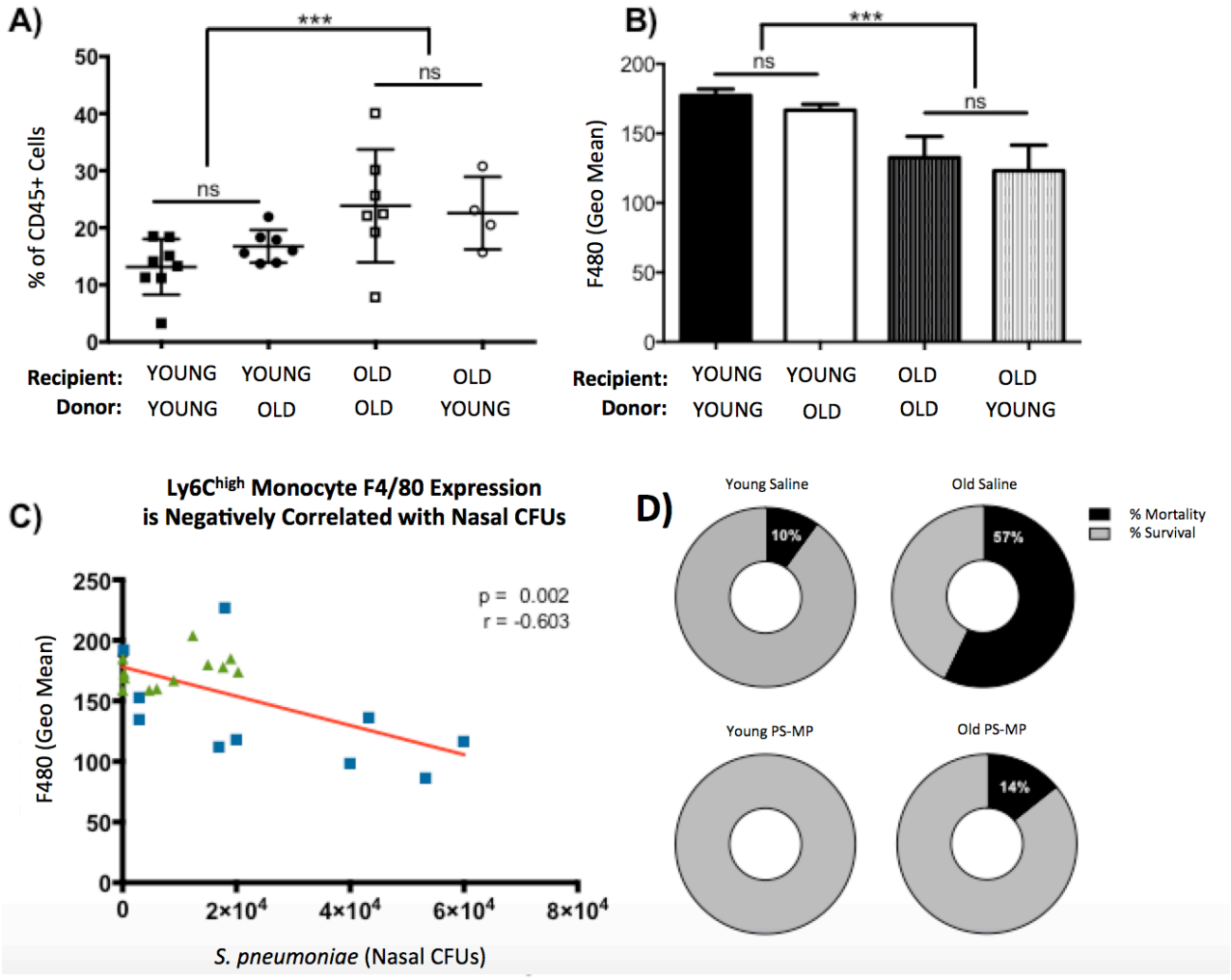


Figure 5. ***Inflammatory Monocyte Maturity is Negatively Correlated with Clearance of S.pneumoniae.***

Heterochronic chimeras were colonized with 1x10^5 CFUs of S.pneumoniae (P1547, serotype 6A) for 5 days via intranasal colonization. At sacrifice (day 5), peripheral blood was collected and flow cytometry performed; n=5-8 per group. Inflammatory Ly6C^{high} monocytes were assessed as **A)** a percentage of total leukocytes (%CD45). Further, **B)** monocyte maturity, as measured by F4/80 expression, was determined. Analysis performed using One-way ANOVA, Multiple Comparisons; p≤0.05(*), p≤0.01(**). Further, at sacrifice, nasal washes were performed with 300uL PBS and plated on blood agar plates. **C)** A negative correlation was observed between circulating Ly6C^{high} monocyte maturity (F4/80) and *S. pneumoniae* present in the nasopharynx. Triangles represent young

microenvironment and squares represent old. Pearson's r correlational analysis was performed using Prism ($p=0.002$, $r=-0.6$, $R^2=0.364$). **D)** Mice depleted of Ly6C^{high} monocytes using PS-MPs had improved survival upon colonization with S. *pneumoniae* for 5 days; n=3-10 per group.

TNF indirectly affects monocyte phenotype.

Prior work from our laboratory showed that TNF knockout macrophages from aged (18-22mo) mice are indistinguishable from those isolated from younger mice[9]. Thus, it appears that age-associated increases in TNF contribute to changes in myeloid cell phenotype and function. We created chimeras in which young wildtype and TNF knockout donor marrow was transplanted into young wildtype and TNF knockout mice. Upon aging these chimeras to 18mo (**Supp. Fig. 2A**), we assessed the contribution of TNF from hematopoietic and non-hematopoietic cells (**Fig. 6A**). If the monocytes derived from TNF KO donors (which can respond to TNF but not produce it) maintain the "young" phenotype when transplanted into wildtype mice, then TNF produced by hematopoietic precursors themselves are not responsible for increases in Ly6C^{high} monocyte frequency with age. If monocytes from TNF KO donors acquire the "old" phenotype, TNF production from non-hematopoietic cells (and signalling to hematopoietic cells) dictates age-associated increases in Ly6C^{high} monocytes. As observed in **Fig. 6A**, monocytes originally derived from TNFKOs acquired the old phenotype when in an old recipient mouse. Thus, it is evident that TNF production from non-hematopoietic cells contributes to age-associated changes in monocytes.

TNF regulates the expression of many cytokines that are also increased with age (e.g. IL6, IL-12) and therefore TNF may not *directly* affect macrophage function but rather *indirectly* by regulating additional factors downstream. We created chimeras in which young wildtype and

TNFR knockout donor marrow was transplanted into young and old wildtype recipients (**Supp. Fig. 2B**). By creating TNF**R**$^{-/-}$ chimeric mice (which can make but not respond to TNF), we could distinguish between sensing TNF directly (which the knockouts *cannot* do) and sensing all other microenvironmental factors (which the knockouts *are able* to do). TNF**R**$^{-/-}$ donor marrow was transplanted into old wildtype recipients, which presumably have all the elements of the aging microenvironment (in addition to the cytokines regulated by TNF) (**Fig. 6B**). If they maintain the "young" phenotype, it would support that *direct* TNF signaling to the hematopoietic precursors is required for age-associated myeloid cell dysfunction. If they acquire the "old" phenotype, cytokines induced by TNF (which would be present in the wildtype recipient) but not TNF itself is required for the aging phenotype (i.e. the effect of TNF is *indirect*). Herein, we see that TNF indirectly influences the age-associated changes in inflammatory monocytes (**Fig. 6B**).

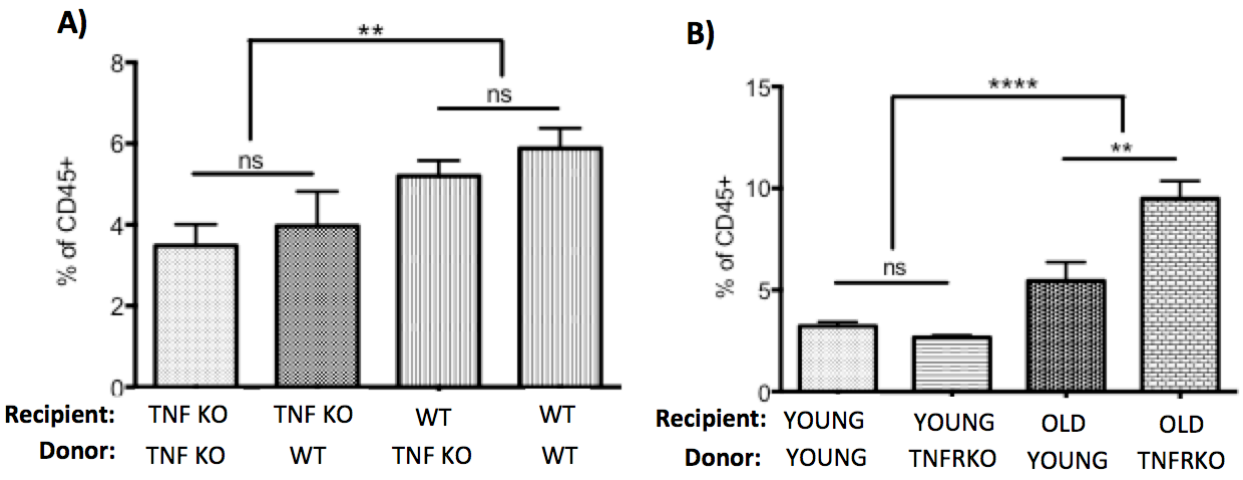


Figure 6. ***TNF indirectly affects monocyte phenotype.*** Ly6C^{high} monocytes were assessed by flow cytometry, as a percentage of CD45+ cells in **A)** TNF KO **B)** TNFR KO chimeras. Statistical significance determined by one-way ANOVA with Fisher's LSD post-test where appropriate, $p \leq 0.01$(**), $p \leq 0.0001$(****), with n=4-10 mice per group ± SEM.

DISCUSSION

Aging is accompanied by an increase in circulating, Ly6C^{high} monocytes which express higher levels of CCR2, resulting in their premature egress from the bone marrow into the circulation. Whether these changes in monocytes were due to the presence of persistent chronic inflammation concomitant with aging, or to intrinsic defects in myeloid progenitors were poorly understood. We demonstrate that inflammatory monocyte frequency and phenotype is under the instruction of the local microenvironment, regardless of whether the progenitor cells were derived from a young or old donor. Further, the increases in Ly6C^{high} monocyte frequency and CCR2 expression observed the blood of aged recipients were paralleled in the bone marrow. These findings suggest the bone marrow microenvironment contributes to increased myelopoeisis with age. Interestingly, in TNF KO mice, these age-related changes in monocytes were not observed, both in the steady and colonized states. As such, it may be concluded that TNF drives the expansion, and increased egress, of the Ly6C^{high} monocytes with age. Ly6C^{high} monocytes produce high amounts of pro-inflammatory cytokines[12-18] and have been shown to contribute to many models of chronic inflammatory disease (e.g. atherosclerosis, myocardial infarction, IBD, neurodegenerative disease and cancer[35–41]). Circulating Ly6C^{high} monocytes correlated with disease progression, and ablation of the subset effectively decreased pathology[36,39–41]. Modulation of the aging microenvironment to reduce circulating levels of TNF may be a therapeutic strategy to reduce Ly6C^{high} monocytes, and their corresponding contribution to age-related pathologies.

Macrophages play an integral role in anti-bacterial immunity but many of their diverse functions are adversely affected with advanced age. Macrophages derived from aged mice (>18mo) demonstrate a diminished capacity to phagocytose and eliminate infectious agents, and produce a hyper-inflammatory response to bacteria or bacterial products, compared to young mice[9,16,33,34]. Murine macrophages are intimately attuned to their microenvironment, demonstrating dramatic functional plasticity in response to extrinsic (tissue derived) signals[42–44]. In the presence of an *old* microenvironment, macrophages derived from *young* myeloid precursors became functionally impaired, demonstrating an inability to eliminate an infectious agent and hyper-inflammatory responses, comparable to that of aged controls. Strikingly, we were able to reverse the age-associated defects of macrophages derived from *old* myeloid precursors by placing them in the context of a *young* microenvironment. These "old" macrophages were able to efficiently phagocytose and eliminate *S. pneumoniae* and coordinate a more controlled inflammatory response.

Interestingly, though extrinsic factors greatly influence macrophage functionality, when we cultured bone marrow-derived macrophages *ex vivo*, these functional changes remain. Intuitively, one would anticipate altered functionality when macrophages are removed from their host microenvironment and cultured in an age-neutral medium. Thus, we propose that myeloid precursors are nurtured by soluble factors (e.g. cytokine milieu) present in the bone marrow microenvironment, which contributes to downstream intrinsic changes in monocyte phenotype and macrophage function. We speculate these gene expression changes are under epigenetic regulation, given that stable, long-term alterations, that are dependent on the

environment, are occurring, and these changes demonstrate reversibility[45]. An emerging body of literature supports that inflammatory stimuli can greatly influence the epigenetic regulation of myeloid cells[46–49]. Chronic exposure of macrophages to pro-inflammatory cytokines, analogous to that of an aged microenvironment, induced changes in the methylation profile of genes regulating differentiation and cell identity[47], and consequently, promptly preferential differentiation into pro-inflammatory M1 macrophages[46]. Further, an age-related decrease in DNA methylation of the TNF promoter has been observed in both human peripheral blood leukocytes and macrophages. Such a change contributes to increased TNF production and higher incidence of inflammatory disease[48]. Together, these findings suggest that chronic inflammation can functionally reprogram macrophages. Once an in-depth understanding of how age-associated changes in the microenvironment contribute to epigenetic modulation of myeloid-derived cells is achieved, targeting of epigenetic mechanisms to modulate macrophage function could emerge as a potential therapeutic tool in treatment of age-related diseases.

Approximately 1/3 of deaths in the elderly occur as a result of infectious disease, with pneumonia being the most frequent and costly infection[50–54]. *Streptococcus pneumoniae* is the most common cause of pneumonia in the elderly[50,55]. However, limited knowledge exists on how colonization of the nasopharynx (defined as asymptomatic and transient occupancy of *S. pneumoniae*) progresses into pneumococcal disease in aged populations. As such, we used an age-discordant chimeric mouse model of *Streptococcus pneumoniae* colonization and infection to uncover fundamental changes in the elderly immune system that predispose to bacterial dissemination. Our data illustrates that in the context of *S. pneumoniae* infection, transition

from nasopharyngeal colonization to symptomatic pneumococcal disease is largely dependent on the microenvironment, as opposed to age-associated intrinsic defects in immune cell function. Irrespective of age of the donor bone marrow (and their myeloid progeny), old recipient mice had an impaired capacity to limit bacterial dissemination, as demonstrated by an increased bacterial burden in lungs and blood (Fig 4). Despite robust Ly6C^{high} monocyte recruitment, bacterial clearance was significantly impaired. Increased levels of developmentally immature Ly6C^{high} monocytes could potentially be more detrimental than beneficial (Fig. 5), given their ability to excessively high levels of pro-inflammatory cytokines (e.g. IL-6, TNF). Prior studies have shown that increased inflammation with age is detrimental to clearance of *S. pneumoniae* from the nasopharynx, given that old TNF knockout mice, which are protected from age-associated inflammation, have lower bacterial loads compared to their WT counterparts[9]. This data is corroborated by a study showing that pre-infection systemic levels of inflammatory cytokines (TNF and IL-6) were associated with a higher risk of pneumonia[56]. Further, when mice young mice were infused with TNF, they became significantly more susceptible to experimental infection with *Streptococcus pneumoniae* as old mice[57]. As such, TNF may be used as a potential target to rescue the "young" phenotype of myeloid cells and improve anti-pneumococcal immunity. Through removal of TNF using genetic knockdowns or pharmacological inhibition, age-associated inflammation was shown to be reduced and Ly6C^{high} monocyte output decreased[9]. When we selectively depleted circulating Ly6C^{high} monocytes using carboxylated polystyrene microparticles in aged mice to the level of their young counterparts, we significantly improved survival in the old. As such, therapeutic manipulation of

the microenvironment by reducing the inflammatory status of the host shows promise in protecting the elderly from morbidity and mortality associated with infectious disease.

While we now understand that age-associated changes in the microenvironment alter monocyte phenotype and macrophage functionality, the next logical step is to determine what exactly is effectuating such changes. Old TNF knockout mice demonstrate a reduction in the frequency of, and CCR2 expression on, inflammatory (Ly6C^{high}) monocytes. Further, prior studies have shown that macrophages isolated from old TNF knockout mice are indistinguishable from young macrophages and maintain their phagocytic and anti-bacterial activity, unlike those isolated from aged WT mice. Given this basis, we believe that an age-related increase in TNF contributes to macrophage dysfunction and increased inflammatory monocyte egress to blood from the bone marrow; however, the source of this TNF (e.g. stroma, leukocytes or their precursors) remains unclear. Using aged TNFKO chimeras, we determined that the source of TNF was non-hematopoietic, as consistent with our previous findings. We postulate that either increases in adipose deposition, changes in mesenchymal stromal cell functionality and/or increased microbial translocation may principally contribute to the inflammatory milieu that transpires with age. During the aging process, hematopoietic tissue is gradually replaced by adipose tissue, a process known as fatty degeneration[50]. Adipose tissue (AT), known to be pro-inflammatory due to secretion of elevated amounts of TNF, is primarily dominated by M1 (classically-activated, pro-inflammatory) macrophages[50]. Further, AT from old mice (>16mo) expressed significantly higher amounts of proinflammatory molecules (e.g. TNF, IL-6), both basally and upon stimulation, compared to young controls. Therefore, increased

bone marrow adiposity with age may possibly contribute to inflammatory microenvironment establishment, and consequent downstream changes in myeloid cell precursors. Given that mesenchymal stromal cells (MSCs) are the major support cells for the HSCs in the bone marrow, they may potentially contribute to the inflammatory aged microenvironment. MSCs are known to exert immunoregulatory activities, however, with age, their homeostatic function may be impaired, and as, such their immunosuppressive activity may decrease[52]. Age-associated chronic inflammation has been show to alter the MSC compartment and promote adipogenesis[58–60]. Alternatively, in animal models, microbial translocation caused by an age-associated loss of intestinal barrier integrity has been proposed to drive inflammaging[61,62], and this is consistent with observations of increased circulating bacterial products found in the elderly[63]. In fact, long-term exposure of very low doses of LPS in young mice parallels many of the changes in myelopoeisis observed with old age[64]. Based on these studies, the etiology of inflammaging is presumably multifactorial.

Age-associated changes in immune function are not exclusively mediated by TNF. TNF regulates the expression of a number of other cytokines increased with age (e.g. IL-6, IL-12, CCL5); therefore TNF may not directly affect myeloid cell function, but rather indirectly through the induction of downstream cytokines. Using $TNFR^{-/-}$ chimeras, we support that indirect TNF signaling to hematopoietic precursors is required for age-associated changes in monocytes and macrophages. Recent studies have shown that aging of certain systems (e.g. muscular, cardiac and central nervous systems[25–28]) can be reversed by providing old animals with young blood through use of heterochronic parabiosis models, where circulating systems of young and aged

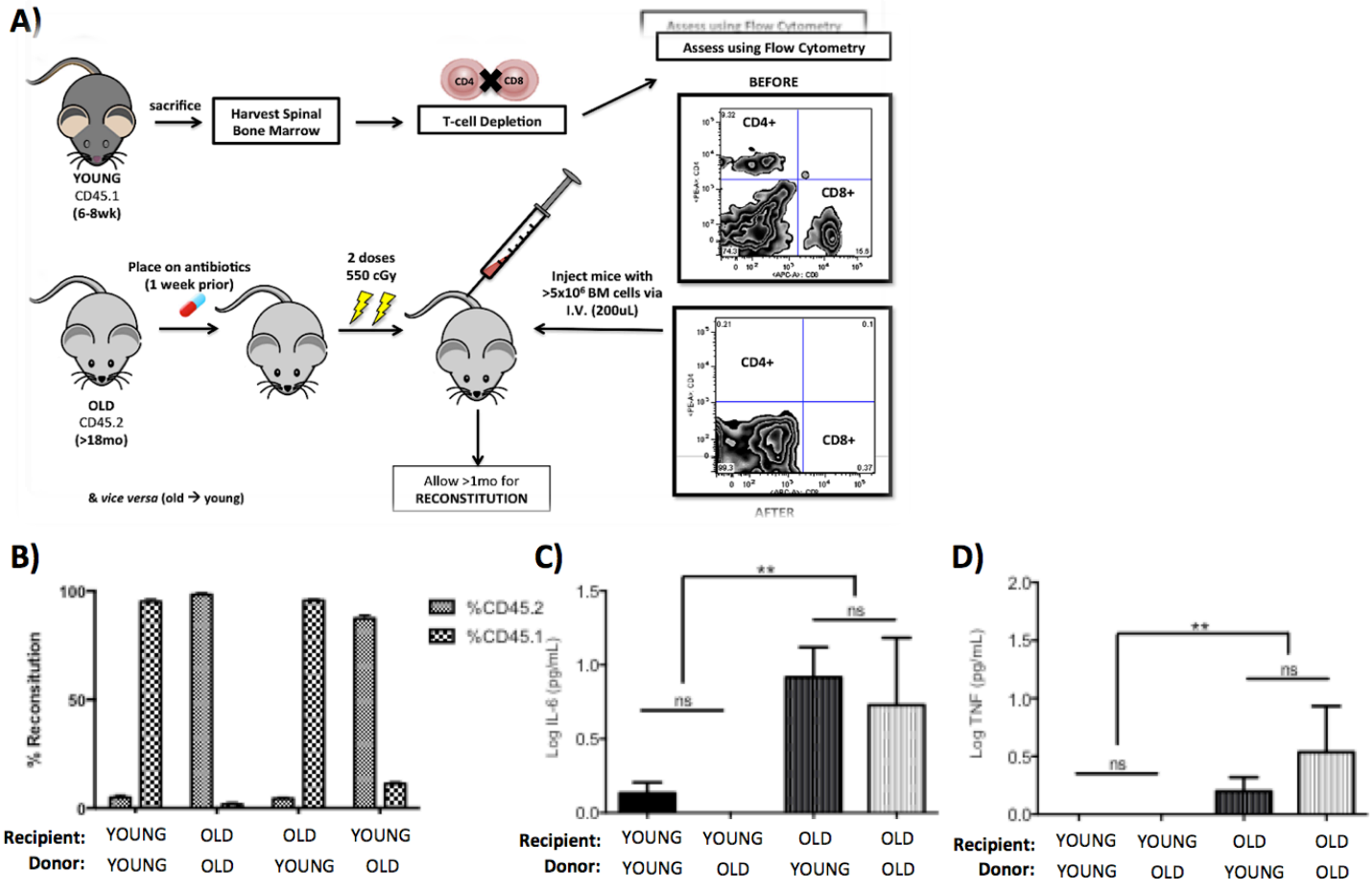
through use of heterochronic parabiosis models, where circulating systems of young and aged mice are joined by surgery or through infusion of young serum/plasma into old animals[65]. In these studies, potential candidates that mediate age-associated changes in these systems include chemokine, CCL11, transforming growth factor-β (TGF-β) superfamily member, GDF11, and microRNAs[25–28,65], however the potential for numerous blood-borne candidates are collectively agreed upon. Further, these parabiosis studies examined how *'pro-youthful'* factors from young blood can reverse age-related impairments. Conversely, from a novel perspective, we assessed how abrogating '*pro-aging factors'* from old blood can counteract impairments in immune cell function. Both approaches warrant further investigation and may each provide a successful strategy to combat the effects of aging in numerous organ systems.

CONCLUSION

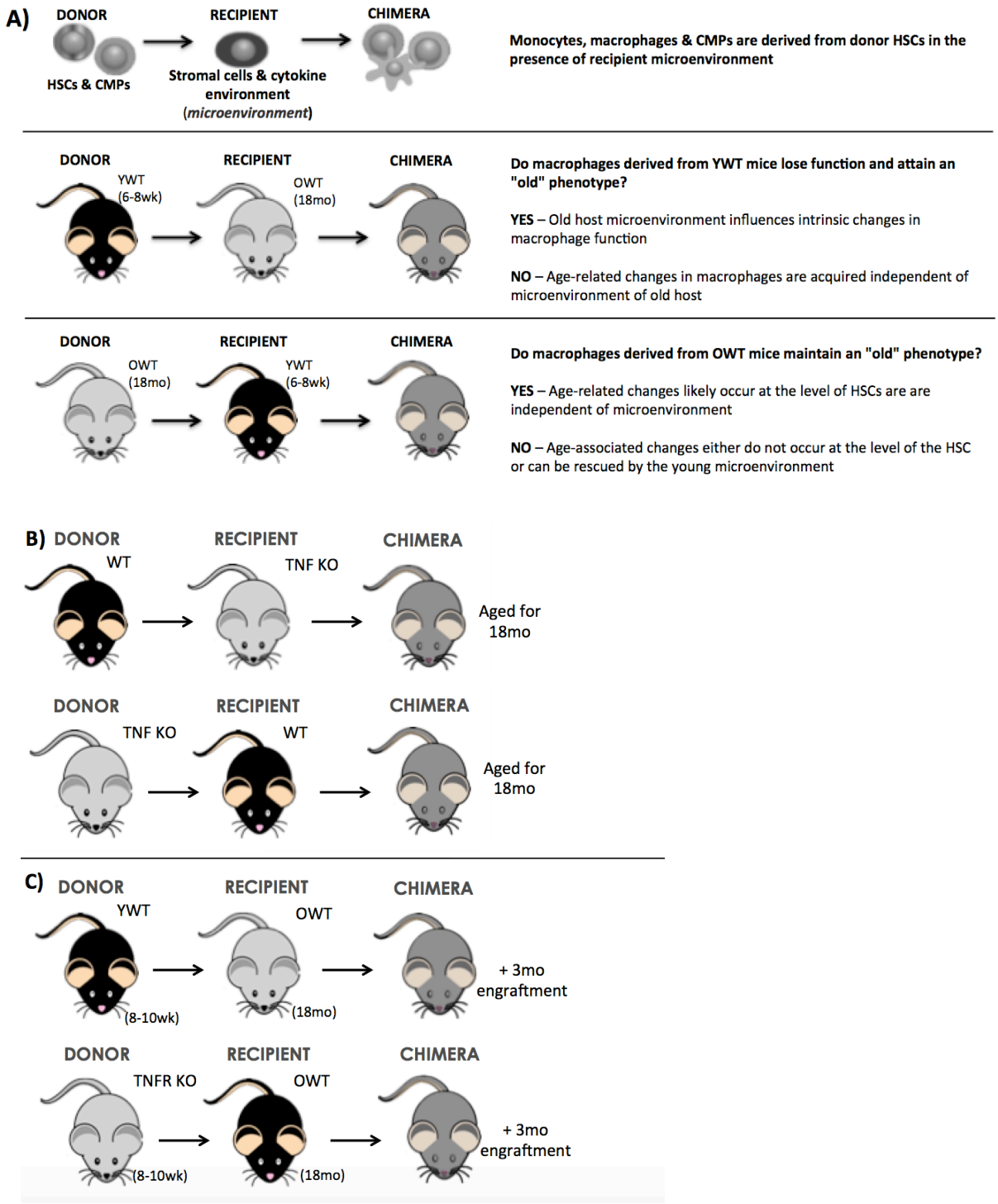
In this study, using heterochronic chimeras, we aimed to elucidate the relative contributions of cell intrinsic and global microenvironmental factors on age-associated changes in myeloid cells, and ultimately, their impact on anti-bacterial immunity. Here, we demonstrate that exposure of aged immune cells to the microenvironment of a young host can counteract and reverse pre-existing effects of monocyte and macrophage aging at the phenotypic and functional level. Fortunately, given that these changes are reversible, and due to the inflammatory microenvironment, rather than intrinsic defects at the level of the hematopoietic stem cell, it means they are amenable to therapeutic manipulation, if not by anti-TNF, than by other anti-inflammatory measures. These findings facilitate an improved understanding of the etiology behind the age-associated changes in myeloid cell phenotype and the consequent

enhanced susceptibility to infectious disease, specifically *S. pneumoniae*. These conclusions expand our fundamental knowledge regarding the aging immune system, and expectantly, will lead to more informed decisions regarding development of novel immunotherapeutics or pneumococcal vaccines in elderly populations.

SUPPLEMENTARY FIGURES



Supplementary Figure 1. *Establishment of Heterochronic Bone Marrow Chimeras.* **A)** To generate chimeras, spinal bone marrow (BM) was harvested from donor mice and T-cell depleted. Recipient mice were treated with antibiotics for 1 week prior to irradiation (2 doses at 550 rads). Within 2hrs of 2nd dose, recipient mice received donor BM via tailvein inject. **B)** Engraftment was determined by assessing the ratios of CD45.1 to CD45.2 cells using flow cytometry and was >95% in all recipients following transplant. Multiplex high-sensitivity ELISAs were uses to assess plasma **C)** IL-6 and **D)** TNF in chimeras.



Supplementary Figure 2A. ***Experimental Outline for Bone Marrow Chimera Experiments.*** **A)** T-cell depleted donor bone marrow from either young (6-8wk) or old (16mo) were adoptively transferred into young or old recipient mice. Differential expression of congenic markers (CD45.1 or CD45.2) between donors and recipients were used to

evaluate degree engraftment (>95%). **B)** T-cell depleted donor bone marrow from either young (12wk) TNF knockout (TNFKO) or wildtype (WT) mice were adoptively transferred into both WT and TNFKO recipient mice (WT→WT, and TNKO→TNFKO were used as controls). Mice were aged 18mo. **C)** Young WT and TNFR KO (12wk) bone marrow were adoptively transferred into young (12 wk) and old (18mo) recipient mice. Mice were allowed to engraft for 3mo prior to sacrifice.

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CHAPTER 4: Monocyte Characteristics Predict Insulin Levels.

Declaration of Academic Achievement:

This research was conducted during the period of September 2014 to December 2015. I am the first author on this manuscript. I performed the majority of experiments, with the exception of Supplementary Figure 2, and analyzed and interpreted all the data. Dr. K. Foley assessed the insulin and HOMA-IR values in mouse cohorts (Supp. Fig. 2), and performed insulin ELISAs on the human plasma samples (Fig. 3G-I). Dr. C. Verschoor performed the human monocyte immunophenotyping required to generate the correlative plots (Fig. 3G-I). Dr. J. Schertzer provided invaluable insight and critical editorial input. Experiments were conceived and designed by Dr. J. Schertzer, Dr. D.M.E. Bowdish, and myself. Dr. D.M.E. Bowdish and Dr. J. Schertzer provided guidance throughout the writing process.

Monocyte Characteristics Predict Insulin Levels

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Running Title: Monocyte phenotype relates to insulin

Keywords: obesity, diabetes, chemokine, cytokine, inflammation, insulin resistance, adipose, liver

ABSTRACT

Inflammation underpins components of obesity-related hyperinsulinemia and insulin resistance, which often leads to type 2 diabetes. Understanding the etiology of metabolic inflammation may identify ways to distinguish obesity from insulin resistance. We found that high fat diet (HFD)-induced obesity altered myelopoiesis and increased the egress of immature, pro-inflammatory ($Ly6C^{high}$) monocytes from the bone marrow into the circulation of mice. Tumor necrosis factor (TNF) regulated monocyte phenotype. HFD-fed $TNF^{-/-}$ mice had lower premature egress of $Ly6C^{high}$ monocytes, which corresponded with less macrophage accumulation in the liver and adipose tissue despite obesity. The number and maturity status of $Ly6C^{high}$ monocytes predicted insulin resistance and circulating insulin levels better than adipocyte size or fat mass in mice. These findings also held true in humans where the number of circulating classical and intermediate monocytes correlated with insulin levels. These data demonstrate that blood monocyte characteristics are predictive markers of insulin levels and that the premature egress of monocytes represents an adjustable mechanism linking diet-induced obesity to hyperinsulinemia and insulin resistance.

INTRODUCTION

The incidence of type 2 diabetes (T2D) is increasing. This phenomenon has been closely linked to soaring obesity rates, which have reached epidemic proportions in the developed world[1]. Obesity is associated with chronic, low-grade inflammation that is compartmentalized in certain tissues[2]. This metabolic inflammation can impair insulin action in tissues, such as skeletal muscle, liver and adipose, which help control blood glucose. The consequent insulin resistance is often matched by hyperinsulinemia, which generally precedes type 2 diabetes and can promote obesity[3]. Metabolic inflammation also promotes pancreatic beta cell failure. Further, in combination with both environmental and genetic factors, metabolic inflammation can contribute to insufficient insulin secretion, which is a key factor in T2D[4]. There are many mediators of metabolic inflammation. The pro-inflammatory cytokine, tumour necrosis factor (TNF), is increased during obesity and promotes insulin resistance[5,6]. Although TNF can be derived from metabolic cells, such as adipocytes, immune cells are the principal contributors to metabolic inflammation[6,7].

Macrophages that accumulate in obese adipose tissue demonstrate augmented pro-inflammatory polarization[8]. Pro-inflammatory, classically-activated (M1) adipose tissue macrophages (ATMs) are primarily derived from circulating monocytes recruited to adipose in response to tissue-derived chemokine signals, such as CCL2[9–11]. The CCR2/CCL2 chemotactic axis promotes monocyte migration and macrophage recruitment into adipose and the liver during obesity[8]. As such, we hypothesized that ATMs would be generated from Ly6C^{high} inflammatory monocytes, known to express

high levels of CCR2[12–15]. These recently recruited monocytes propagate inflammation and can interfere with insulin signaling[8,16]. Furthermore, we hypothesized that metabolic inflammation during diet-induced obesity is linked to premature egress of inflammatory Ly6C^{high} monocytes and that this correlates with insulin resistance. We observed that monocyte reprogramming during obesity begins in the bone marrow and is marked by increased circulating Ly6C^{high} monocytes. Using TNF$^{-/-}$ as a model of reduced systemic inflammation, we demonstrated that recruitment of Ly6C^{high} monocytes and tissue macrophage infiltration was reduced despite diet-induced obesity. More importantly, this allowed for an assessment of fasting blood glucose and insulin resistance in mice that had low, intermediate and high levels of Ly6C^{high} monocytes during obesity. We demonstrate that blood monocyte characteristics, such as monocyte maturity and quantity, served as a better predictor of hyperinsulinemia and insulin resistance than adipocyte size or adipose tissue mass during diet-induced obesity. This relationship held true in humans where specific monocyte characteristics were better predictors of insulin levels compared to circulating cytokines.

- An increase in body fat is generally associated with increased risk of metabolic diseases such as type 2 diabetes mellitus, hypertension and dyslipidaemia. However, not all overweight or obese patients have metabolic diseases, and vice versa.
- Body mass index: Calculated from height and weight (kg/m2) measured using standardised examination protocols [BMI = mass/h^2]
- >25 (overweight) and >30 (obese)

MATERIALS AND METHODS

Animals

Male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). $TNF^{-/-}$ were originally attained from The Jackson Laboratory (Bar Harbor, ME) and bred at the McMaster Central Animal Facility (Hamilton, ON, Canada). All animals were housed under specific pathogen-free conditions. At 8 weeks of age, mice fed *ad libitum* standard chow diet (Harlan Teklad no. 7913, 6.2% kcal from fat) or a high-fat diet (Research Diets D12492, 60% kcal from fat) for 24 weeks. All experiments were performed in accordance with Institutional Animal Utilization protocols approved by McMaster University's Animal Research Ethics Board following the recommendations of the Canadian Council for Animal Care.

Mouse Flow Cytometry

Monoclonal antibodies with the following specificities were used in this study: F4/80 (APC), Ly6C (FITC), CD45 (eFluor 450), CD11b (PE-Cy7), CD3 (Alexa Fluor 700), NK1.1 (Alexa Fluor 700), CD19 (Alexa Fluor 700), CCR2 (PE) TNF (BV650) or IL-6 (PerCP-efluour 710). Bone marrow flushed from femurs or peripheral blood (100 μL) collected retro-orbitally from mice were analyzed by flow cytometry for $Ly6C^{high}$ monocytes, as previously published[17]. Figure S1 shows the gating strategy. IL-6 was detected using intracellular staining following permeabilization of cells. Flow cytometry was performed on a BD Biosciences Fortessa and analysis occurred using the FlowJo v9

software (Treestar).

Immunohistochemistry

Adipose tissue derived from epididymal fat pads and liver samples were fixed in 10% formalin at room temperature and embedded in paraffin. Five-micron sections cut at 50-μm intervals were mounted on charged glass slides, deparaffinized in xylene, and stained for macrophage detection with an anti-F4/80 (1:500) monoclonal antibody on the Leica Bond RX automated staining system using an enzyme-pretreatment. The total number of F4/80-expressing cells was counted and averaged using ImageJ.

Mouse Insulin and Glucose Measurement

Mice were fasted for 6 hours prior to blood collection via tail vein. Fasting blood glucose was measured by using the Roche Accu-Chek Inform II system. Blood (50 μL) was collected, incubated at room temperature for 20min, and spun at 7500xg for 5 min at 4°C. Serum was collected and stored at -80°C. Insulin was detected in mouse serum samples by ELISA according to the manufacturer's protocol (Millipore Cat #EZRMI-13K).

Human Samples

To compare monocyte frequency to insulin concentration in humans, whole blood and serum was collected from elderly individuals (n=27; 25 female) between the ages of 80 and 100 (average, 89.5), from nursing homes in Hamilton, Ontario, Canada. As previously described[18], the rate of metabolic disorders is high in this population, and

furthermore, are known to have elevated systemic inflammation and dysregulated metabolism[19,20]. Written informed consent was obtained from all participants or their legally appointed guardian in the event they were not competent to provide consent themselves. These studies, consent procedures and documents were approved by the McMaster Research Ethics Board.

Human Insulin and Monocyte Immunophenotyping

Monocyte immunophenotyping was performed using flow cytometry as published [13]. Monoclonal antibodies with the following specificities were used in this study: CD2-PE, CD3-PE, CD19-PE, CD56-PE, NKp46-PE, CD15-PE, CD14-APC-Alexa750, CD16-PE-Cy7, HLADR-PerCp-Cy5.5, CD45-eFluor605NC. Monocytes were defined as lineage (CD2, CD3, CD19, CD56, NKp46, CD15) negative, and CD45, HLADR, and CD14 positive. Classical monocytes were defined as CD16 negative, whereas intermediate and non-classical monocytes were defined as CD16 positive. Absolute counts were measured using 123count beads (eBioscience).

Associations between log-transformed insulin concentration and log-transformed monocyte subset frequency were determined by multiple linear regression, adjusting for age and sex. Each monocyte subset was included in separate models. Regression was performed in R v3.2.1. Random-fed human insulin was detected in plasma samples by ELISA according to the manufacturer's protocol (Mercodia Iso-Insulin ELISA kit).

Statistical Analyses

Data was analyzed with GraphPad Prism 6 software to determine statistical significance as indicated in figure legends. Statistical significance was assessed using one-way ANOVA using Fisher's LSD post hoc test or nonparametric Mann-Whitney t-tests. Data presented as bar graphs are presented as means ± SEM, unless otherwise noted. A p value <0.05 was considered statistically significant.

RESULTS & DISCUSSION

Inflammatory Ly6C^{high} monocytes are increased in the blood and bone marrow of HFD-fed mice but are phenotypically and functionally different.

ATMs associate with degree of obesity and contribute to inflammation and insulin resistance[7,8,21]. CCL2 and CCR2-dependent monocyte migration to adipose tissue has been associated with ATM accumulation[10,22]. We found that the percentage of Ly6C^{high} inflammatory monocytes was increased in both the bone marrow and blood of mice fed a HFD compared to chow-fed controls (Fig. 1A-B). Since Ly6C^{high} monocytes represent an intermediate stage in monocyte-to-macrophage differentiation, we investigated their maturity using the monocyte/macrophage maturity marker, F4/80. In HFD-fed mice, Ly6C^{high} monocytes in the bone marrow and circulation express lower levels of F4/80, indicating they are less mature (Fig. 1C-D). CCR2 is necessary for egress of Ly6C^{high} monocytes from the bone marrow into the blood[14] and we found that increased CCR2 expression on Ly6C^{high} monocytes corresponds with premature egress from the bone marrow to blood during diet-induced obesity (Fig. 1E). Intracellular staining of Ly6C^{high} inflammatory monocytes from HFD-fed mice had higher levels of the pro-inflammatory

cytokine IL-6 (Fig. 1F). Together, the data suggests that HFD-feeding altered myelopoeisis in mice, where Ly6C^{high} monocytes are less mature, prematurely leave the bone marrow and enter the blood. These pro-inflammatory monocytes are postulated to contribute to metabolic inflammation and could be a marker of insulin resistance during obesity.

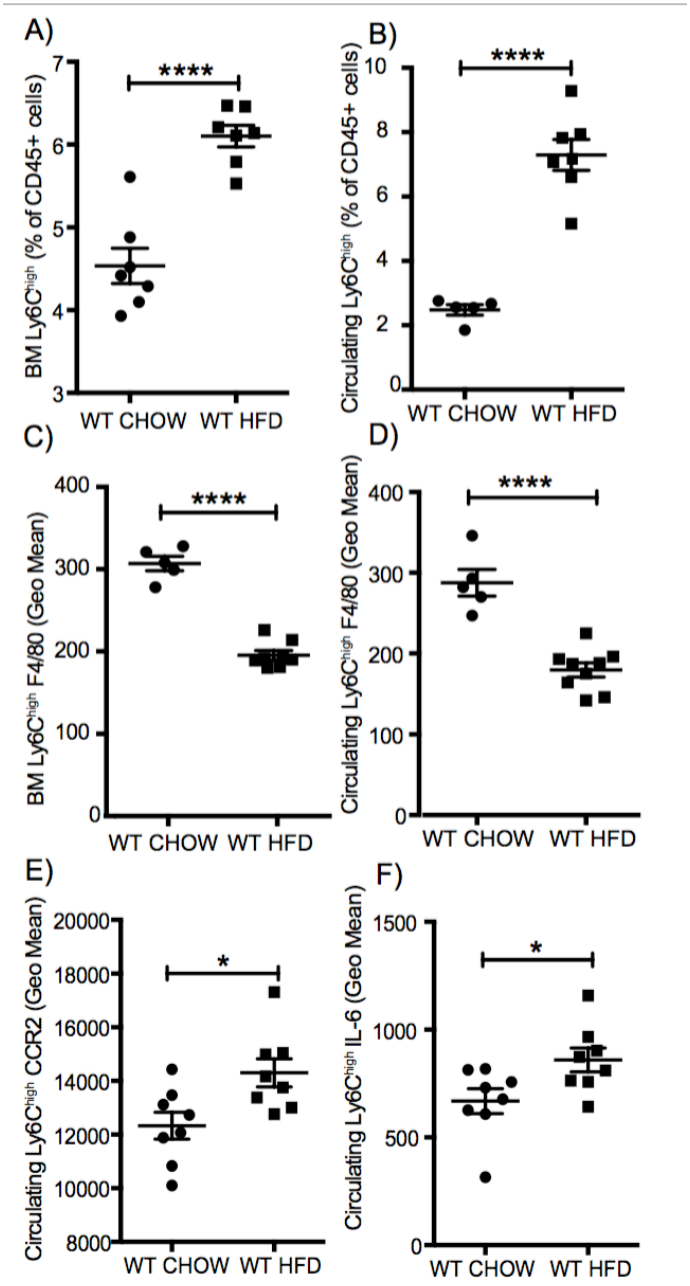

Figure 1: ***HFD alters Ly6C^{high} inflammatory monocytes.*** The percentage of Ly6C^{high} inflammatory monocytes was increased in both the **A)** bone marrow and **B)** blood of mice fed a HFD compared to chow-fed controls. Further, we investigated Ly6C^{high} monocyte maturity using F4/80 expression in **C)** bone marrow and **D)** circulation, in addition to the **E)** egress (CCR2) of circulating, inflammatory monocytes. **F)** Monocytes isolated from HFD diet-fed mice are more pro-inflammatory, as indicated by increased intracellular IL-6. Data representative of 2 individual experiments, where n=5-7 mice per group ± SEM. Statistical significance determined by two-tailed Mann-Whitney-Wilcoxon tests. *$p \leq 0.05$, ****$p \leq 0.0001$.

TNF-associated inflammation contributes to monocyte phenotype during obesity.

As it is well established that TNF contributes to insulin resistance during obesity[23], we hypothesized that systemic TNF-mediated inflammation contributes to obesity-associated Ly6C^{high} monocyte egress from the bone marrow into the blood. To test this, first, we confirmed that TNF$^{-/-}$ mice are partially protected from HFD-induced hyperinsulinemia and insulin resistance (measured using the HOMA-IR index) (Fig. 2). Compared to HFD-fed WT mice, TNF$^{-/-}$ mice had a lower percentage of Ly6C^{high} monocytes in the bone marrow (Fig. 3A), which also had higher levels of F4/80 (Fig. 3B). Further, a lower percentage of Ly6C^{high} monocytes were observed in the blood from HFD-fed TNF$^{-/-}$ mice (Fig. 3C). Circulating Ly6C^{high} monocytes in the blood from HFD-fed TNF$^{-/-}$ mice had lower CCR2 levels, but no difference in monocyte maturity, as measured by F4/80 levels (Fig. 3D-E).

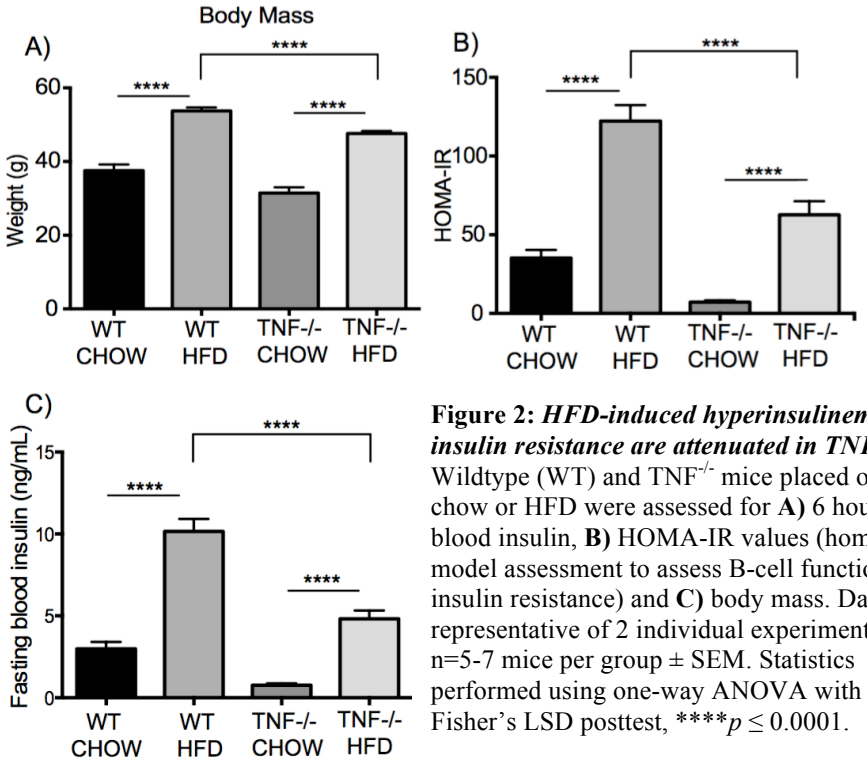

Figure 2: ***HFD-induced hyperinsulinemia and insulin resistance are attenuated in TNF$^{-/-}$ mice.*** Wildtype (WT) and TNF$^{-/-}$ mice placed on either chow or HFD were assessed for **A)** 6 hour fasting blood insulin, **B)** HOMA-IR values (homeostatic model assessment to assess B-cell function and insulin resistance) and **C)** body mass. Data representative of 2 individual experiments, where n=5-7 mice per group ± SEM. Statistics performed using one-way ANOVA with the Fisher's LSD posttest, ****$p \leq 0.0001$.

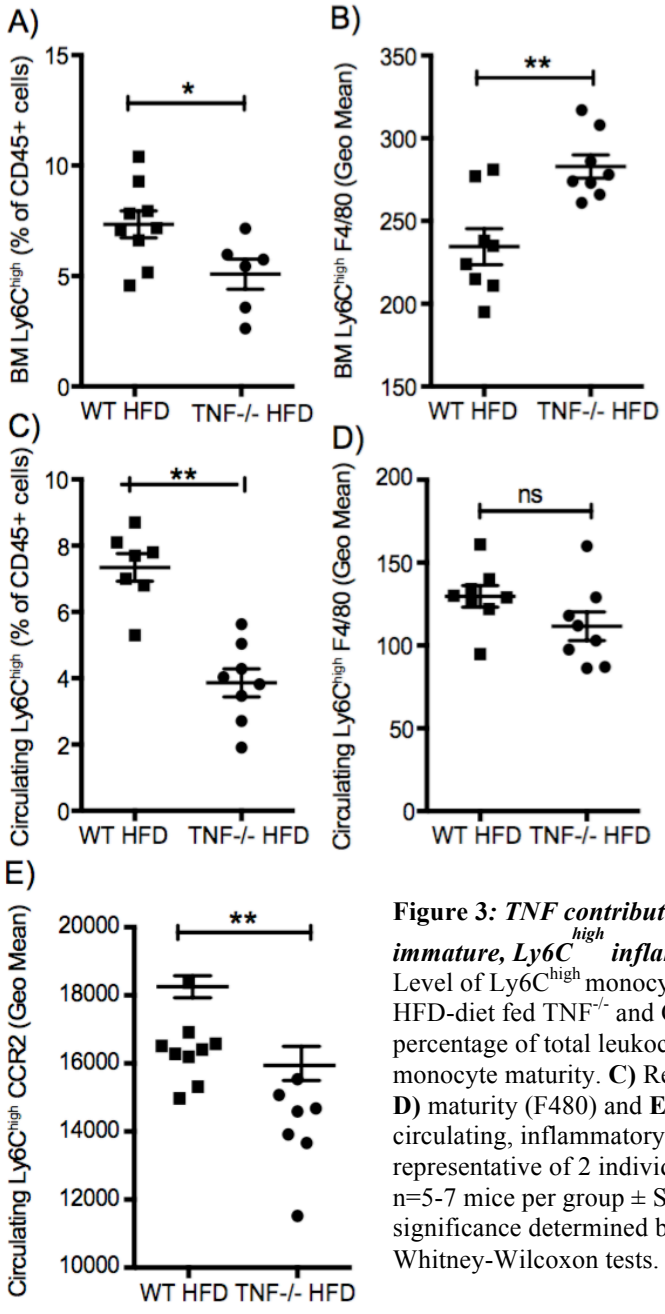

Figure 3*: TNF contributes to the egress of immature, Ly6C^{high} inflammatory monocytes.* Level of Ly6C^{high} monocytes in the bone marrow of HFD-diet fed TNF$^{-/-}$ and C57Bl/6 mice as a **A)** percentage of total leukocytes (CD45+ cells) & **B)** monocyte maturity. **C)** Relative extent (%CD45), **D)** maturity (F480) and **E)** egress (CCR2) of circulating, inflammatory monocytes. Data representative of 2 individual experiments, where n=5-7 mice per group ± SEM. Statistical significance determined by two-tailed Mann-Whitney-Wilcoxon tests. *$p \leq 0.05$, **$p \leq 0.01$.

Circulating inflammatory monocytes predict onset of insulin resistance.

We confirmed that both the adipose tissue and liver accumulated more macrophages after HFD-feeding of mice (Fig. 4). HFD-fed TNF$^{-/-}$ mice had fewer adipose and liver resident

macrophages compared to HFD-fed WT mice, but adipocyte size was similar in all HFD-fed mice (Fig. 4). We next showed that ATM accumulation positively correlated with fasting blood insulin in mice (Fig. 5A). Our results suggest that obesity-related changes on monocytes occur in the bone marrow niche. These monocytes then transit through the blood and precipitate inflammatory effects in metabolic tissues. We hypothesized that the percentage and maturity of circulating Ly6C^{high} monocytes would correlate with the extent of insulin resistance and hyperinsulinemia. Indeed, the quantities of blood Ly6C^{high} monocytes were positively correlated with fasting blood insulin and HOMA-IR, an index of insulin resistance in mice (Fig. 5B-C). In fact, the number of circulating Ly6C^{high} monocytes explained ~74% of the variance in insulin and HOMA-IR (Fig. 5B-C). In addition, Ly6C^{high} monocyte maturity (defined by F4/80 levels) was negatively correlated with fasting blood insulin (Fig. 5D). We hypothesized that these circulating monocyte characteristics would correlate with insulin better than the level of obesity defined by adipose tissue mass or adipocyte size, defined by cell cross-sectional area (CSA). Indeed, the epididymal adipose tissue mass and adipocyte CSA did not correlate with fasting blood insulin (Fig. 5E-F).

This prompted us to investigate analogous inflammatory monocyte characterises in humans. Human monocytes can be categorized based on the levels of CD14 and CD16. It has been shown that obesity coincides with increased circulating non-classical and intermediate monocytes, as opposed to classical monocytes[24,25]. However, little is known about human monocyte characteristics that relate to insulin levels. Given our results in mice showing that Ly6C^{high} monocytes correlated better with insulin compared to

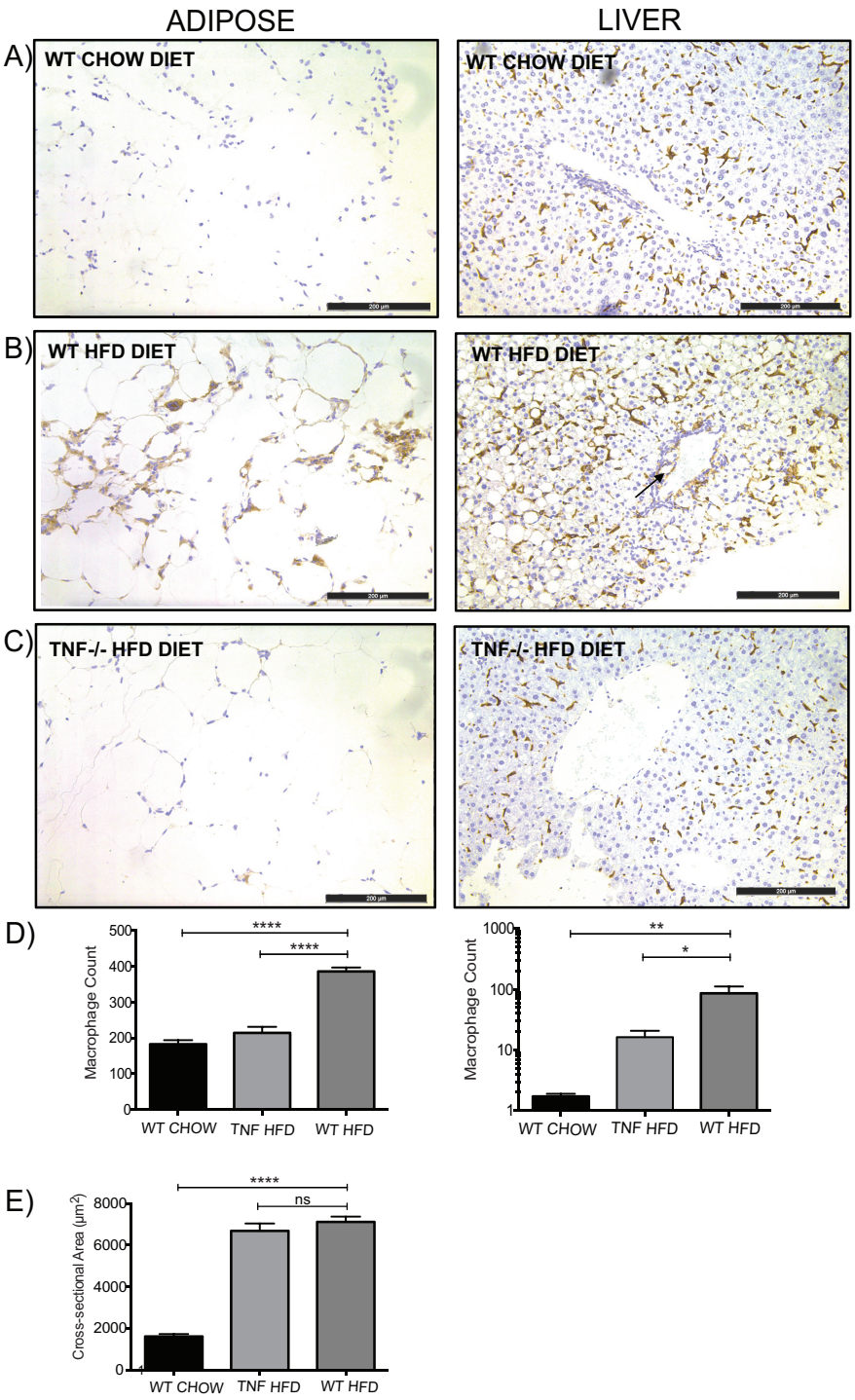

Figure 4: ***HFD-induced macrophage accumulation in adipose tissue and liver are attenuated in TNF$^{-/-}$ mice.*** Representative photomicrographs (**A-C**) of epididymal F4/80 immunohistochemistry in liver and epididymal adipose tissue. **D)** Quantification of macrophages was based on F4/80-positive cells using 10 images, where counts for were averaged per individual mouse. **E)** Cross-sectional areas of adipocytes were quantified using ImageJ, where 300 adipocytes per mouse were measured. Data representative of 2 individual experiments, with n=5 mice per group ± SEM. Statistics performed using one-way ANOVA with the Fisher's LSD posttest. *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$.

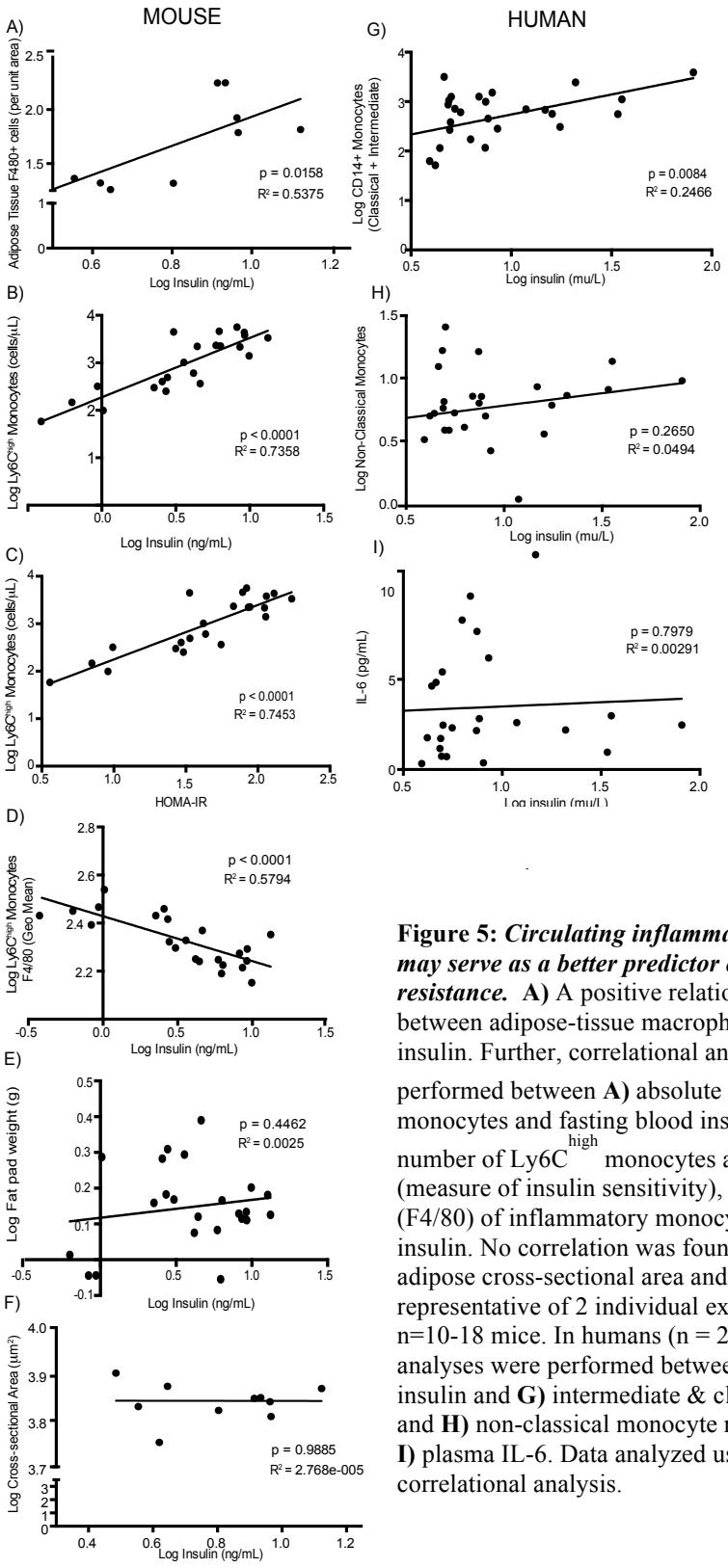

Figure 5: ***Circulating inflammatory monocytes may serve as a better predictor of insulin resistance.*** **A)** A positive relationship was observed between adipose-tissue macrophages and fasting insulin. Further, correlational analyses were performed between **A)** absolute number of Ly6C^{high} monocytes and fasting blood insulin, **B)** absolute number of Ly6C^{high} monocytes and HOMA-IR (measure of insulin sensitivity), and **C)** maturity (F4/80) of inflammatory monocytes and blood insulin. No correlation was found between **E)** adipose cross-sectional area and insulin. Data is representative of 2 individual experiments, with n=10-18 mice. In humans (n = 27), correlational analyses were performed between random-fed insulin and **G)** intermediate & classical monocytes, and **H)** non-classical monocyte numbers, as well as **I)** plasma IL-6. Data analyzed using Pearson's correlational analysis.

adiposity/obesity, we hypothesized the human monocyte populations that more closely resemble Ly6C^{high} monocytes (i.e. intermediate and classical monocytes[26]) would correlate with insulin levels. We found that both intermediate and classical monocyte numbers correlated with random-fed insulin levels in humans (Fig. 5G). Non-classical monocyte numbers did not correlate with insulin levels (Fig. 5H). In fact, classical and intermediate monocyte characteristics correlated with insulin levels even though circulating IL-6 concentrations did not correlate with insulin levels (Fig. 5I).

CONCLUSION

We demonstrate that obesity influences egress of immature, inflammatory Ly6C^{high} monocytes from the bone marrow into the circulation. This corresponds with an accumulation of macrophages in liver and adipose tissues. TNF is known to promote insulin resistance, but we expand on the pleiotropic role of this pro-inflammatory cytokine by demonstrating its involvement in driving inflammatory monocyte egress during obesity. Comparison of blood monocyte phenotype and circulating insulin levels demonstrated that immature, inflammatory Ly6C^{high} monocytes were better predictors of circulating insulin when compared to the extent of adiposity. Further, circulating levels of classical and intermediate monocyte subsets in humans correlated with insulin levels. Our results suggest that inflammatory monocyte characteristics could serve as predictive biomarkers of type 2 diabetes risk and may represent a mechanism linking immunity and regulation of insulin.

ACKNOWLEDGMENTS

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Disclosures:

The authors have no financial conflicts of interest.

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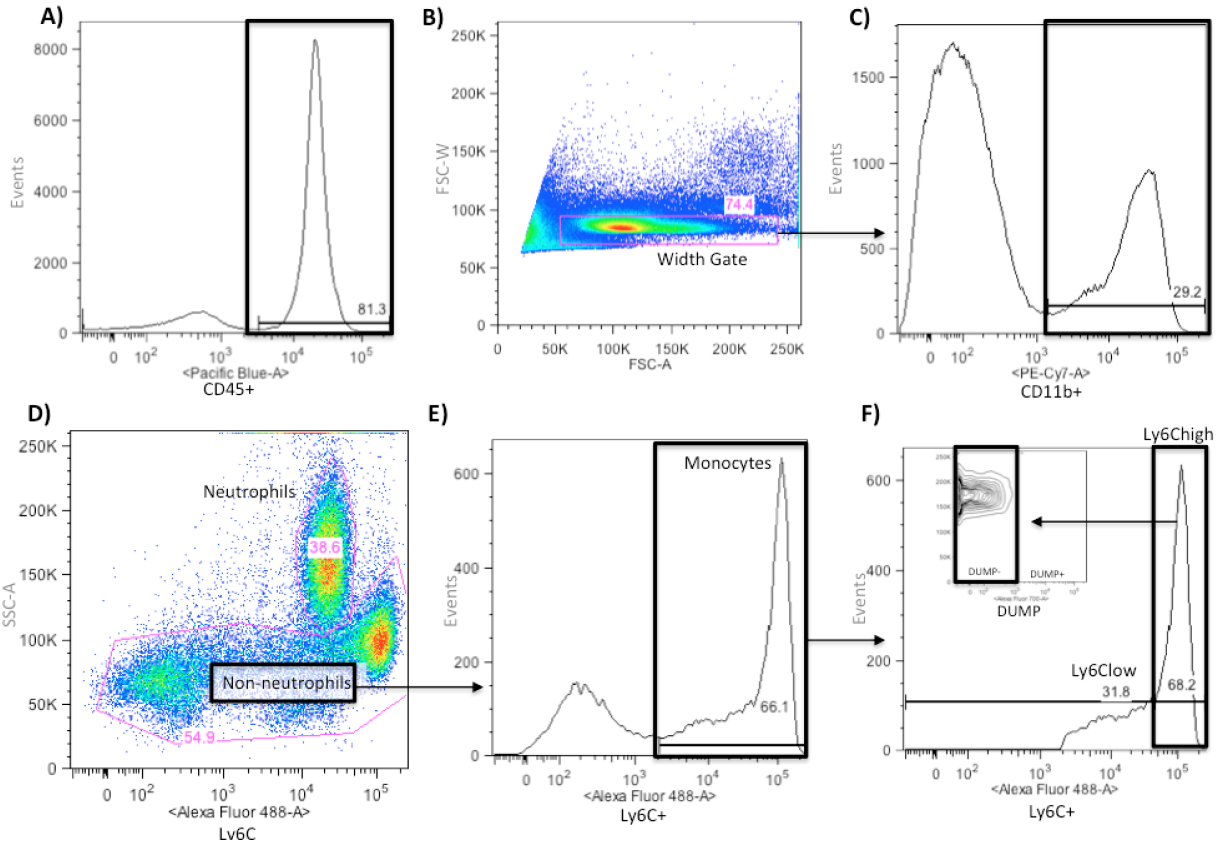
SUPPLEMENTARY



Supplementary Figure 1: ***Flow cytometry gating strategy for Ly6C^{high} monocytes.*** To gate on Ly6C^{high} monocytes (bone marrow-resident and circulating) first **A)** CD45+ cells (leukocytes) are gated. Subsequently, a **B)** width gate is created to exclude cell aggregates, and **C)** CD11b+ cells are selected. Using this population, cells can be divided into **D)** neutrophils and non-neutrophil using SSC and Ly6C surface expression. **E)** Monocytes are gated as Ly6C^{+}/SSC^{low} cells, allowing definition of **F)** Ly6C^{high} monocytes. Using a dump gate positive for NK1.1, CD19, and CD3, it is apparent that no NK cells, B cells or T cells are found in this population.

CHAPTER 5: Identification of the Etiology Behind Inflammaging.

Declaration of Academic Achievement:

This research was conducted during the period of August 2013 to March 2014. I pioneered the bioassays, performed the majority experiments, and analyzed and interpreted the data. Dr. C. Verschoor performed the human monocyte immunophenotyping required to generate the correlative plots. Dr. Z. Xing generously provided the TNF KO mice, and Dr. J. Schertzer kindly gave us the HEK293T/mNod2 cell line as a gift. Experiments were conceived and designed by Dr. D.M.E. Bowdish

INTRODUCTION

Increased levels of circulating, pro-inflammatory cytokines (e.g. TNF, IL-6, IL-1β) accompany the aging process[1], however, the cause of this cytokine production remains poorly understood. Inflammaging is postulated to be a consequence of the cumulative lifetime exposure to an unknown antigen load, which results in the continuous stimulation of immune cells[1]. The result of the chronic stimulation is a low-grade inflammatory response, which involves tissue damage and release of oxygen radicals, thereby leading to the further release of additional cytokines. A vicious cycle that drives immune system remodeling results and perpetuates a chronic inflammatory state[2–4]. Identifying the source triggering inflammaging will allow for a better understanding of the progression of, and ultimately, circumvent, the adverse health outcomes concomitant with age-associated inflammation.

Age-associated inflammation, in multiple ways, parallels the state of low-grade inflammation observed in HIV-infected individuals. The inflammatory state associated with HIV is attributed to the translocation of bacteria, or their components, across the mucosal epithelium into circulation[5]. HIV is characterized by the rapid depletion of CD4 T-cells early in infection that is primarily localized to the gastrointestinal tract[6–8], leading to an early breach in mucosal barrier integrity[9]. Breakdowns in intestinal epithelial integrity favour microbial translocation from the intestinal lumen into the systemic circulation, where these products (e.g. LPS) activate various innate immune pathways and exert a sustained pro-inflammatory effect[10–12]. Based on these findings and studies by Ma et al. demonstrating a deterioration of intestinal barrier function with age, elevated levels

of bacterial products in the blood could contribute, in part, to age-associated inflammation. Though various assays that measure intestinal permeability using blood currently exist, these assays largely depend on detection of antibodies against specific microbial antigens originating from the gut lumen, such as anti-laminaribioside (ALCA), and anti-outer membrane porin C (OmpC) antibodies[13]. These assays are highly specific for certain species and would likely only capture a fraction of the total circulating bacterial products. Thus, *in lieu* of these assays, we decided to investigate the presence of bacterial products that are broadly expressed by gram-positive and gram-negative bacteria in blood.

To support the hypothesis that increased microbial translocation contributes to age-associated inflammation, high-sensitivity, novel bioassays to detect muramyl dipeptide (MDP), a peptidoglycan constituent present in both Gram-positive and Gram-negative bacteria, and lipopolysaccharide, a major component of the outer membrane of Gram-negative bacteria, were developed[14,15]. MDP and LPS are recognized by the NOD2 receptor and TLR4/CD14/MD2 receptor complex, respectively[14,15]. LPS binds to LPS Binding Protein (LBP), which delivers LPS to CD14, an anchored protein on the cell surface without a transmembrane domain that signals through TLR4[15]. Using cell lines expressing these receptors, along with a NFκB reporter, we assessed the presence bacterial products in circulation. Although bacterial products can be quantitated using the standard silkworm larvae plasma (SLP) test and limulus amoebocyte lysate (LAL) assay, these tests do not provide the necessary sensitivity for our experimental model. Furthermore, the LAL assay cannot detect Gram-positive bacteria, thereby limiting the

scope of bacterial product detection[16]. Conversely, the SLP test can only be used in cases of severe bacterial infections; in healthy subjects, the amount of peptidoglycan is below the level of detection[16].

In this chapter, we demonstrate that aged mice (>18mo) have significantly higher levels of circulating bacterial products when compared to the young (10-12wks), and these differences are paralleled by a loss of gut barrier integrity. Further, mice protected from age-associated inflammation (e.g. TNF knockouts) and mice lacking microbiota (e.g. germ-free) do not demonstrate these age-associated changes. When human samples were assessed, a similar increase in circulating bacterial products (CBPs) was observed in old compared to young adults. Further, levels of bacterial products in plasma correlated with increased systemic inflammation and mortality in the elderly.

METHODS

***Assessment of Circulating Bacterial* Products.** To quantify the circulating levels of bacterial products, we developed and optimized a novel and sensitive reporter cell line used to detect the presence of MDP via intracellular receptor NOD2[14]. Low passage HEK293T cells were stably transfected with mNOD2 (a kind gift from Dr. Jonathan Schertzer) and pNifty2-SEAP plasmids (Invivogen) plasmids. The latter plasmid is composed of genes encoding an ELAM proximal promoter, 5 NF-κB repeated transcription factor binding sites (TFBS) and the SEAP reporter gene. Upon recognition of the NOD2 agonist, MDP, a signal transduction is induced, leading to the activation of NFκB, a transcriptional regulator of inflammation[14], and subsequent expression and

expression and release of SEAP (secreted embryonic alkaline phosphatase) into the surrounding media. SEAP is a form of human placental alkaline phosphatase (PLAP) in which the GPI anchor is deleted. Unlike intracellular reporters, SEAP is secreted into the cell culture supernatant, allowing for easy detection without adverse effects on the cell. The lipopolysaccharide (LPS) detection assay was created in the same manner using low passage HEK293 cells transfected to stably express TLR4, MD2 & CD14 on the cell surface (Invivogen), which were later transiently transfected with the SEAP reporter. The stable cell line was passaged in Dulbecco's Modified Eagle Media (DMEM), supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 mg/ml), and 10 mM L-glutamine, and maintained under constant antibiotic selection (25 μg/mL zeocin & 50 μg/mL blasticidin).

To assess circulating MDP and LPS in mouse plasma, 96-well plates were seeded with 4000 cells/well 24 hours prior to addition of heat-inactivated mouse plasma (plasma diluted 1:5 in PBS, then 1:1 in endotoxin-free water [1:10 final dilution], followed by heat inactivation at 75℃ for 5 min). Heat-inactivated mouse plasma (10 μL; 30 μL for human plasma) was added to HEK Blue Detection Media (Invivogen) to a final volume of 200 μL. The HEK Blue Detection Media consists of a SEAP substrate. In the presence of alkaline phosphatase activity, the media undergoes a colour change, allowing for quantification via colourimetric analysis. After 24hrs, the colourimetric changes were quantified at 630 nm via absorbance spectroscopy. Levels of bacterial products were assessed in young (10wk) and old (22mo) mice. In mouse experiments, germ-free (GNU) plasma was used as a negative control (*GNU data not shown)* and purified bacterial

products (MDP, LPS) were used as positive controls. TNF knockout (TNFKO) mice were included since these mice do not undergo age-associated inflammation. Levels of circulating bacterial products were quantified in the plasma of young adults (>45yrs) and community dwelling elderly (CDE; >65yrs). Doses responses were performed for the MDP (0-5 μg/mL) and LPS (0-100 ng/mL) assays to ensure that bacterial product values fell within the range of detection and to allow interpolation of concentrations from absorbance values.

FITC-Dextran Assessment of Intestinal Permeability. To non-terminally evaluate intestinal permeability *in vivo*, young and old mice were orally gavaged with 200 μl of FITC-labeled dextran (3-5 kD) at 0.8 mg/mL (Sigma-Aldrich). Food was removed 4 hrs prior to gavage, and both food and water were removed for 4 hrs following gavage. Dextran of 3-5kD was chosen to allow for translocation across the epithelial layer from the gastrointestinal lumen into the lamina propria[17]. The purpose of withholding food and water was to remove the effects of consumption on intestinal permeability. After the 4 hrs, we collected blood retro-orbitally, and added 50 μL of the sample to 50 μL of sterile PBS. Immediately after, we measured fluorescence using a spectrofluorometric plate reader (excitation at 403nm and emission at 518nm).

Participants and Cytokine Quantification. Advanced-age, frail elderly participants (n=135, median age=88 years, range 68–99, male to female ratio 21:114) were recruited from 5 nursing homes in Hamilton, Ontario, between November 2010 and January 2011. Individuals on immunosuppressive medication, chemotherapy or undergoing radiation therapy were excluded. A sample of 35 young adults (19–45 years old) was also recruited

in the same time frame. The McMaster Research Ethics Board approved these studies, and written informed consent was obtained for all participants. Cytokines were quantified in cryopreserved plasma by CP Verschoor from heparinized venous blood using the Milliplex High Sensitivity Human Cytokine Kit (Millipore, MA, USA)

Human Monocyte Immunophenotyping. Monocyte immunophenotyping was performed by CP Verschoor by flow cytometry as published[18]. Monoclonal antibodies with the following specificities were used in this study: CD2-PE, CD3-PE, CD19-PE, CD56-PE, NKp46-PE, CD15-PE, CD14-APC-Alexa750, CD16-PE-Cy7, HLADR-PerCp-Cy5.5, CD45-eFluor605NC. Monocytes were defined as lineage (CD2, CD3, CD19, CD56, NKp46, CD15) negative, and CD45, HLADR, and CD14 positive. Classical monocytes were defined as CD16 negative, whereas intermediate and non-classical monocytes were defined as CD16 positive[19]. Absolute counts were measured using 123count beads (eBioscience).

RESULTS

Aging is associated with elevated circulating bacterial products and increased intestinal permeability. Old mice (>18mo) have significantly higher levels of circulating MDP than young mice, most of which did not have any detectable MDP (Fig. 1A). TNF knockout mice were included since TNF is both a key pro-inflammatory cytokine that increases with age and has a well-supported role in inducing intestinal epithelial tight junction permeability[20]. Strikingly, circulating MDP did not increase with age in old TNFKO mice. Similarly, young and old WT plasma was assed using the LPS detection assay.

While a general trend of increased levels of the microbial products in the old age cohorts versus the young existed and was repeatedly observed (in 5 independent experiments), differences between groups were not significant (Fig. 1C).

Consisting with our findings that circulating MDP is increased with age, we observed old wildtype mice to have increased intestinal permeability compared to young mice. Our data demonstrate that aging is associated with compromised intestinal barrier integrity, as determined by increased (FITC-dextran) fluorescence in the blood of old mice orally gavaged with FITC-Dextran, compared to young mice (Fig. 1B). Further, mice protected from age-associated inflammation (e.g. old TNF knockouts) and germ-free (GF) mice do not demonstrate a loss of gut integrity with age, suggesting that both TNF and the presence of microbiota contributes to increased age-associated intestinal permeability.

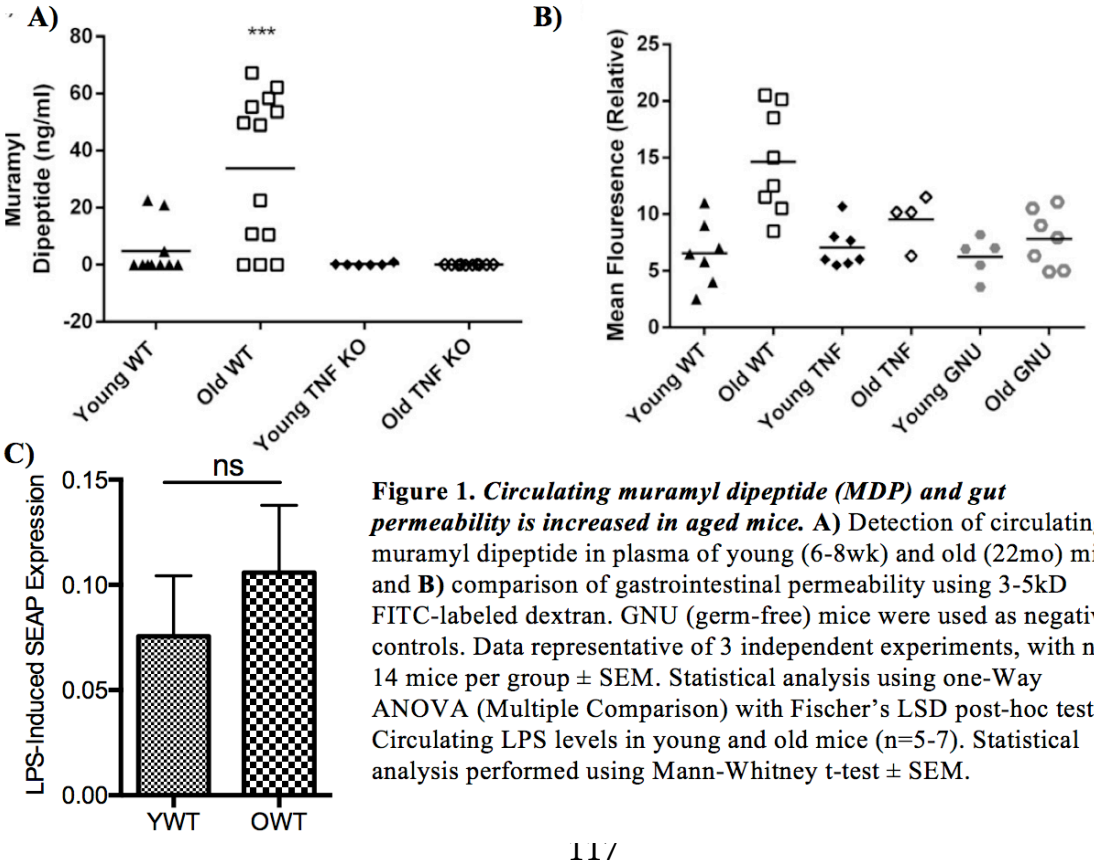


Figure 1. ***Circulating muramyl dipeptide (MDP) and gut permeability is increased in aged mice.*** **A)** Detection of circulating muramyl dipeptide in plasma of young (6-8wk) and old (22mo) mice and **B)** comparison of gastrointestinal permeability using 3-5kD FITC-labeled dextran. GNU (germ-free) mice were used as negative controls. Data representative of 3 independent experiments, with n=5-14 mice per group ± SEM. Statistical analysis using one-Way ANOVA (Multiple Comparison) with Fischer's LSD post-hoc test. **C)** Circulating LPS levels in young and old mice (n=5-7). Statistical analysis performed using Mann-Whitney t-test ± SEM.

Complementary studies by Dr. A. Puchta and K. Lee demonstrated using transcriptional profile analysis that a significant downregulation of gene expression associated with tight junction regulation, cell adhesion, and adhesion junction integrity in the colons of old mice (*data unpublished*). Hence, increased permeability of the colon of old mice, which may be attributed to decreased expression of genes responsible for regulating intestinal integrity, allows for increased translocation of microbial products into the systemic circulation of the host.

HUMAN STUDIES

Old adults have higher levels of muramyl dipeptide (MDP) in circulation. We quantified circulating levels of MDP in human plasma. It was apparent that a greater proportion of the young cohort (<45yrs) had lower levels of MDP, when compared to the MDP distribution among the elderly (>65yrs) (**Fig. 2A**). Conversely, a large proportion of old individuals had very high levels of circulating MDP, with ~30% having over 50 ng/mL. Further, we observed significantly higher circulating MDP levels in older adults, when compared to younger counterparts (**Fig. 2B**). Interestingly, we found that the distributions of MDP in old humans and old mice (**Fig. 2C**) tend to cluster into 2 groups – a high and a low.

Plasma MDP correlates with circulating inflammatory cytokines. In support of our hypothesis that microbial translocation contributes to increased inflammation with age,

plasma levels of MDP negatively correlated with circulating IL-10 (**Fig. 3A**), an anti-inflammatory cytokine, and positively correlated with IL-12p70 (**Fig. 3B**), a pro-inflammatory cytokine produced largely by monocytes and macrophages[21]. Further, we found a statistically significant correlation (p=0.0008) between MDP levels in the elderly & the Barthel score, a measure of frailty, where a higher level of MDP was associated with a low score (**Fig. 3C**). The Barthel score includes ten parameters which assess performance in activities of daily living and mobility[22,23]. A higher number (up to 100) is associated with a greater likelihood of being able to live at home with some degree of independence[24]. Together, these data suggest that MDP associates with increased systemic inflammation and poor health outcomes in the elderly.

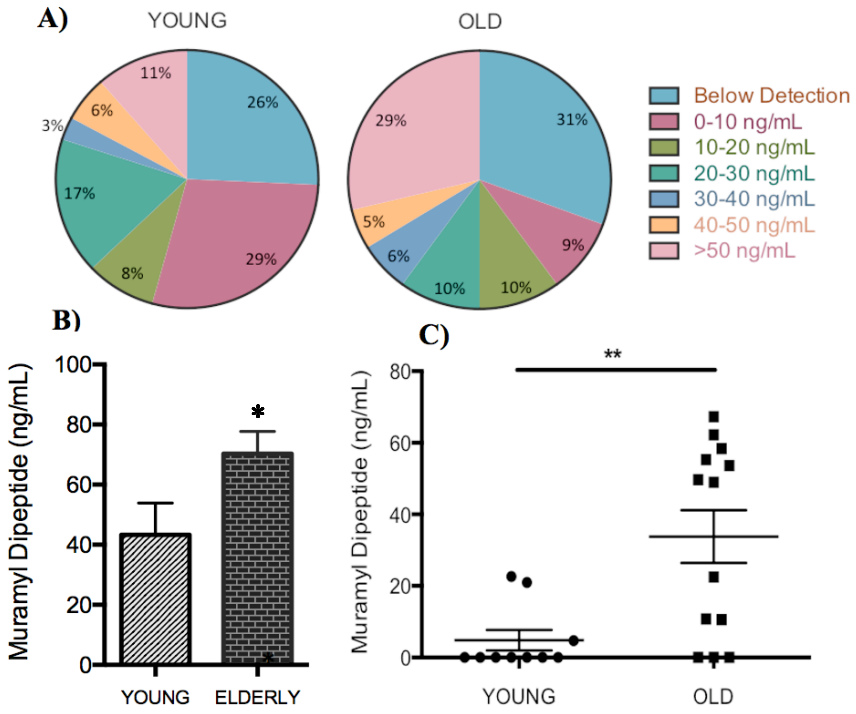


Figure 2. ***Circulating muramyl dipeptide (MDP) levels in old humans parallels mouse data.*** **A)** Distribution of muramyl dipeptide in plasma in human young (<45yr, n=35) and old (>65yr, n=160) age cohorts. **B)** Elderly humans (>65yr, n=80) have higher plasma levels of MDP than young adults (<45yr, n=35). **C)** MDP levels in old mice cluster into high and low groups, mirroring human data. Data representative of 3 independent experiments, ± SEM.

Plasma lipopolysaccharide (LPS) both positively correlates with circulating IL-12 and induces production of IL-12 in mononuclear phagocytes. We quantified circulating levels of LPS in human plasma. Like MDP, LPS positively correlated with IL-12 (**Fig. 4B**), a pro-inflammatory cytokine produced largely by monocytes and macrophages[21]. Further, when whole blood was stimulated with LPS, a dose-dependent increase in IL-12 production was observed in monocytes and dendritic cells (**Fig. 4B**; performed by Dr. C. Verschoor). These data suggest that increased bacterial products in the elderly induce mononuclear phagocytes to produce higher levels of inflammatory cytokines, which may contribute to increased systemic inflammation.

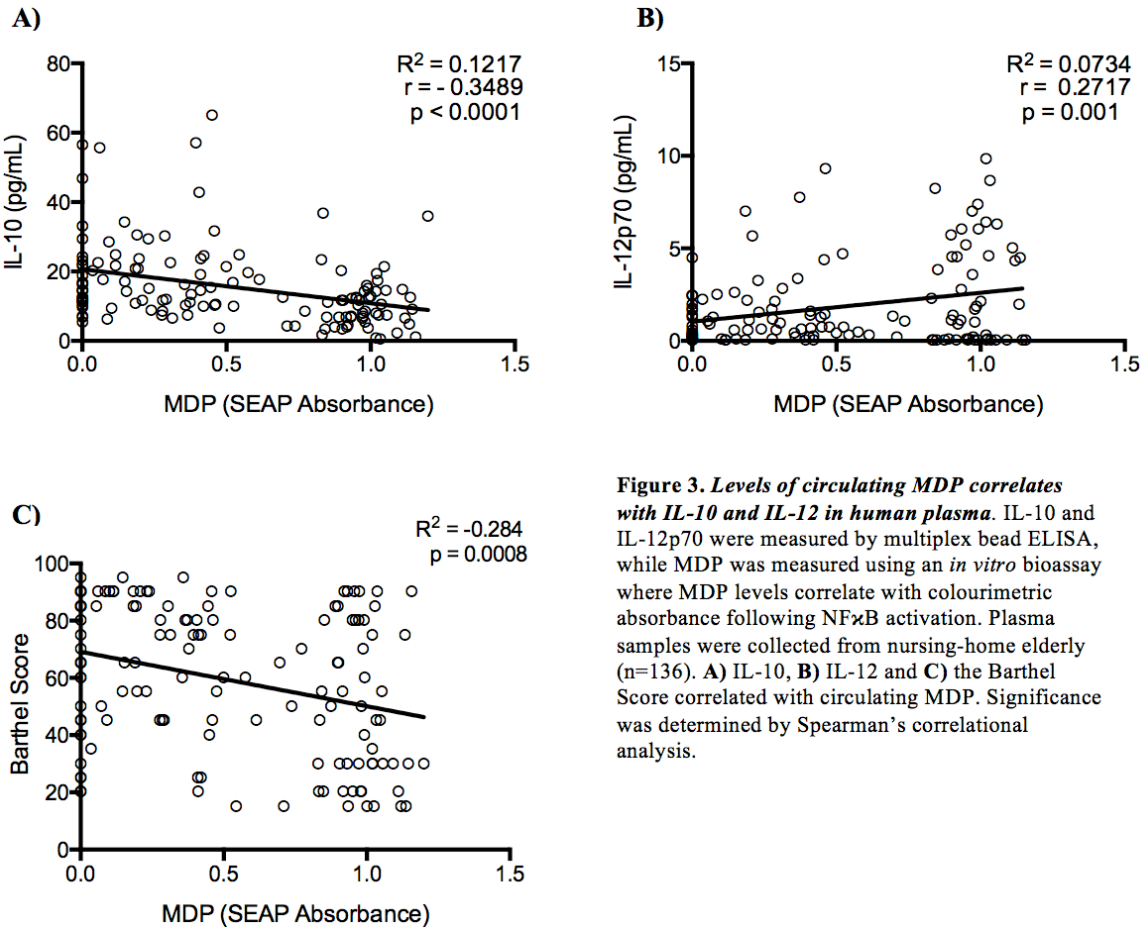


Figure 3. ***Levels of circulating MDP correlates with IL-10 and IL-12 in human plasma.*** IL-10 and IL-12p70 were measured by multiplex bead ELISA, while MDP was measured using an *in vitro* bioassay where MDP levels correlate with colourimetric absorbance following NFκB activation. Plasma samples were collected from nursing-home elderly (n=136). **A)** IL-10, **B)** IL-12 and **C)** the Barthel Score correlated with circulating MDP. Significance was determined by Spearman's correlational analysis.

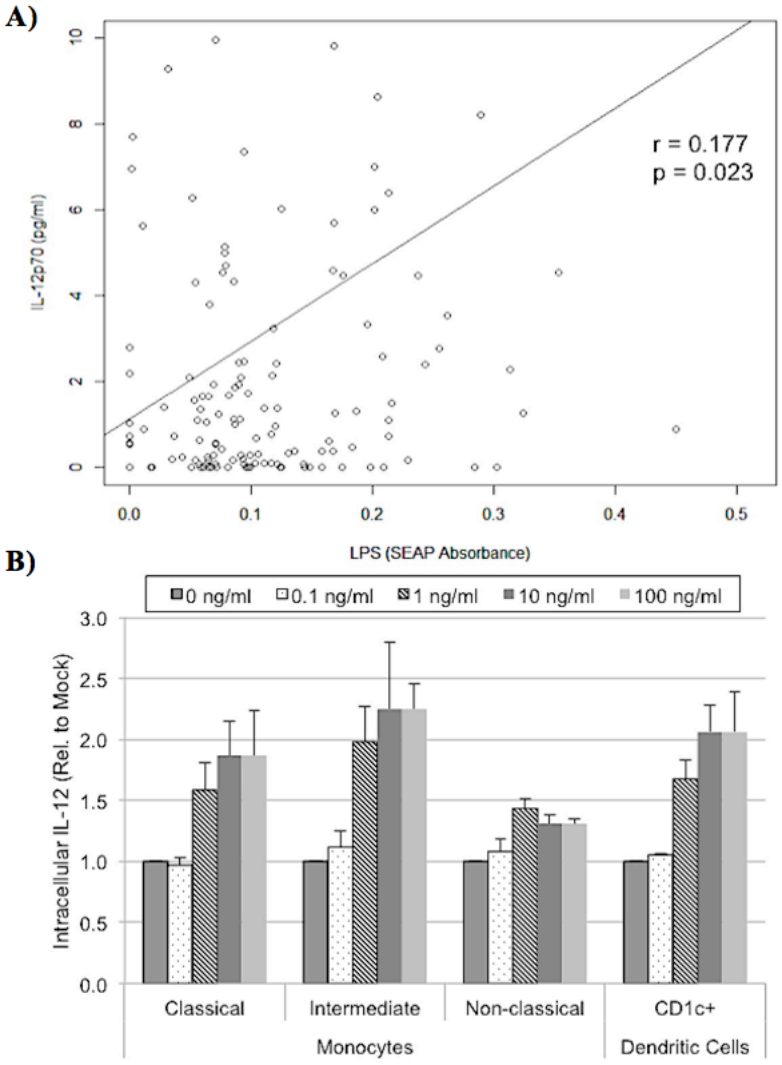


Figure 4: ***LPS positively correlates plasma IL-12 levels, and induces IL-12 production in peripheral blood monocytes and dendritic cells in vitro.*** **A)** A correlation was observed between plasma LPS and IL-12. **B)** The production of intracellular IL-12 in peripheral blood classical ($CD14^{++}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{+}$) monocytes and CD1c+ myeloid dendritic cells in response to LPS was determined by flow cytometry (n=136). Correlations (r) represent Pearson's product-moment correlation coefficient, and significance (p) determined by linear regression, fitting all log-transformed cytokine measurements, and adjusting for age and sex.

LPS Associates with Age when Adjusted for One-Year Mortality. No correlation was observed between circulating LPS levels and age within the elderly cohort, as indirectly determined by SEAP expression (**Fig. 5A**). However, we attained a statistically significant correlation when one-year mortality was taken into account (**Fig. 5B**). One-year mortality refers to individuals who died within 1 year of blood collection.

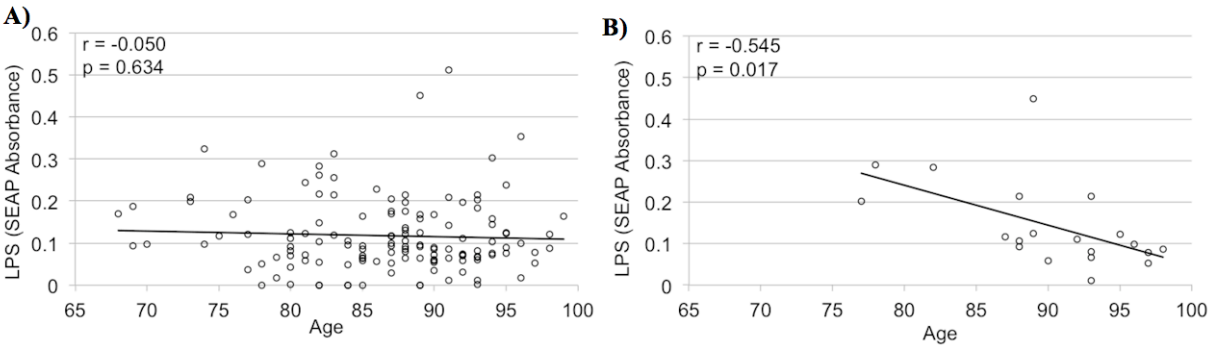


Figure 5: ***LPS associates with age when adjusted for one-year mortality.*** The relationship between plasma LPS levels and age were compared in **A)** all donors (n=136) and **B)** those that passed away within one year of sampling (n=22). Correlations (r) represent Pearson's product-moment correlation coefficient, and significance (p) determined by linear regression on log-transformed values, adjusting for sex.

DISCUSSION

Aging is accompanied by increasing levels of pro-inflammatory cytokines in the serum and changes in the phenotype and function of leukocytes. This "inflamm-aging" is believed to contribute to the wide spectrum of diseases associated with aging (e.g. cardiovascular disease, diabetes, dementia) that have an inflammatory etiology. However, the cause of increasing levels of inflammatory cytokines with age is not known. In a study by Ma et al., researchers concluded that intestinal permeability increases with age. Further, it has been shown that breakdowns in epithelial integrity promote translocation of microbial products from the intestinal lumen into the systemic circulation, where these products activate various innate immune pathways and exert a sustained pro-inflammatory effect[14]. Consequently, we hypothesized that increased circulating levels of bacterial products (e.g. MDP and LPS) increases with age. Chronic exposure to bacterial components could explain the increased level of inflammation[1,4,25] and reduced responsiveness to bacterial products (e.g. downregulation of TLRs)[26,27] that occurs with age. To validate this hypothesis, we established novel and sensitive bioassays to

quantitate circulating microbial components such as LPS (lipopolysaccharide) and MDP (muramyl dipeptide). Using these assays, we demonstrate that aged mice and humans had significantly higher quantities of microbial products, which parallel changes observed in intestinal permeability. Further, in humans, these circulating bacterial products correlate with pro-inflammatory cytokine IL-12 (LPS & MDP) and negatively correlate with anti-inflammatory cytokine IL-10 (MDP). Therefore, leakage of these bacterial constituents from the gut lumen may contribute to the inflammatory state that occurs with age. However, since these associations are correlative, we cannot yet establish causation.

Studies on microbial translocation in HIV-positive patient populations corroborate our findings. Age-associated inflammation, in many ways, parallels the state of low-grade inflammation observed in HIV-infected individuals[5]. HIV-infected individuals suffer from accelerated or premature aging[9]. This is accompanied by an increased burden of age-associated co-morbidities, a shortened lifespan, immune cell fatigue, shortened telomeres in certain cell populations, and low-grade, chronic inflammation[9,28,29]. Accelerated aging in this cohort has been attributed microbial translocation of bacteria, or its components, across the mucosal epithelium, which contributes to a systemic, inflammatory state. HIV is characterized by the rapid depletion of CD4 T-cells with the first few weeks of infection that is primarily localized to the gastrointestinal tract[6–8]. Thus, there's an early breach in mucosal barrier integrity[9]. Breakdowns in intestinal epithelial integrity favour microbial translocation from the intestinal lumen into the systemic circulation, where these products activate various innate immune pathways and exert a sustained pro-inflammatory effect[10–12]. Circulating LPS is significantly increased

in chronically HIV-infected individuals, and correlates with measures of innate and adaptive immune activation[28]. Interestingly, circulating bacterial products do not return to normal levels when T-cells are replenished during antiviral therapy[28]. As such, it is plausible that similar mechanisms may be involved in aging. We show that gut integrity is compromised with age. This finding is consistent with our previous findings that colons from old mice have reduced expression of genes involved in cellular adhesion than young controls. Whether this age-associated increase in gut permeability occurs due to intrinsic changes in immune cells or structural cells of the GI mucosal surface, or whether it is prompted by compositional changes in the gut microbiome is unclear. Our germ-free mouse data, however, suggests that the microbiome does contribute to age-associated changes in intestinal permeability.

Our findings in humans are consistent with our mouse data. Interestingly, both the mouse and human samples clustered into 2 groups – high MDP and low MDP. The etiology behind this divergence is unknown and requires further investigation. However, these findings support that mice can serve as suitable models to reflect age-associated changes in humans. Upon assessment of plasma LPS levels, we observed elevated levels of LPS in the old, which correlated with IL-12. Further, LPS induced IL-12 production in monocytes and macrophages. Prior studies in our laboratory have shown that aged monocytes and macrophages produce hyper-inflammatory responses upon stimulation with LPS[30]. These data suggest that increased bacterial products could contribute to the systemic low-grade inflammation that characterizes old age.

Strikingly, we observed a negative correlation between circulating LPS in human plasma and age, when the variable of 1-year mortality was accounted for. Individuals who have aged successfully (e.g. centenarians), as determined by increased longevity, had lower plasma levels of LPS than those that died at a younger age. We speculate that these individuals had reduced inflammation and consequently, reduced susceptibility to age-associated morbidities. As aforementioned, individuals with higher-than-average levels of systemic inflammation are at an increased risk for chronic disease and infection[31]. Based on these data, circulating LPS could potentially serve as a predictive biomarker of mortality in the elderly.

The etiology of inflammaging may be multifactorial, inclusive of abnormally activated responses to commensal bacteria due to an diminished mucosal immune tolerance, expression of virulence factors by bacteria, breaks in the mucosal barrier, and changes in composition of the gut microbiome[32]. As we age, the intestinal microbiome changes[33]. Differences exists in the composition of microbiota between young and elderly individuals, with the latter demonstrating increases in *Enterobacteriacea* and endotoxin-producing Gram-negative species, decreases in *Ruminococcus* and *Blautia* species, and a reduced abundance of butyrate-producing species[33]. Studies have shown that composition of the microbiota is predictive of gut barrier dysfunction and age-related microbial dysbiosis increases risk of pathogen infection, promotes growth of harmful pathobionts, and contributes to inflammatory disease[34,35]. Further research to elucidate whether inflammaging contributes to microbial dysbiosis, or altered composition of intestinal bacteria contributes to the age-related inflammatory state, is required. Restoring the

homeostasis of the host–microbiota interactions in the elderly, however, could be a way to reduce intestinal inflammation and function, and in turn, decrease the systemic inflammatory status. Thus far, prebiotics, probiotics and synbiotics have shown some capacity to modulate microbiota and correct inflammatory conditions in the elderly[36]. An improved understanding of the mechanisms underlying host-intestinal microbiota cross-talk would aid in the design of novel nutritional strategies to reduce age-associated inflammation.

CONCLUSION

Herein, we attempt to resolve a major disparity in the field by identifying circulating bacterial products as a potential source of inflammaging. We demonstrate that aged mice and humans exhibit significantly higher quantities of microbial products (e.g. LPS and MDP), which is accompanied by changes in intestinal permeability, thus proposing these products as contributors to age-associated inflammation. As populations age, a better understanding of these processes associated with inflammation is essential in identifying older people who are at risk of aging-related chronic diseases, in order to facilitate successful, early, targeted interventions.

To address and validate our hypothesis, we pioneered novel and high-sensitivity bioassays to detect specific bacterial products, such as MDP and LPS, in the circulation of mice and humans. Prior to this establishment, limited experimental tools could reliably quantitate bacterial constituents in the plasma of healthy, non-septic individuals. In addition to aging research, these innovative assays have proven to be applicable in other

models of chronic inflammation (e.g. obesity, ulcerative colitis, celiac disease, autism spectrum disorder), where translocation of bacterial products may play a causative role. Given the reliability, sensitivity, and specificity of the assay and the minute sample volume required, these valuable experimental tools have practical applications in numerous models of disease.

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CHAPTER 6: The Role of TNF on Muscle Integrity with Age

Declaration of Academic Achievement:

This research was conducted during the period of January 2014 to June 2015. I performed the experiments and analyzed the data, with the assistance Jun Lu from the lab of Dr. P. Bercik, who conducted the behavioural assessments.

INTRODUCTION

Aging is characterized by a predictable increase in pro-inflammatory cytokines, resulting in a state of chronic inflammation. Increased levels of circulating cytokines, such as TNF, serve as strong independent risk factors for morbidity and mortality in older people[1]. Tumor necrosis factor (TNF) is a well-known potent inflammatory cytokine that exerts its pleiotropic effects on a wide array of immunological and metabolic processes. Although many age-associated diseases, such as sarcopenia, neurodegeneration, type II diabetes and cardiovascular disease, have been linked to elevated levels of TNF[2–5], the causative role is of TNF is incompletely understood. In this chapter, we characterize the role of TNF on the age-associated deterioration of muscle strength in mice using behavioural studies to assess functional impairments.

Sarcopenia, or the degenerative age-related loss of muscle mass and strength, is a major risk factor for physical disability and frailty in older adults[6]. Severe muscle loss is widespread in the elderly[7]; the prevalence of sarcopenia in individuals aged 70 or older is approximately 40% [8]. Sarcopenia is operationally defined as muscle mass corrected for height being 2 standard deviations below the level of healthy young persons[7]. This loss of skeletal muscle mass beyond this critical threshold contributes to functional impairments, resulting in a reduced quality of life, loss of independence, an increased need for hospital and long-term care, and premature death[9,10]. Sarcopenia imposes a significant economic burden on healthcare. In the US, $19 billion in direct healthcare costs was attributed to sarcopenia in 2000[11]. Furthermore, sarcopenia is associated with multiple comorbidities including osteoporosis[12], obesity[13], and type II diabetes[14]. Given the growing number of

older adults, the personal and public costs associated with sarcopenia will escalate unless research aimed at reducing the occurrence of sarcopenia is implemented[11]. The pathological mechanisms underlying this age-associated muscle wasting is incompletely understood[15]. Many factors have been proposed to contribute to sarcopenia[16,17], inclusive of an age-related loss of motor neurons[18], a decline in steroid hormones[19,20], a decrease in dietary protein[21], and a reduced level of physical activity[22]. Increased levels of pro-inflammatory cytokine, tumour necrosis factor (TNF), has been postulated to contribute to the development of sarcopenia, however the primary data is limited[23–25]. Although previous studies have assessed the impact of TNF on muscle catabolism, we used a novel approach to evaluate how TNF contributes to the age-associated deterioration of muscle strength in mice by using behavioural studies to assess functional impairments. We hypothesized that TNF knockout mice would maintain their muscle strength and mobility with age, unlike their wildtype counterparts. Using a battery of behavioural tests to assess muscle strength, locomotor activity and mobility, we demonstrate that TNF drives age-related defects in muscle function and mobility with age.

In addition to our studies on sarcopenia and mobility, we investigated the impact of pleiotropic cytokine TNF on age-related impairments in behaviour and cognition. In humans, increased levels of circulating TNF has been associated with the onset of mild cognitive impairment (MCI) and Alzheimer's Disease (AD)[26]. AD is a progressive, neurodegenerative disorder, associated with cognitive, memory and behavioural impairments[27], and by 2050, is expected to affect over 150 million people[28]. Persons clinically diagnosed with MCI progress to dementia at a an accelerated rate compared

with healthy age-matched individuals[29]. Using a behavioural assessment known as the novel object recognition test, we examined whether aged mice demonstrate cognitive deficits and whether recognition memory could be rescued in mice deficient in TNF. Although TNF was not found to drive memory impairments, we demonstrate age-related cognitive defects in aged C57Bl/6 mice.

METHODOLOGY

Cage Hang Testing. The cage hang test is a broadly used assessment that evaluates motor function and deficit in rodent models of central nervous system (CNS) and skeletal muscle disorders & serves as a measure of muscle strength[30]. The test consists of placing mice on elevated wire cage top, then inverting and suspending top above a padded cage. The latency to when the mouse falls is recorded. Individual mouse performance for each session is presented as the average of three trials.

Open Field Testing. Locomotor deficits, such as muscle wasting, decreased muscle mass, and stiffness in joints and ligaments, often accompany aging[31]. To assess locomotor activity[32–36] and willingness to explore[37], an open field test (OFT) was performed on young and old wildtype and TNF knockout mice. Although open field tests were originally designed to evaluate anxiety-like behaviour in rodent models[38,39], this behavioural assessment has been increasingly utilized to reliably evaluate locomotor activity[32–37]. A lack of movement of mice to the peripheral region when placed in the centre of the field may be indicative of either motor impairment or weakness[40] or anxiety-like behaviour[40]. During testing, individual mice were placed in an arena and allowed to

freely move for 10 min. Mouse movement was measured using a fully automated system (arena 27.3 × 27.3 cm; Med Associates Inc., St. Albans, VT, USA) with parameters including total-distance traveled, velocity & time spent in pre-defined zones[38]. All experiments were conducted in a quiet, well-lit room, with one handler throughout[41].

Novel Object Recognition Test. Aging mice may suffer from progressive deficits in learning and memory. As a common assessment used to evaluate cognition in murine models, particularly recognition memory, the novel object recognition test serves as readout for cognitive impairment. The novel object test represents a test for dorsal hippocampal function based on the tendency of mice to investigate a novel object, rather than a familiar one[42]. The test consists of a habituation phase (day 1), followed by training (day 2) and testing (day 3). During training, individual mice are placed in a cage and are permitted to explore two identical objects. In the subsequent testing phase, a novel object replaces one of the two identical objects. To avoid object bias, preliminary studies tested objects for equal preference. Exploratory behaviour was recorded by video camera. Duration of time spent exploring the novel object is indicative of better memory of familiar object[27,43]. If mice however, explore novel and familiar objects for a similar amount of time, this would indicate a loss of memory for the familiar object presented during the initial trial.

Establishment of TNF KO Chimeras. Wildtype (CD45.1, 3mo) and TNFKO (CD45.2, 3mo) were treated with prophylactic antibiotics (e.g. sulfamethoxazole/trimethoprim; Septra, Pfizer, New York, NY, USA) for 5 days prior to receiving two doses of 550 Rads of gamma irradiation (1100 Rads total), to eradicate recipient hematopoietic stem cells

(HSCs). Within 2 hrs of the second irradiation, mice were injected intravenously with >5x10^6 cells of T cell-depleted donor bone marrow. T cell-depletion was performed using the EasySep™ Mouse CD90.2 Positive Selection Kit II (StemCell, Vancouver, BC, Canada) to eliminate risk of graft vs. host disease. Reconstitution was determined at 4 weeks post-engraftment by assessing the ratios of CD45.1 to CD45.2 cells in the blood using flow cytometry. Engraftment was determined to be >95% in all recipients. Chimeras were aged for 18mo. To protect from age-related obesity, aging mice are fed with a low-protein diet (Teklad Irradiated Global 14% protein Maintenance Diet) and provided with an exercise wheel. The average final weight of aged mice was on average, 27g±2.5g.

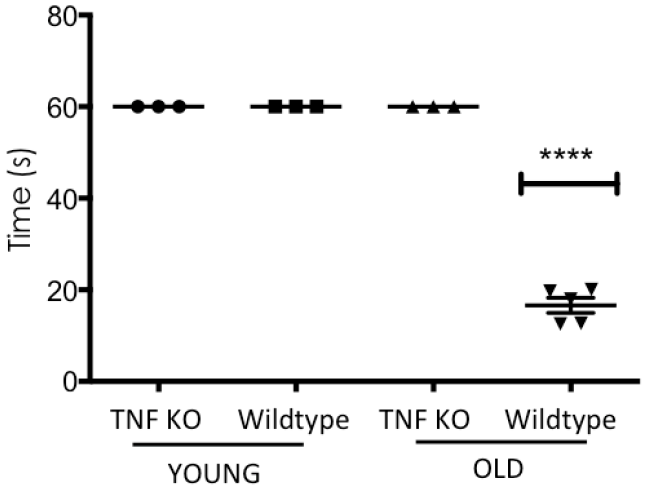

Figure 1. ***TNF contributes to decreased muscle strength with age.*** Young and old TNF KO and wildtype mice were subjected to a cage hang test to determine grip strength. A maximum grip time (up to t= 60s) was recorded after 3 trials. Mice were allowed rest periods in between trials. Data representative of 3 individual experiments. Statistical analysis using One-Way ANOVA (Multiple Comparison) with Fischer's LSD post-hoc test ± SEM.

RESULTS

TNF Contributes to Reduced Muscle Strength with Age. Sarcopenia, or reduction in muscle mass and or function with age, is frequently correlated with high levels of pro-inflammatory cytokine, TNF[31]. To directly assess the role of TNF on muscle strength with age, cage hang tests were performed in young (10-12wks) and mid-to-old (14-15mo) TNF knockout and wildtype mice. Unlike old TNF KO mice, old wildtype mice

demonstrate considerably reduced muscle strength, as determined by the lower maximum grip strength time (**Fig. 1**). The duration of maximum grip was comparable between old TNF KO mice and young controls.

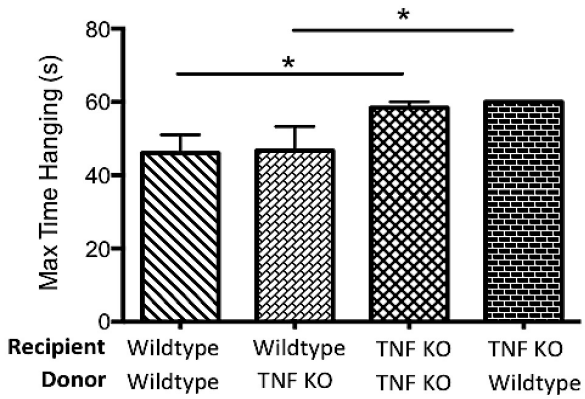

Figure 2. ***TNF from non-hematopoietic sources influence muscle grip strength.*** TNF KO and WT recipient mice that were adoptively transferred with either donor TNF KO or WT BM (and aged for 18 mo) were subjected to a cage hang test to determine grip strength. A maximum grip time (up to t=60s) was recorded after 3 trials. Mice were allowed rest periods in between trials. Statistical analysis using One-Way ANOVA (Multiple Comparison) with Fischer's LSD post-hoc test, with n=3-8 mice per group ± SEM.

Age-related increases in TNF from non-hematopoietic cells influence muscle grip strength. In order to determine whether TNF from hematopoietic or non-hematopoietic sources contribute to sarcopenia, we established TNF KO and wildtype chimeras and aged them to 18 mo. At this time point, cage hang tests were performed to assess whether the source of age-related increases in TNF was of hematopoietic (e.g. blood cells) or non-hematopoietic (e.g. microenvironmental) origin. As observed in **Fig. 2**, when muscle strength was assessed in TNF KO recipient mice adoptively transferred with WT bone marrow, the maximum hang time was equivalent to old TNF-concordant chimeras. Further, when cage hang tests were performed in aged WT recipients adoptively transferred with TNF KO bone marrow, their maximum grip time was comparable to WT concordant BM, which demonstrate reduced muscle strength compared to TNF KOs. Muscle strength was not influenced by the source of donor marrow. Thus, increased TNF

in the aging microenvironment contributes to diminished muscle strength with age.

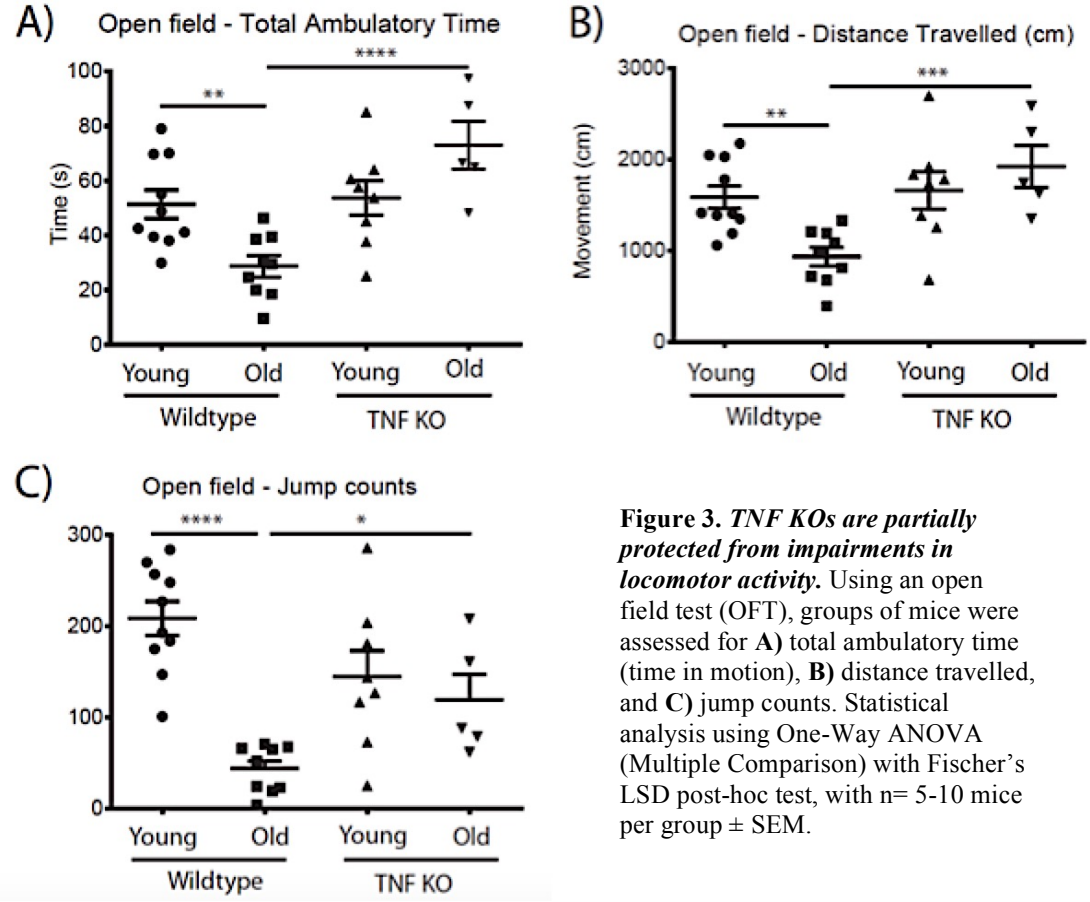

Figure 3. ***TNF KOs are partially protected from impairments in locomotor activity.*** Using an open field test (OFT), groups of mice were assessed for **A)** total ambulatory time (time in motion), **B)** distance travelled, and **C)** jump counts. Statistical analysis using One-Way ANOVA (Multiple Comparison) with Fischer's LSD post-hoc test, with n= 5-10 mice per group ± SEM.

TNF drives age-related impairments in locomotor activity. We evaluated locomotor activity and mobility in young (10-12wk) and old (>18mo) wildtype and TNF knockouts using a battery of behavioural assessments as part of the open field testing (**Fig. 3**). Old wildtype mice demonstrated reduced movement, as measured by their total ambulatory time (**Fig. 3A**) and distance travelled (**Fig. 3B**), in comparison to young wildtypes. Old TNF knockouts, however, do not demonstrate impairments in movement. Further, we show that old wildtype mice have reduced mobility when compared to young controls

(**Fig. 3C**), as assessed by quantifying the frequency of jumps during the 10 min open field period. Old TNF knockouts were partially protected from the age-related loss of mobility. Thus, TNF plays a key role in driving diminished locomotor activity and mobility that accompanies aging in mouse models.

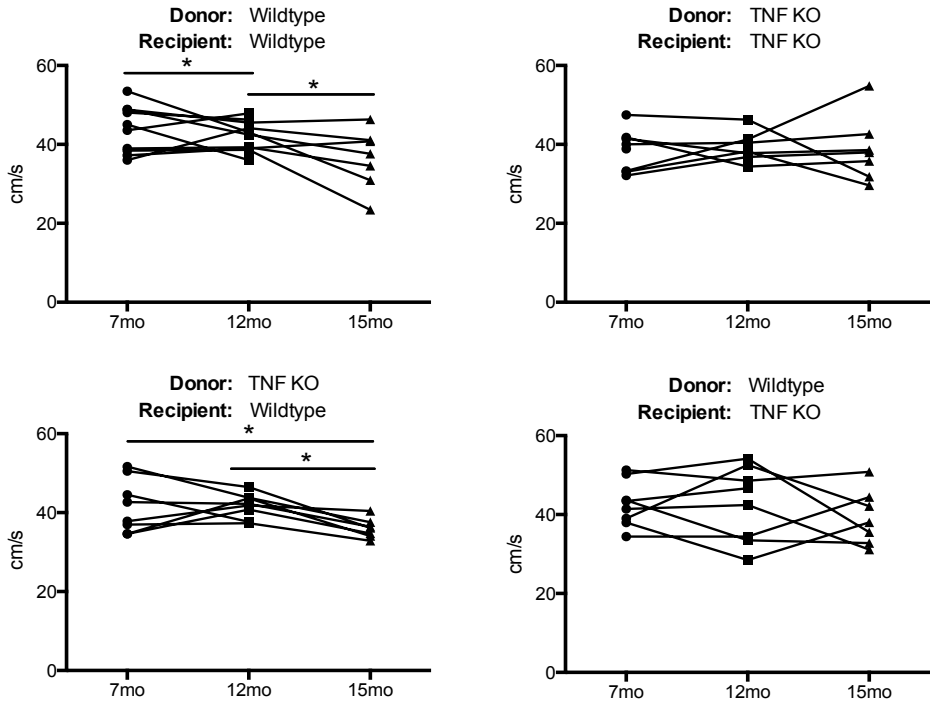

Figure 4. ***TNF from non-hematopoietic sources accelerates decline in locomotor activity.*** Bone marrow chimeras and controls were subjected to an open field assessment to evaluate locomotor activity at 3 time points over their lifetime. Statistical analysis was performed using the Mann-Whitney t-

TNF from non-hematopoietic sources accelerates the age-related decline in locomotor activity and mobility. We evaluated the progressive decline in locomotor activity, as determined by changes in average velocity (cm/s) during the mouse lifespan, in aging TNF knockout chimeras. Mice were assessed at 3 time points: 7mo, 12mo and 15mo. T-tests were performed to assess differences in groups among the time points. In WT recipients adoptively transferred with either WT or TNF KO bone marrow, a steep decline in movement was observed over time (**Fig. 4A/C**). These findings demonstrate

that TNF derived the aging microenvironment contributes to the progressive decline in movement observed with age. Conversely, no decline in locomotor activity was observed in aging TNF KO recipient mice adoptively transferred with either WT or TNF KO bone marrow (**Fig. 4B/D**). Thus, locomotor activity was not influenced by TNF derived from donor marrow. A similar observation was seen when mobility was assessed by quantifying frequency of jumps, which serves as a measure of stiffness in joints and ligaments, and locomotor deficits[31]. A lower jump count is indicative of greater locomotor deficit. Decline in mobility was accelerated in recipient mice where TNF was present in the microenvironment (**Fig. 5B/D**). Such a decline was considerably less dramatic in TNF KO recipients. Together, these findings support that the steady increase in TNF in the aging microenvironment leads to the waning mobility and locomotor activities that accompanies aging.

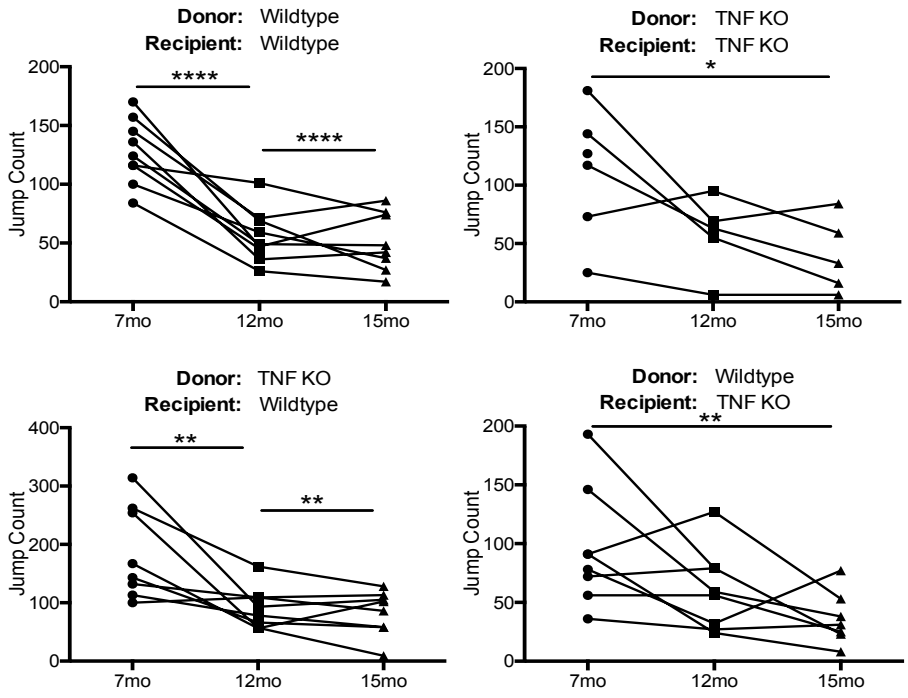

Figure 5. ***TNF from non-hematopoietic sources accelerates decline in mobility*** Bone marrow chimeras and controls were subjected to an open field assessment to evaluate locomotor activity at 3 time points over their lifetime. Statistical analysis was performed using the Mann-Whitney t-test, with n=6-7 mice per group ± SEM.

TNF knockout mice may be protected from age-related impairments in motor activity. Young (10-12wk) and old (>18mo) wildtype and TNF knockout were placed in the central region of the open field apparatus and the time that they spent in the centre was measured. Old mice spent significantly more time in the centre than young mice (**Fig. 6**). A lack of movement of mice to the peripheral regions when placed in the centre of the field may be indicative of motor impairment or weakness[40] or a deficiency in anxiety-like behaviour[38]. Since prior studies demonstrate that aged animals display a decline in explorative behaviour that is independent of changes in anxiety levels[37], and since locomotor deficits accompany aging in humans[31], this observation in old wildtype mice is likely due to motor impairments. Aged TNF knockouts did not demonstrate a deficit in motor activity and spent a greater proportion of time in the peripheral regions.

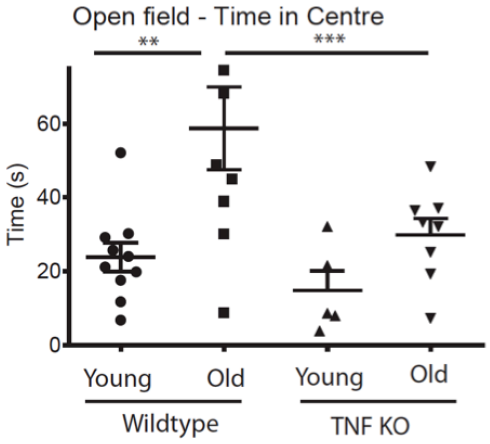

Figure 6. ***TNF KOs are protected from age-related motor impairment.*** Mice were placed in the open field apparatus and duration of time spent in the central region was measured. Statistical analyses was performed using the One-Way ANOVA (Multiple Comparison) with Fischer's LSD post-hoc test, with n=5-10 mice per group ± SEM.

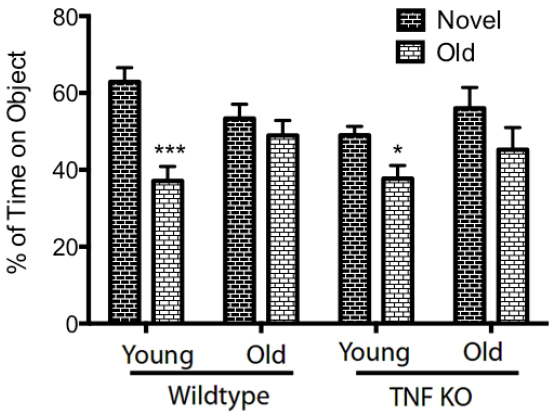

Figure 7. ***Aging, but not TNF, impairs recognition memory.*** A novel object recognition test was performed to assess recognition memory. Increased time spent exploring the novel object compared to the old object was reflective of a better memory. Statistical analyses was performed using a Mann-Whitney t-test, with n=3-10 mice per group ± SEM.

Aging, but not TNF, Impairs Recognition Memory. Increases in systemic and cerebrospinal levels of pro-inflammatory cytokine, TNF, has been associated with development of mild-to-severe cognitive impairment[44,45]. We investigated the role of TNF on memory impairment with age using the novel object recognition test (**Fig. 7**). This test assesses dorsal hippocampal function based on the tendency of mice to investigate a novel object, rather than a familiar object[42]. Individual mice were placed in a cage and were permitted to explore two identical objects. In the subsequent testing phase, a novel object replaces one of the two identical objects. A greater duration of time spent exploring the novel object is indicative of better memory of the familiar object[27]. As demonstrated in figure 7, old mice (>18 mo) demonstrated poor recognition memory, as compared to young (<3 mo) controls. Since old mice, regardless of genotype, explore novel and familiar objects for a similar amount of time, this would indicate a loss of memory for the familiar object presented during the initial trial[43]. These findings indicate aging itself, but not age-related increases in TNF, lead to impairments in recognition memory.

DISCUSSION

Aging is accompanied by the progressive decline in muscle mass and strength, a condition known as sarcopenia[46]. Impaired muscle strength is a powerful risk factor of physical disability, poor quality of life, and all-cause mortality in advanced age[47–49]. The prevalence of sarcopenia in individuals aged 70 or older is approximately 40%[8]. Despite the huge economic burden associated with sarcopenia, the pathological mechanisms

underlying muscle wasting, and eventually, physical disability, are poorly understood[46]. Pro-inflammatory cytokines, such as TNF, IL-1β and IL-6, have been postulated contribute to the pathogenesis of sarcopenia, however the primary data is limited[23–25]. Here, we show for the first time that TNF plays a causative role in the onset and progression of muscle wasting, loss of mobility and reduced locomotor activity with age using behavioural testing. Consistent with our findings, TNF-mediated inflammation has been associated with decline in muscle mass and strength with aging in several studies. TNF administration was found to accelerate muscle breakdown in rodents through stimulation of proteolysis, or protein degradation[50,51]. The ability of TNF to directly act on muscle cells to stimulate proteolysis was shown to be mediated by transcription factor, NFκB, which increases activity of the ubiquitin/proteasome pathway, known to accelerate the regulated degradation of muscle proteins and promote muscle weakness[52]. *In vitro* treatment of muscle with of recombinant TNF, however, failed to increase proteolysis, thereby suggesting that TNF mediates its effects on sarcopenia an indirect manner[50]. TNF regulates the expression of a number of cytokines that increase with age (e.g. IL-6, IL-12, CCL5), therefore any of these cytokines could mediate the downstream effects of TNF on sarcopenia[53]. In addition to activating catabolic pathways, TNF can influence muscle metabolism by downregulating anabolic pathways. For instance, LPS-induced muscle wasting is associated with elevated levels of TNF, which leads to inhibition of AKT/mTOR signal transduction. Enhancers of AKT activity, however, can counteract TNF-induced wasting[54]. Further, antibody-mediated TNF blockade can prevent muscle breakdown in rodent models associated with significant muscle deterioration, such as

tumour-bearing mice[54]. Evidence for TNF's effect on muscle integrity, along with knowledge of increases in this pro-inflammatory mediator with age, provides further rationale for inflammation as contributory to physical disability in the elderly[55].

Using aged TNF knockout chimeras, we demonstrate that age-related increases in TNF are derived from the aging microenvironment, and not of hematopoietic origin. Although further studies are required to pinpoint the source of TNF, age-related increases in adiposity[56,57] and dysregulation of mesenchymal stromal cells[58] have been shown to contribute. One of the major limitations to murine and human sarcopenia studies is that they are cross-sectional, and therefore, underlying mechanisms for the association between inflammatory mediators and physical function cannot be clearly elucidated[55]. Compared to cross-sectional studies, longitudinal studies allow for more causal inferences. Further, in rodent studies, they avoid the challenge of cage effects, which can considerably confound data interpretation[59–61]. Using a long-term aging colony of TNF knockout chimeras and wildtype controls, we tested mice longitudinally to evaluate progressive changes in muscle function that occur within individual mice over their lifespan. We observed that the steady increase in TNF from the aging microenvironment, and not hematopoietic origin, accelerates the progressive decline in mobility and locomotor activities that is observed with age. Given that a decline in mobility occurs naturally with aging, even when TNF is absent, this finding suggests that there is some factor in addition to TNF that drives the age-related loss in muscle integrity.

Aging in accompanied by decline in cognitive capacity. Cognitive decline encompasses a large spectrum of clinical manifestations, with a continuum that ranges

from the cognitive changes of normal aging (age-associated cognitive decline (AACD)), through mild cognitive impairment (MCI), and finally, dementia[29,62]. MCI refers to the condition where individuals experience memory loss greater than one would expect for age, as determined by the Mini-Mental State Examination (MMSE), but do not meet the criteria for clinical dementia[63]. MCI incidence was found to increase with age, reaching 10 new cases per 100 in individuals 90 years and older, and correlate with mortality[64]. Those with a clinical diagnosis of MCI progress to dementia at a an accelerated rate compared with healthy age-matched individuals[29]. Currently, there exists a large volume of literature supporting the association between subclinical inflammation and cognitive impairment in older adults[65–67]. Although the etiology remains unclear, TNF has been postulated to contribute to pathogenesis of both MCI and AD[26]. Human studies measuring plasma levels of TNF found significantly higher levels of TNF in centenarians compared to younger control groups, and this high concentration was strongly associated with AD[26]. Further, in patients with Alzheimer's, concentrations of TNF in the cerebrospinal fluid (CSF) were found to be 25-fold higher when compared to controls, with higher levels correlating with increased clinical deterioration[45].

Though age-related cognitive impairments are normally modeled in mice using the APP/PS1 and SAMP8 transgenic strains[68], we for the first time demonstrate that naturally aged mice can suffer from progressive deficits in memory recognition. We believe that this model could better recapitulate the impact of age on cognitive impairments. Using the novel object recognition test, it is clear that old mice have an impaired recognition memory, given that they explore both old and novel objects for

equal durations. When we tested old TNF knockouts, we observed no improvement in memory. This was surprising given the large volume of literature supporting the association between subclinical inflammation and cognitive decline with advanced age[65–67,69]. Thus, we postulate that other pro-inflammatory cytokines that are increased with age (e.g. IL-1β, IL-6, IL-12 or CXCL10) may contribute to memory impairments. In a mouse model of brain aging that undergoes early onset cerebral neurodegeneration, increased levels of IL-6 and CXCL10 associated with deterioration of cognitive function; as such, these pro-inflammatory mediators may act as etiological agents[70]. Further studies involving larger sample sizes and auxiliary assessments of cognitive deficits, such as the Morris water maze[71] and radial arm maze[40,72], are required to validate our findings.

CONCLUSION

Our findings suggest that higher levels of pro-inflammatory cytokine, TNF, may be a marker of functional limitations in old persons across several age-associated diseases. Our data demonstrate that age-related increases in TNF contribute to muscle wasting, loss of mobility, and reduced locomotor activity. Given the prevalence of low-grade inflammation in older persons, interventions aimed at reducing TNF concentrations could help to circumvent the decline of physical function with age.

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DISCUSSION

The research presented in this thesis focuses on models of chronic inflammation; with emphasis on its etiology, impact on host immune responses, specifically focusing on monocytes and macrophages, and role in disease onset and severity. Since individual chapters presented within the thesis were accompanied by their own discussion, the aim of the following discussion section is to briefly integrate and interpret our collective findings and propose avenues that warrant further investigation.

ORIGINS OF INFLAMMATION IN THE AGING MICROENVIRONMENT

In this thesis, we demonstrate that age-related changes in monocyte and macrophage development, phenotype and function are due to changes in the aging microenvironment. Old TNF knockout mice have fewer Ly6C^{high} monocytes and macrophages isolated from old TNF knockouts did not demonstrate any impairment in phagocytic and anti-bacterial activity, unlike those isolated from old wildtype mice. As such, we concluded that increases in TNF in the aging microenvironment drive monocyte and macrophage dysfunction; however, the source of this TNF is unknown. Using bone marrow chimeras of wildtype and TNF knockout mice, we determined the source of TNF to be of non-hematopoietic origin. TNF regulates the expression of a number of other pro-inflammatory cytokines increased with age (e.g. IL-6, IL-12); therefore TNF may not directly affect myeloid cell function, but rather indirectly through the induction of downstream cytokines. Following an exhaustive literature review, we hypothesize that either increases in adipose deposition, changes in mesenchymal stromal cell functionality and/or increased microbial translocation may principally contribute to the age-related inflammatory microenvironment.

I. Age-Related Increases In Adiposity

As we age, our bone marrow is gradually replaced by adipose tissue, leading to both an increase in size and number of adipocytes[196,216]. This process is known as the *fatty degeneration* of the marrow[48]. Fatty generation has been attributed to enhanced

adipogenesis versus osteo-blastogenesis with age[217]. The presence of adipocytes is rare in the bone marrow of neonatal mammals, however, with age, bone marrow adiposity increases, resulting in the appearance of fatty marrow[218]. In fact, in humans, the majority of the femoral cavity is occupied by fat by the third decade of life[218]. These bone marrow adipocytes have been speculated to be the primary source of the age-related increase of adipose-derived inflammatory cytokines and chemokines[219,220]. Bone marrow adipocytes secrete adipokines, such as TNF, IL-6, IL-1β, MCP-1, leptin and adiponectin, which shape the bone marrow microenvironment[221]. Adiponectin and TNF can directly impair hematopoiesis by inhibiting hematopoietic progenitor expansion. Adipocyte-rich bone marrow demonstrates a diminished ability to support hematopoietic progenitor proliferation and stem cell quiescence[222]. Further, leptin, a key adipokine involved in the regulation of the inflammatory response by adipocytes[148,223], has been implicated in the differentiation of monocytes and macrophages and their increased production of IL-6 and TNF[126]. Increased pro-inflammatory cytokine secretion by adipocyte precursors has been shown to induce a 'pro-inflammatory state' in macrophages, as determined by higher levels of TNF mRNA[152]. Further, adipocyte precursors promote tissue infiltration of M1 (classically-activated, pro-inflammatory) macrophages[220]. In fact, macrophage infiltration correlates with adiposity in aging mice[151]. In Chapter 4, we provide novel data demonstrating that adipose tissue-associated macrophage accumulation is dictated by TNF rather than increases in adipose mass or size. In addition to bone marrow adiposity, age-related changes in peripheral adipose depots can contribute to inflammaging. Increasing evidence suggests that body fat distribution may be more important to health

than total body fat[224–226]. Adipose tissue accumulates in the central regions of the body in older adults, even in individuals with 'healthy' body weights[227]. This visceral adipose tissue (VAT) is associated with an increased risk of heart diseases, diabetes, cancer, and all-cause mortality[224–226,228]. Visceral adipose tissue (AT) from old C57Bl/6 mice has been shown to produce significantly more pro-inflammatory cytokines (e.g. TNF, IL-6), both basally and upon stimulation, than young AT[220]. Further, culture medium from adipocytes (ACM) isolated from visceral fat pads of old mice increased IL-6 and TNF production by young and old peritoneal macrophages significantly more than young ACM[220]. Together, these findings suggest that the expansion of adipose tissue with age in the bone marrow, and in the periphery, may contribute to the establishment of the inflammatory microenvironment and downstream changes in macrophage function.

II. MSC Dysregulation With Age

Mesenchymal stromal cells (MSCs) support the growth and differentiation of myeloid progenitors in the bone marrow and may contribute to age-related developmental defects in monocytes and macrophages. MSCs, known as non-hematopoietic stem cells, generate the stromal bone marrow components, such as adipocytes, reticular cells, and osteoblasts[229] and play a critical role in the regulation of hematopoiesis[52]. According to the seed-and-soil theory, mesenchymal stromal cells serve as the soil and generate a niche that regulates the proliferation and differentiation of stem cells, or the seeds[230,231]. It is widely postulated that age-related defects in immune cells primarily arise from the stem-cell niches, which are unable to provide appropriate signals or provide incorrect signals to

the stem cells, rather than defects in the stem cells *per se*[3,231,232]. In Chapter 3, we assert that the bone marrow microenvironment dictates age-related changes in immune cell phenotype and function. The impact of age on the bone marrow niche remains poorly understood.

MSCs are known to exert immunoregulatory activities, however, these functions become compromised with age[52]. Through direct cellular contact and/or the production of soluble factors (e.g. TGFβ, HGF, IL-10, HLA-G, PDL-1, PGE_2[233]), MSCs can inhibit the inappropriate activation of B and T lymphocytes and generate a tolerogenic environment to promote immune homeostasis[234–236]. For instance, MSC secretion of indoleamine 2,3-dioxygenase (IDO) leads to tryptophan depletion in lymphocytes and results in inhibition of their activity and proliferation[221]. Furthermore, prostaglandin E_2 (PGE_2) by MSCs induces immunosuppression[221]. This immunosuppressive activity, however, becomes reduced with age. In a study by *Bustos et al.*, human MSCs isolated from cadaveric vertebral bones of elderly donors were found to secrete lower levels of IL-10 (an anti-inflammatory cytokines) than young donors[237]. Further, MSCs isolated from old mice secreted lower levels of IL-1RN, an anti-inflammatory factor that competes for IL-1R1 binding, and had a profound decrease in their ability to resolve inflammation. Perhaps dysregulation of the MSC immunosuppressive activity contributes to the inflammatory microenvironment that results with old age. MSC aging is further accompanied by a senescent secretory phenotype associated with increased production of pro-inflammatory cytokines, chemokines and reactive oxygen species[238]. MSCs isolated from old rats have been shown to producer higher levels of TNF and IL-6 than those isolated from young

rats[219]. In a mouse model of RA, it was observed that the inflammatory microenvironment presence could reverse the immunosuppressive capacity of MSCs[239]. Together, these findings demonstrate that aged MSCs can both contribute to, and be influenced by, the inflammatory microenvironment associated with aging.

Age-related changes in MSC function have been attributed to both cellular senescence and epigenetic modifications. In humans, the decline in differentiation and proliferation of aged MSCs has been attributed to telomere erosion[229]. Microarray analyses demonstrated that MSC from aged animals differentially express genes associated with cell activation, differentiation and migration compared to young counterparts[237]. Strikingly, 90% of these genes were decreased in MSCs from old mice, suggesting age-related MSC exhaustion (progressive loss of MSC function)[237]. By failing to tightly regulate inflammation, these findings suggest that age-related MSCs exhaustion may contribute to the persistent inflammatory state concomitant with age. Further, it has been proposed that age-related changes in gene expression in MSCs is modulated by histone acetylation, specifically H3 acetylation on K9 and K14 [240]. *Wagner et al.* recently reported differential DNA methylation patterns in the gene loci of the IFNγ β-chain, the IL-1 receptor antagonist isoform 3 and mediators of TNF signaling. These findings support that intrinsic alterations in MSCs may impair their function with age.

III. Microbial Translocation

In this thesis, we hypothesized that age-associated inflammation is at least partially caused by elevated levels of circulating bacterial products, which result from

age-associated alterations in intestinal permeability, such as a loss of integrity in the gut epithelia. The basis of such a hypothesis was derived from Elie Metchnikoff, who in the early 1900's, theorized that microbial translocation occurs with age, due to an increased penetrability of the gastrointestinal tract[135]. In Chapter 5, we clearly demonstrate that circulating bacterial products, muramyl dipeptide (MDP) and lipopolysaccharide (LPS), increase with age, in both mice and humans. Increased bacterial translocation in mice coincided with an age-associated deterioration of intestinal barrier integrity. Immune cell stimulation by bacterial components leads to the release of pro-inflammatory cytokines, such as TNF. We have shown that monocytes and macrophages isolated from aged hosts secrete higher levels of IL-6 and TNF upon stimulation with bacterial products (e.g. LPS) compared to young counterparts. This inflammatory response likely contributes to tissue damage and release of oxygen radicals, leading to the further release of additional cytokines. What results is a positive feedback loop that perpetuates the chronic inflammatory state, known as inflammaging.

Recent studies demonstrate that bone marrow HSCs can directly detect bacterial products[241,242]. TLR4 ligation by haematopoietic progenitors induces proliferation and differentiation, leading to an increase in common myeloid progenitors (CMPs), preferential differentiation of common lymphoid progenitors (CLPs) into dendritic cells (DCs) and arrest of B lymphopoiesis[27,242,243]. In contrast to steady-state HSC differentiation, myeloid progenitors stimulated with TLR4 ligands *in vitro* differentiate into monocytes and/or macrophages in the absence of exogenous differentiation and growth factors[242]. In a similar manner, lymphoid progenitors differentiated into DCs *in*

vitro by circumventing normal growth and differentiation requirements[242,243]. Consistent with these findings, *Esplin et al.* observed that long-term exposure of mice to low-dose LPS lead to reduced numbers of CLPs and increased myeloid skewing[244]. Further, *Ueda et al.* have shown that injected LPS quickly diffuses into the bone marrow and depletes B lineage lymphocytes[245]. These studies suggest that the age-related increase circulating bacterial products drive the skewing towards myeloid cell differentiation observed in old age[246]. Other studies have demonstrated using labeled bacteria that gut-derived peptidoglycan can translocate to the bone marrow and enhance innate immunity (e.g. neutrophil anti-bacterial killing and inflammatory responses)[241], however, the effects of peptidoglycan on HSC differentiation is not well understood.

Together, we propose that increases in circulating bacterial products with age have dual roles in promoting the inflammaging phenotype. First, an age-related deterioration of gut barrier integrity allows the translocation of microbial products from the intestinal lumen into circulation and ultimately, the bone marrow. Here, these products direct hematopoietic progenitors to preferentially differentiate and proliferate into cells of the myeloid lineage, such as monocytes and macrophages. Monocytes, which may be primed by LPS or MDP in the bone marrow, then migrate into the periphery, where they secrete high levels of pro-inflammatory cytokines in response to the circulating bacterial products, and thereby facilitate the perpetuation the age-related chronic inflammatory state.

TRANSLATION OF FINDINGS

Community-acquired pneumonia (CAP) is the highest cause of mortality among infectious diseases in developed countries[212]. Elderly individuals with higher than age-average levels of circulating, pro-inflammatory cytokines, TNF and IL-6, have a considerably increased risk of acquiring, becoming hospitalized with, or dying from *S. pneumoniae* than those with lower than average levels[105,107,203,206]. Premature mortality in patients with community-acquired pneumonia is not attributed to failure to eradicate the etiological agents, but rather, a dysfunctional host reponse[202,207]. In this thesis, we establish that age-related chronic inflammation induces changes in monocyte and macrophage development, phenotype and function, and ultimately, impairs anti-pneumococcal immunity. A poorly regulated immune response contributes to increased disease severity due to enhanced dissemination and greater immunopathology[212]. Further, we show that lowering levels of TNF may be an effective strategy in improving host responses against *S. pneumoniae* with old age[60]. Our findings in mice are consistent with several clinical studies that recommend use of anti-inflammatories, such as corticosteroids, in combination with conventional antibiotic treatments to improve pneumococcal disease prognosis in the elderly[212–214]. Thus, strategies to suppress the excessive inflammatory responses during pneumonia infection are being considered. A recent meta-analysis conducted by *Siemieniuk et al.* evaluated the use of corticosteroids in treatment of CAP in 13 clinical trials[214]. Use of combination therapy was associated with approximately 3% lower mortality, a 5% decrease in mechanical ventilation, and a shorter hospital stay for older adults with community-acquired pneumonia[214]. The benefits arising from corticosteroid usage were large enough to be considered clinically

significant. In these trials, the median age was only ~64[214]. Based on our research, one would expect that those with higher levels of chronic inflammation, such as the very old, would have greater benefited from these treatments, making the results even more dramatic. Conceivably, a biomarker, such as circulating TNF, could be used to identify CAP patients with strong inflammatory responses that would better respond to combination therapy. It is necessary that the benefits outweigh the potential harms of systemic corticosteroid therapy to provide optimal care for patients with severe CAP.

As mentioned above, although numerous studies have shown that immunosuppressive steroid use in combination with antibiotics reduces pneumonia-associated mortality in the elderly[212,214,247–249], the application for this therapy has been limited. Until now, the mechanistic underpinnings and cells involved in such a phenomenon were unknown. We demonstrate that suppression of inflammation can reverse the impact of age on monocytes and macrophages to better coordinate controlled immune responses to *S. pneumoniae.* Thus, our research supports that administration of corticosteroids could better refine inflammatory immune responses in the elderly and facilitate clearance of pneumonia, or other infectious diseases. It may appear counterintuitive to limit inflammatory responses during a bacterial infection, however, clinical observations and our animal studies confirm that anti-pneumococcal strategies must be tailored to the age of the host.

CONCLUDING REMARKS

The data presented in this thesis emphasizes models of chronic inflammation; focusing on its etiology, impact on host immune responses, specifically focusing on monocytes and macrophages and their role in infectious and chronic disease onset and severity. Chapter 2 concentrates on how TNF, a key mediator of inflammaging, contributes to changes in Ly6C^{high} monocyte development, phenotype and function, and ultimately, impaired anti-pneumococcal immunity. Chapter 3 characterizes the role of aging microenvironment on age-related defects in monocytes and macrophages, and emphasizes the functional plasticity of these myeloid cells. Chapter 4 demonstrates how circulating Ly6C^{high} monocytes can be better predictors of insulin resistance than conventional biomarkers. Chapter 5 investigates increased microbial translocation with age as a potential etiological agent contributing to the inflammaging phenotype. Lastly, Chapter 6 focuses on the role of TNF on age-associated sarcopenia. Together, these studies increase the breadth of our understanding of the cross talk between inflammatory microenvironment and host immunity, and their impact on age-associated diseases. Although there are many more details to be unraveled, these studies indeed have made great progress toward rejuvenating the immune systems of aged hosts and these findings could be used to develop novel and much-needed therapeutic strategies to reduce the risk of infectious and chronic disease and improve the quality of life in the aging population.

Chapter 8. Methodology

MOUSE STRAINS, CARE AND DIET

Wildtype and Aging Mice: C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and aged in-house. To protect from age-related obesity, aging mice are fed with a low-protein diet (Teklad Irradiated Global 14% protein Maintenance Diet) and provided with an exercise wheel, as were young controls. The average weight of young mice (10-12wks) in these studies was 20g+/-1g and old mice (≥18mo) were on average, 27g ± 2.5g.

TNF Knockout: Knockout mice (*B6;129S-Tnft^{m1Gkl}/J*) on a C57/Bl6 background were a generous gift from Dr. Zhou Xing and/or purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were bred in the barrier unit at the McMaster University Central Animal Facility (Hamilton, ON, Canada) as previously described. All mice were housed in specific pathogen-free conditions.

TNF Receptor (TNFR) Knockout: C57/Bl6 mice (*B6.129S-Tnfrsf1^{atm1Imx} Tnfrsf1^{btm1Imx}/J*) deficient in both the p55 and p75 subunits of the TNF receptor superfamily fail can produce but cannot respond to TNF. Mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed in specific pathogen-free conditions.

CD45.1 Congenic: Transgenic mice (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). This C57BL/6 congenic strain was used in bone marrow transplant studies because it carries the differential *Ptprc^{a}* pan leukocyte marker commonly known as CD45.1 or Ly5.1. Wildtype C57/Bl6 mice instead express CD45.2. Mice were housed in specific pathogen-free conditions.

HFD Diet Treatment. At 8 weeks of age, mice (male C57Bl/6 and $TNF^{-/-}$) were fed *ad libitum* standard chow diet (Harlan Teklad no. 7913, 6.2% kcal from fat) or a high-fat diet (Research Diets D12492, 60% kcal from fat) for 24 weeks. All experiments were performed in accordance with Institutional Animal Utilization protocols approved by McMaster University's Animal Research Ethics Board.

*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 (CCAC). The pathogen-free status of mice was ensured through continuous monitoring of sentinel mice and specific testing of fecal samples for common murine pathogens.

Murine Model Of Pneumococcal Colonization.

S. pneumoniae strain P1547 (a clinical isolate, serotype 6A)[250] was used for all experiments. Bacteria were grown in tryptic soy broth (Life Technologies) at 37°C and 5% CO_2 until cultures reached log phase, OD_{600} between 0.45 and 0.50, then placed on ice to inhibit further growth. A 1 mL aliquot of bacteria in TSB was transferred to 1.5mL tube, centrifuged at 15,000 rpm for 1 min, then resuspended in 100 µL PBS. Unanaesthetized mice were inoculated intranasally with 10 µl bacterial suspension containing ~1×10^7 CFU of *S. pneumoniae*. At sacrifice, the trachea was cannulated, and 300 µl of cold, phosphate-buffered saline (PBS) was instilled[251]. Nasal lavage fluid was

collected, serially diluted in PBS, and plated on tryptic soy agar (Becton Dickinson, cat:236950) plates containing 5% sheep's blood and neomycin (10 μg/ml). Colonies were counted after overnight incubation at 37°C and 5% CO_2.

FLOW CYTOMETRY

Murine Samples.

Monoclonal antibodies with the following specificities were used in these studies: **F4/80** (APC, eBioscience), **Ly6C** (FITC, BD Pharmingen), **CD45** (eFluor 450, eBioscience), **CD11b** (PE-Cy7, eBioscience), **CD3** (Alexa Fluor 700, eBioscience), **NK1.1** (Alexa Fluor 700, eBioscience), **CD19** (Alexa Fluor 700), **CCR2** (PE, R&D Systems), **CD11c** (PerCP-Cy5.5, eBioscience), **TNF** (BV650, BioLegend) or **IL-6** (PerCP-efluour 710, BioLegend), **CD45.2** (APC, eBiosciences), and **CD45.1** (PerCP-Cy5.5, eBiosciences). Blood and single cell suspensions of tissue were stained according to previously published procedures[60]. All experiments included unstained and isotype controls to account for non-specific binding. Flow cytometry was performed on a BD Biosciences Fortessa and acquired FACS data files were analyzed using FlowJo v9 software (Treestar).

Intracellular Staining.

Intracellular cytokine staining was performed on peripheral blood. Immediately following collection, 100 uL of heparinized blood in 1:1 RPMI-1640 media (supplemented with 10% fetal bovine serum) were treated with PBS (unstimulated) or 10 ng/mL LPS (Sigma,

MO, USA) and 1x Protein Transport Inhibitor (eBioscience, CA, USA) for 4 hours at 37°C and 5% CO_2. Surface staining to identify Ly6C^{high} monocytes was performed for 30 min at room temperature with the aforementioned conjugated antibodies, and fixed with 1x 1-Step Fix/lyse buffer (eBioscience, CA, USA) for 10 min. Cells were permeabilized for 30 min with 1x Permeabilization Buffer (eBioscience, CA, USA) at room temperature, and stained with the conjugated antibodies TNF- BV650 and IL-6-PerCP-efluour 710 (BioLegend, USA) for 30 min at room temperature. Cells were fixed with 2% paraformaldehyde, centrifuged and resuspended in FacsWash prior to flow cytometric analysis.

Preparation of Bone Marrow.

Bone marrow was harvested from femurs and tibias of mice. Once bones were cleaned of tissue and sterilized using 70% ethanol, a syringe was used to flush bone marrow into a 15mL tubes. Bone marrow was sheared to attain a single cell suspension, washed and resuspended in PBS. Prior to staining, a RBC lysis was performed using 1x RBC Fix/Lyse Buffer (eBioscience) and cells were incubated with anti-CD16/32.

Preparation of Lung Tissue

To attain a single-cell suspension of mouse lung tissue, half a lung was collected from each mouse and kept on ice. Immediately following, each lung was mechanically dissociated and enzymatically degraded using a Miltenyi Biotec Lung Dissociation Kit (Cat#: 130-095-927) along with the gentleMACS Octo-Dissociator with Heaters (Cat#:

130-096-427). Following dissociation as per protocol, cell suspensions were filtered (70 μM cell filter) and centrifuged at 300 x g for 10 min. Subsequently, single-cell suspensions were resuspended in phosphate-buffered saline & processed for flow cytometry

Human Monocyte Immunophenotyping

To compare monocyte frequency in humans, whole blood was collected from young (≤45 yrs and) elderly individuals (≥65 yrs). Monoclonal antibodies with the following specificities were used in this study: CD2-PE, CD3-PE, CD19-PE, CD56- PE, NKp46-PE, CD15-PE, CD14-APC-Alexa750, CD16-PE-Cy7, HLADR-PerCp-Cy5.5, CD45-eFluor605NC. Monocytes were gated as previously published[252]. Monocytes were defined as lineage (CD2, CD3, CD19, CD56, NKp46, CD15) negative, and CD45, HLADR, and CD14 positive. Classical monocytes were defined as CD16 negative, whereas intermediate and non-classical monocytes were defined as CD16 positive. Absolute counts were measured using 123count beads (eBioscience).

Written informed consent was obtained from all participants or their legally appointed guardian in the event they were not competent to provide consent themselves. These studies, consent procedures, and documents were approved by the McMaster Research Ethics Board.

IN VIVO EXPERIMENTS

Chimera Establishment. Mice were treated with prophylactic antibiotics (e.g.

sulfamethoxazole/trimethoprim; Septra, Pfizer, New York, NY, USA) for 3 days prior to receiving two doses of 550 Rads of gamma irradiation (1100 Rads total), to eliminate recipient hematopoietic stem cells (HSCs). Within 2 hrs of second irradiation, mice will be injected intravenously with >5x10^6 cells of T-cell depleted bone marrow (CD45.1 or CD45.2). T-cell depletion was performed using the EasySep™ Mouse CD90.2 Positive Selection Kit II (StemCell, Vancouver, BC, Canada). Bone marrow was harvested from spines of donors since spinal HSCs have greater proliferative and reconstitutive capacities[253]. Reconstitution was determined at 4 weeks post-engraftment by assessing the ratios of CD45.1 to CD45.2 cells in the blood using flow cytometry. Engraftment was determined to be >95% in all recipients.

Ly6C^{high} Monocyte Depletion

FITC Fluoresbrite 500 nm carboxylated polsytrene microparticles (PS-MPs) were obtained from Polysciences. Microparticles were diluted in saline to a concentration of 5 x 10^9 particles in 200 µl, and PS-MPs were injected intravenously, as previously described[254]. Injections were performed every other day during timespan of the experiment. Monocyte depletion was confirmed by flow cytometry.

Quantitation Of Monocyte-Bound *S. pneumoniae*

100 µL samples of peripheral blood were incubated with TRITC-labeled *S. pneumoniae* (MOI 20) resuspended in 100µL of complete RPMI-1640 media at 4°C to allow binding, but not uptake, and at 37°C to allow uptake. Following 30 min of incubation, cells were

stained for flow cytometry. After RBC lysis (1x 1-Step Fix/Lyse Solution eBioscience; ref: 00-5333-57) was performed for 10 min, fixed cells were washed 2x with PBS to remove excess stain and non-adherent bacteria, and resuspended in FACS wash (10% fetal bovine solution in sterile PBS). Flow cytometry was performed and the amount of *S. pneumoniae* bound by Ly6C^{high} monocytes was quantitated based on the mean fluorescent intensities of TRITC.

Administration of anti-TNF

Mice were administered with adalimumab (HUMIRA, Abbott Laboratories), a humanized anti-TNF antibody, or the human IgG isotype control, diluted in sterile saline. A dose of 50 ng per gram of body weight was given intraperitoneally in a volume of 200 µl every other day, for a period of 3 weeks to young (10-12 wks) and old (≥18 mo) wildtype mice.

Insulin and Glucose Measurement

Mice were fasted for 6 hours prior to blood collection via tail vein. Fasting blood glucose was measured by using the Roche Accu-Chek Inform II system. Blood (50uL) was collected, incubated at room temperature for 20min, and spun at 7500g for 5min at 4°C. Serum was collected and stored at -80°C. Insulin was detected in mouse serum samples by ELISA according to the manufacturer's protocol (Millipore Cat #EZRMI-13K).

FITC-Dextran Assessment of Permeability

To evaluate intestinal permeability *in vivo* and non-terminally, mice were orally gavaged

with 200 µl of 3-5kD of FITC-labeled dextran at 0.8 mg/mL (Sigma-Aldrich). Mice were fasted 4hrs prior to, and 4 hrs following gavage. After the 4hrs, blood was retro-orbitally collected, diluted 1:1 in sterile PBS, and fluorescence intensity was measured (excitation at 403nm and emission at 518nm).

HISTOLOGY

Lung Immunohistochemistry. Histologic analysis was performed on lungs from chimeric cohorts. Upon collection, lungs were fixed in 10% neutral-buffered formalin at room temperature for a minimum of 24 hrs prior to embedment in paraffin. Tissue blocks were cut into 3mm sections and stained with H&E at the Core Histology Facility, McMaster Immunology Research Centre. Images were acquired with a DM LB2 microscope at a magnification of x20 (Leica, Bannockburn, IL, USA).

Adipose & Liver Immunohistochemistry. Adipose tissue derived from epididymal fat pads and liver samples were fixed in 10% neutral-buffered formalin at room temperature for a minimum of 24 hrs and embedded in paraffin. Five-micron sections cut at 50-µm intervals were mounted on charged glass slides, deparaffinized in xylene, and stained for macrophages with an anti-F4/80 (1:500) monoclonal antibody on the Leica Bond RX automated staining system using an enzyme-pretreatment. The total number of F4/80-expressing cells was counted and averaged using ImageJ.

IN VITRO EXPERIMENTS

Macrophage Culture and Stimulation

Bone marrow-derived macrophages were attained by flushing femurs and tibias of mice

with cold PBS. Flushed bone marrow was washed and resuspended in 25mL RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100mg/ml), 10 mML-glutamine (R10 media) and 15% L929-cell conditioned medium (contains M-CSF). Macrophage progenitors are cultured for 7 days, with fresh media changes at day 3 and day 6. To isolate peritoneal macrophages, peritoneal lavages were performed with 10 ml ice-cold PBS. Cells were then washed once with RPMI-1640 and resuspended in R10 media and plated in 10 mL on 10 cm tissue culture dishes. Cells were allowed to adhere for 2 h and then non-adherent cells were removed by washing with warm media, and adherent cells were incubated at 37℃ overnight.

All differentiated macrophages were cultured and maintained in a humidified environment at 37℃ with 5% CO_2 in R10 media. To lift macrophages, cells were incubated with 10 mL of cold PBS for 10 minutes at 4°C and gently lifted using a cell lifter. Cells were then centrifuged, counted and re-suspended in medium at a concentration appropriate for measurement of cytokine production, bacterial uptake, flow cytometry or bacterial killing assays.

Macrophage Killing Assays.

Macrophages (bone marrow-derived or peritoneal) were suspended in 1 ml HBSS at a concentration of $1x10^6$ cells/ml. Live *S. pneumoniae* (strain P1547, serotype 6A) in the log phase of growth (OD: 0.5) was added at an MOI of 10, and solution was mixed on a nutating mixer at 37℃ for 30min. The cells were then separated from unbound bacteria by centrifuging at 1500 rpm for 5 min, and resuspended in 1mL HBSS. Macrophages

were incubated with bound bacteria for 30 min. To measure macrophage killing of *S.pneumoniae*, aliquots were collected at 30 min and macrophages were lysed by osmotic rupture in sterile H_2O. Serial dilutions were performed in H_2O and plated on sheep's blood agar supplemented with 10 mg/ml neomycin. Colonies were counted the next day to assess viable bacteria remaining.

Spinal Bone Marrow Isolation

As opposed to standard bone marrow isolation procedures using femurs and tibias, isolation of spinal marrow allows for a 5x higher cell yield. Upon sacrifice, spines were collected under sterile conditions and connective tissues were removed. Cervical dislocation was avoided during sacrifice to prevent potential contamination of bone marrow cells. Once bones were cleaned, spines were cut open using bone scissors and spinal cord was carefully removed. Subsequently, bones were places into a mortal with 10 mL of cold PBS and a pestle was used to crush bones. The bone marrow suspension was filtered using 40uM cell strainer placed over 50 mL conical tube. Cells were spun down at 1500 rpm for 5min and resuspended in RPMI-1640 supplemented with 10% FBS,

Cytokine ELISA

ELISAs for IL-6 were performed as per the manufacturer's directions (eBioscience). Macrophage supernatants were diluted 1:2. To assess plasma levels of IL-6 and TNF, high-sensitivity Milliplex kits (EMD Millipore, Billerica, MA, USA) were used, as per the manufacturer's instructions. Plasma was collected by spinning blood samples from

young and old collected into heparin at 15000 x g for 10 min.

BACTERIAL PRODUCT DETECTION

Muramyl Dipeptide (MDP) Detection Assay

To quantify the circulating levels of bacterial products, I developed and optimized a novel and sensitive reporter cell line used to detect the presence of MDP (constituent of gram-positive and gram-negative bacteria) via intracellular receptor $Nod2^{255}$. Low passage HEK293T cells were stably dual-transfected with mNod2 (a kind gift from Dr. Jonathan Schertzer) and pNifty2-SEAP plasmids (Invivogen) plasmids. The latter plasmid is composed of genes encoding an ELAM proximal promoter, 5 NF-κB repeated transcription factor binding sites (TFBS) and the SEAP reporter gene. Upon recognition of the NOD2 agonist, MDP, a signal transduction pathway is induced, leading to the activation of NFκB, a transcriptional regulator of inflammation, and subsequent expression and release of SEAP (secreted embryonic alkaline phosphatase) into the surrounding media. SEAP is a truncated form of human placental alkaline phosphatase (PLAP) through the deletion of a GPI anchor. Unlike intracellular reporters, SEAP is secreted into the cell culture supernatant, allowing for easy detection without adverse effects on the cell. The stable cell line is passaged in Dulbecco's Modified Eagle Media (DMEM), supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 mg/ml), and 10 mML-glutamine, and maintained under constant antibiotic selection (of 10 mg/mL Zeocin & 100 uL of 1 mg/mL in 20 mL media).

To assess circulating MDP in mouse plasma, 96-well plates were seeded with

4000 cells/well 24 hours prior to addition of heat-inactivated mouse plasma (plasma diluted 1:5 in PBS, then 1:1 in endotoxin-free water, followed by inactivation at 75℃ for 5min). Use of specialized HEK-Blue Detection Media (Invitrogen) allows for quantification via colorimetric analysis. Heat-inactivated mouse plasma (10 uL; 30 uL for human plasma) was diluted 1 in 20 in HEK Blue Detection Media (Invivogen) to a final volume of 200uL. Readings were performed at 630 nm, 24 hours subsequent to stimulation.

Lipopolysaccharide (LPS) Detection Assay

Lipopolysaccharide (LPS) is a constituent of the outer membrane of Gram-negative bacteria, which acts as an endotoxin and elicits strong immune responses. The lipopolysaccharide (LPS) detection assay was created in the same manner using low passage HEK293s transfected to stably express TLR4, MD2 & CD14 on the cell surface, which were later transiently transfected with the SEAP reporter. LPS is captured by LPS Binding Protein (LBP), which delivers LPS to CD14, an anchored protein on the cell surface without a transmembrane domain that signals through TLR4[256]. The assay is almost identical to the aforementioned MDP assay, with the exception that the HEK Blue Detection Media is supplemented 1% FBS (due to the presence of LBP).

BEHAVIOURAL EXPERIMENTS

Open Field (OF) Testing

Locomotor deficits, such as muscle wasting, decreased muscle mass, and stiffness in joints and ligaments, often accompany aging[257]. The purpose of the open field test (OFT) is to assess locomotor activity and mobility, willingness to explore, and anxiety in mice. Upon testing, individual mice are placed in an arena and allowed to freely move for 10 min. Behavior in a novel OF was measured using a fully automated system (arena 27.3 × 27.3 cm; Med Associates Inc., St. Albans, VT, USA). Parameters include: total-distance traveled, velocity & time spent in pre-defined zones[258]. Further, anxiety-like behaviour may be measured by including additional parameters of defecation and duration spent in the center of the field[258] during first few minutes of activity. All experiments were conducted in a quiet, well-lit room, with one handler throughout.

Novel Object Recognition Test

Aging mice can suffer from progressive deficits in learning and memory. As a common assessment used to evaluate cognition in murine models, particularly recognition memory, the novel object recognition test serves as readout of cognitive impairment[259]. The novel object test represents a test for dorsal hippocampal function based on the tendency of mice to investigate a novel object, rather then a familiar one[260]. The test consists of a habituation phase (day 1), followed by training (day 2) and testing (day 3). During training, individual mice are placed in a cage and are permitted to explore two identical objects. In the subsequent testing phase, a novel object replaces one of the two identical objects. To avoid object bias, preliminary studies tested objects for equal preference. Exploratory behaviour was recorded by video camera (Sony, Toronto,

Ontario, Canada). Duration of time spent exploring the novel object is indicative of better memory of familiar object[163]. If mice however, explore novel and familiar objects for a similar amount of time, this would indicate a loss of memory for the familiar object presented during the initial trial[261].

Cage Hang

Human and murine aging is characterized by skeletal muscle wasting; a debilitating condition that direct affects quality of life. This sarcopenia, or reduction in muscle mass/and or function, has frequently been associated with high levels of pro-inflammatory cytokines (e.g. TNF, IL-6)[257]. The cage hang test is a well-known assessment that seeks to evaluate motor function and deficit in rodent models of CNS disorders & serves as a measure of muscle strength. The test consists of placing mice on elevated wire cage top, then inverting and suspending top above a padded cage. The latency to when the mouse falls is recorded. Individual mouse performance for each session is presented as the average of three trials[262].

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**Please note these are references associated with the Introduction, Discussion and Methodology. Each thesis chapter has its own reference section.*

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APPENDIX I: Immunosenescence: implications for vaccination programs in the elderly – *A Review*

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REVIEW

Immunosenescence: implications for vaccination programs in the elderly

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Abstract: Worldwide, infectious disease is responsible for much of the morbidity and mortality in the elderly. As the number of individuals over the age of 65 increases, the economic and social costs of treating these infections will become a major challenge. Vaccination is the most effective and least costly preventative measure in our arsenal; however, vaccines that are effective in children and young adults are often ineffective in older adults. This is a result of the deterioration in immune function that occurs with age, referred to as immunosenescence. Age-associated changes in leukocyte phenotype and function impair primary vaccine responses and weaken long-lasting memory responses. In this review, we discuss current vaccination approaches in the elderly and strategies to improve responsiveness in older adults, which include increasing vaccine immunogenicity and overcoming the fundamental immune defects that prevent optimal immune responses.

Keywords: immunosenescence, vaccination, elderly, influenza, pneumonia, zoster

Introduction

By the year 2050, more than 25% of the world's population will be 65 years of age or older.[1] Susceptibility to infectious disease increases with age and in addition to other age-related health issues, poses an enormous challenge to health care systems in the developed world. In the US, pneumonia and influenza were the eighth leading causes of death in 2005, and the elderly (aged ≥65 years) accounted for an estimated 90% of these deaths.[2–4] Combined, these diseases cost the US economy $40.2 billion due to direct and indirect health care expenditures and mortality-related losses in productivity.[5] The economic and social costs of infection include acute treatment and long-term health outcomes. For example, having pneumonia in midlife to late life accelerates development of dementia, respiratory, and cardiac conditions, as well as fall-related injuries that require hospitalization. Consequently, calculations on the costs of acquiring pneumonia in midlife to late life must include the costs of long-term consequences of infection.[6–8]

Infectious diseases account for roughly 20% of hospitalizations in the elderly.[9] Vaccines are the most successful tools we have in preventing infectious disease. Four vaccines are currently recommended for use in the elderly: the seasonal influenza vaccine, the pneumococcal vaccine, the tetanus-diphtheria-pertussis vaccine, and the vaccine to prevent shingles, which is caused by reactivation of *Herpes zoster* virus (Table 1).[10] Despite the importance of preventing these infections and relatively high vaccination rates, protection is still suboptimal due to decreasing immune function with age.[11]

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Table 1 Current vaccine recommendations in the elderly

Vaccine	Type	Adjuvant	Site	Schedule
Diphtheria-tetanus acellular pertussis (DTAP)	Subunit (Adacel®, Boostrix®)	Alum	IM	One dose administered if patient has not received in adulthood (age >18 years) and boost every 10 years. Adults not previously vaccinated receive at least three doses at 0 (DTAP), 8 weeks (DT), and 6–12 months (DT).
Herpes zoster	Attenuated (Zostavax®)	N/A	SC	One dose administered regardless of prior history of shingles.
Influenza	Inactivated split virus (Fluviral®, Vaxigrip®, Fluzone®, Agriflu®, Influvac®, Intanza®)	N/A	IM or ID (Intanza®)	One high dose trivalent vaccine administered every flu season.
	Subunit (Fluad®)	MF59	IM	
Pneumococcal	Conjugate (Prevnar® 13)	Alum	IM	Vaccine-naïve individuals receive one dose PCV13, followed by one dose PPSV23 6–12 months later. Individuals that previously received PPSV23 at age ≥65 years, receive one dose PCV13 ≥1 year later. Individuals that received PPSV23 before age 65 years and now are aged ≥65 years receive one dose PCV13 and one dose PPSV23 6–12 months later.
	Polysaccharide subunit (Pneumovax® 23, Pneumo 23®)	N/A	IM/SC	

Abbreviations: N/A, not applicable; IM, intramuscular; SC, subcutaneous; ID, intradermal; DT, diphtheria-tetanus; PCV, pneumococcal conjugate vaccine; PPSV23, 23-valent pneumococcal polysaccharide vaccine; PCV13, 13-valent pneumococcal conjugate vaccine.

Respiratory tract infections, particularly influenza and pneumonia, account for the majority of hospitalizations due to infectious disease in the elderly. Due to widespread use and careful monitoring of influenza and pneumococcal vaccines, data on the efficacy of these vaccines are well understood. Despite reasonably high vaccination rates in the elderly (61.3% influenza, 59.9% pneumococcal), influenza and pneumonia infections are still associated with serious adverse events leading to hospitalization, debilitating complications, and mortality in the elderly.[9,12,13]

Seasonal influenza causes moderate illness in healthy adults that is generally resolved within 2 weeks; however, children, those with comorbidities, and the elderly are at increased risk of complications (ie, pneumonia, bronchitis, and/or sinus infection) that may result in hospitalization and mortality. Consequently, in many countries influenza vaccination efforts focus on children (aged 6 months–17 years) and the elderly (aged ≥65 years). Vaccination rates are as high as 56.6% in children and 61.3% in the elderly, while coverage in adults (aged 1–64 years) is 35.7%.[13] Even though rates of coverage may be the same in the young and old, protection rates are very different. Studies measuring vaccine efficacy monitor "influenza-like illness", which is used for a proxy of influenza infection without virology testing to confirm the infection. In studies that monitor the effectiveness of the trivalent inactivated vaccines, there were marked differences in protection from influenza-like illnesses between these two age groups. For example, a recent meta-analysis suggests that 58% of vaccinated children (aged <16 years) were protected from influenza-like illness.[14] In a large meta-analysis of influenza vaccination in the nursing home or community-dwelling elderly, there was no significant protection from influenza despite the use of antigen-matched vaccines.[15] Age-related changes in immunity are believed to contribute to the disparity in protective efficacy of trivalent inactivated influenza vaccine (TIV) in these two populations.[15]

Bacterial pneumonia is a common consequence of seasonal and pandemic influenza infection, and is a major cause of morbidity and mortality in the elderly.[16] To prevent community-acquired pneumonia, nursing home-acquired pneumonia, and ventilator-acquired pneumonia, vaccines against the major causative agent, *Streptococcus pneumoniae,* have been developed. In the elderly, *S. pneumoniae* generally causes pneumonia, but more rarely, can cause invasive pneumococcal disease (IPD) (eg, meningitis or septicemia). The most recent Cochrane meta-analysis of the efficacy of the 23-valent pneumococcal polysaccharide vaccine (PPSV23) vaccine demonstrated that there was significant protection from IPD (odds ratio [OR], 0.26; 95% confidence interval [CI], 0.15–0.46), but there was no evidence for protection against pneumonia (including community-acquired pneumonia, nursing home-acquired pneumonia, and ventilator-acquired pneumonia) in the elderly.[17] With few exceptions, other studies have confirmed that there is some protection against IPD, but not pneumonia, in the elderly.[18–21] Current vaccines are clearly not sufficient to protect the elderly from the infectious diseases that they are most susceptible to, and this is likely due to waning immune function.[22] With age, there

is an increase in inflammatory mediators in serum and tissues, accompanied by phenotypic and functional changes to leukocytes, which affect all elements of the immune response necessary to mount a response to vaccination (Figure 1). This review will focus on age-related immune changes relevant to the vaccine response and will provide commentary, based on current data, as to how vaccines can be tailored to provide increased protection in the elderly.

Influence of immunosenescence on vaccine-elicited immune responses

Anatomy of a vaccine response

Vaccine effector responses

Vaccines prime the adaptive immune system to produce a rapid, robust, and protective immune response upon subsequent exposure to an infectious agent. This "memory" response is mediated by antigen-specific lymphocytes (ie, B and T cells). Antigen-specific antibodies produced by B cells bind and neutralize viruses and extracellular bacteria and also mediate their uptake and clearance by macrophages and neutrophils. T cell-mediated responses act to directly or indirectly kill infected cells. Although vaccine effector mechanisms are executed by the adaptive immune system, their generation depends on the innate immune response.

Antigen uptake and antigen-presenting cell activation

Antigen-presenting cells (APCs), primarily dendritic cells (DCs), ingest the vaccine antigen at the site of administration, become activated, and later present the antigen to B and T cells. Activation of APCs is required to initiate production of pro-inflammatory cytokines that upregulate homing receptors, which are required for the DC to migrate to the draining lymph node and present antigens to T cells. An inflammatory response is also required to increase co-stimulatory molecule expression that is needed to activate T and B cells. The degree of APC activation is highly dependent on the type of vaccine that is administered (Table 2).[23–25] For example, live attenuated vaccines are potent immune activators because they are briefly able to replicate, leading to increased and prolonged exposure to antigens and immunostimulatory viral components such as nucleic acids. In contrast, immune responses to purified antigens (ie, protein, polysaccharide, glycoconjugate, and

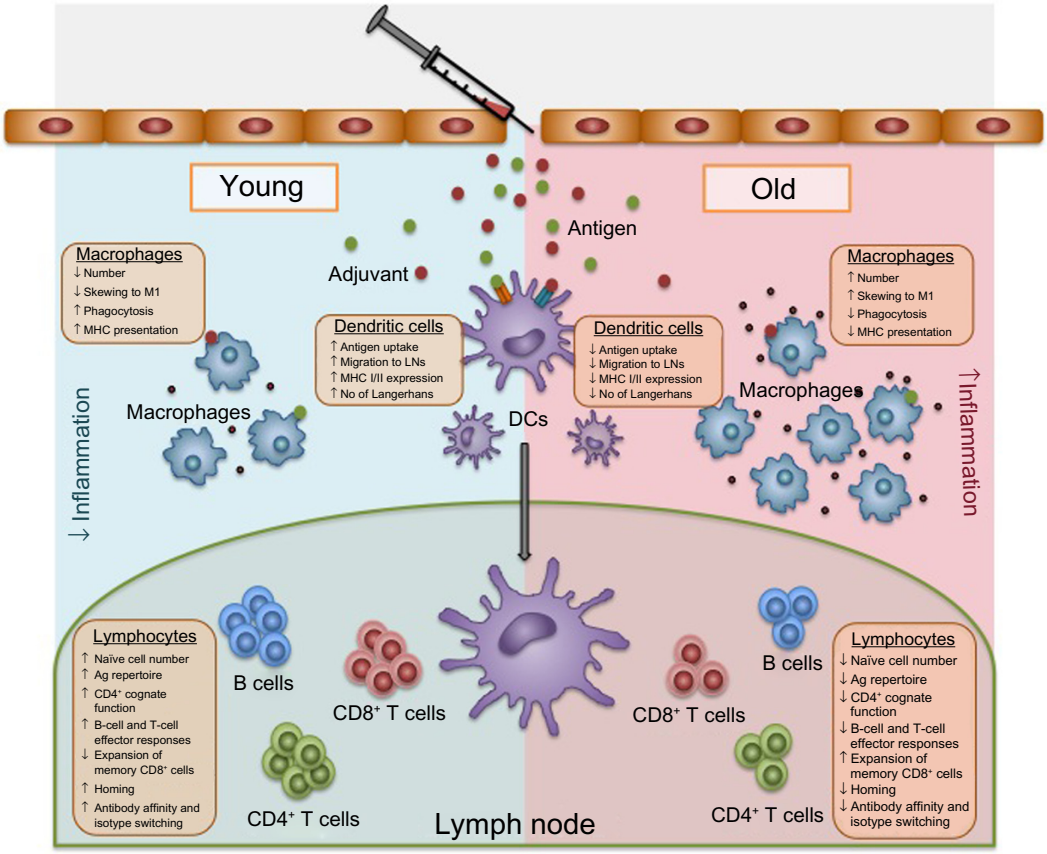

Figure 1 All elements of the immune response necessary to mount a response to vaccination.
Abbreviations: MHC, major histocompatibility complex; No, number; LNs, lymph nodes; DCs, dendritic cells.

Table 2 Vaccine types and immune responses elicited

Vaccine type	Components	Response elicited
Non-live vaccines		
Subunit	Purified pathogen proteins, isolated toxins Surface polysaccharides isolated from bacterial capsule	T cell-dependent antibody production Minimal CTL responses T cell-independent antibody production Minimal isotype class switching Poor recall response following booster
Polysaccharide conjugate	Covalently attached polysaccharide to carrier protein	T cell-dependent antibody production Minimal CTL responses
Inactivated	Whole pathogens inactivated by chemical or physical treatment	Antibody production No CTL responses Poor memory responses
Live vaccines		
Live, attenuated	Replicative pathogens with reduced virulence Retains some pathogenicity	T cell-dependent antibody production Long-lasting circulating antibodies Effective CTL responses Good memory responses
Live	Low doses of actual pathogen	Most effective protection T cell-dependent antibody production Long-lasting circulating antibodies Effective CTL responses Good memory responses

Abbreviation: CTL, cytotoxic T lymphocyte.

inactivated microbes) are more fleeting as they are rapidly cleared from the vaccination site. Purified protein (eg, viral capsid proteins) or carbohydrate (eg, the polysaccharide capsule of *S. pneumoniae*) antigens are poorly immunogenic on their own, except in very high doses. Adjuvants enhance immunogenicity of these proteins and carbohydrates by enhancing antigen presentation or co-stimulation by the APCs.[26] Adjuvants such as alum and oil-in-water emulsions (ie, MF59) trap antigens at the site of injection, creating a depot from which antigen is slowly released, providing longer antigen exposure and increasing recruitment of APCs. In unadjuvanted vaccines, antigen doses are increased considerably (ie, from 3.75 μg to 15 μg for influenza vaccine).[27]

Protein subunit, conjugate, and inactivated vaccine responses

As mentioned in the "Antigen uptake and antigen-presenting cell activation" section, APCs play a crucial role in linking the innate and adaptive arms of immunity, because they present processed antigen to T cells.[28] Antigens are presented to T cells in the context of major histocompatibility complex (MHC) molecules (human leukocyte antigen in humans). Proteins and inert particles are taken up and processed into peptides for presentation on MHC class II. Presentation by APCs activates $CD4^+$ T cells in secondary lymphoid tissue.[29] $CD4^+$ T cells support the differentiation of B cells, $CD8^+$ T cells, and macrophages that act to directly eliminate microbes. Naïve B cells can also take up antigen, which when presented in the context of co-stimulation from activated APCs and $CD4^+$ T cells, leads to their differentiation into low-affinity IgM-producing plasma cells. B cells also present antigen-to-antigen-specific $CD4^+$ T cells, which provide cognate help through co-stimulation. $CD4^+$ T cells help drive immunoglobulin class switching and affinity maturation, and result in plasma cells that produce high-affinity, antigen-specific antibodies. This T cell-dependent antibody response is slow and requires 10–14 days; however, it generates long-lasting, high-affinity antibodies and a memory B cell response.[30]

Live attenuated vaccines

By infecting and replicating in host cells, antigens from live attenuated viruses will be presented on MHC class I molecules to $CD8^+$ T cells. Also referred to as cytotoxic T lymphocytes, $CD8^+$ T cells kill infected cells by destroying them with mediators such as perforins and granzymes. Peptide antigens from live attenuated vaccines may also be presented on MHC class II and may elicit $CD4^+$ T cell and antibody production from B cells.[30]

Polysaccharide vaccines

Polysaccharide antigens act through T cell-independent pathways to generate an antibody response. Polysaccharides interact with marginal zone B cells in secondary lymphoid tissues. The polysaccharide cross-links the B cell receptor, causing activation and differentiation into a plasma cell-producing IgM. Some isotype switching from IgM to intermediate-affinity IgG occurs. In contrast to the T cell-dependent response, this response is rapid, transient (1 week long), produces low-affinity antibodies, and does not result in a memory response. In order to circumvent this short-lived response, polysaccharide vaccines are conjugated to immunogenic carrier proteins (ie, tetanus and diphtheria toxoids) to elicit a T cell-dependent B cell response.[31,32]

Memory responses

The generation of memory responses is the ultimate goal of vaccination. Following the primary response to T cell-dependent antigens, antigen-specific B and T cell numbers decline. The efficacy of a memory response is dictated by

the primary immune response and the number of exposures to antigen. The primary immune response is highly dependent on antigen dose and persistence, which is why live attenuated vaccines generate long-lasting memory responses. Most other inactivated and subunit vaccines require booster vaccinations. A small fraction of B cells, now memory B cells, will migrate to long-term survival niches such as the bone marrow where they continue to undergo affinity maturation for 4–6 months, increasing their antibody affinity.[33] Thus, boosters are generally administered 4–6 months following primary responses, allowing time for the generation of memory B cells. Antigen-specific effector T cells are short-lived and also decline following primary response. Those that persist can become either an effector memory T cell (T_{em}) or a central memory T cell (T_{cm}). T_{em}s migrate through non-lymphoid organs, patrolling tissues for their antigens, and have high cytotoxic activity once reactivated. Conversely, T_{cm}s traffic through lymphoid organs and have high proliferative capacity, generating a large surge of effector cells.[34] The T_{em} response relies on the magnitude of T cell expansion following primary vaccination, which is increased with higher antigen load and persistence.[30] Increased numbers of T_{em}s will result in more T_{em}s following the contraction phase of the T cell response.

Reactivation of memory cells can occur through natural colonization by microbes with cross-reacting epitopes, infection, or by booster immunizations. This leads to activation of memory B cells, which do not require cognate $CD4^+$ T cell help, and leads to rapid proliferation and secretion of high-affinity antibodies. Memory $CD4^+$ and $CD8^+$ T cells are also activated by cognate antigen and do not require costimulation. Generally, booster immunizations include higher antigen content than primary immunizations to increase the activation and proliferation of memory B cells. Upon multiple or prolonged exposures to antigens, activated memory B cells undergo further affinity maturation and produce higher affinity antibodies.[30]

Changes in vaccine responses with age

Both humoral and cellular immune responses to primary immunization and boosters decrease with age.[35–38] These antibody- and T cell-mediated specific immune responses depend on priming by competent APCs, such as DCs and macrophages. APCs from aged individuals, however, are less able to take up antigens by the process of micropinocytosis, have decreased capacity to present antigens due to decreased MHC class I and MHC class II expression,[39–43] and are less responsive to the chemokine CCL19, which is required for migration to the lymph nodes.[44] The impairment of DC migration is due to both intrinsic age-associated defects in the DCs themselves, in addition to the presence of changes in cytokine levels in the aging microenvironment.[44] Human DCs also have decreased expression of the co-stimulatory molecules, CD86 and CD80, which impairs T cell activation.[44–47]

With age, the number of naïve B and T cells produced by the bone marrow decreases,[48] due in part to changes in the aging microenvironment.[49] This results in a decreased ability to respond to new infections or vaccinations,[50] although a study shows that in some viral infections (eg, West Nile virus) the elderly are capable of mounting a de novo response.[51] Elevated levels of tumor necrosis factor (TNF) in the bone marrow decrease B cell lymphopoiesis and plasma cell survival.[52] Antibodies produced in aged individuals tend to be of lower affinity due to reduced isotype switching and somatic hypermutation, and consequently their neutralizing or opsonizing functions are decreased. This is due to impaired $CD4^+$ T cell help because of decreased germinal center formation in peripheral lymph nodes, which are necessary for an efficient, high-affinity humoral response.[53–55] The T cell compartment also undergoes significant changes with age. Levels of IL-7, a cytokine that promotes development of T cells in the thymus and thymic involution, result in decreased peripheral naïve T cells ($CD45RA^+CD28^+$).[56,57] Aged naïve T cells are increasingly difficult to prime.[58] Naïve $CD4^+$ T cells from aged animals show decreased effector cytokine production (ie, IL-2), less clonal expansion, and decreased expression of activation markers (CD25, CD62L, and CD154) following primary antigen presentation by APCs.[58,59] $CD4^+$ T cell function is impaired with age, leading to weaker humoral and $CD8^+$ T cell responses, which can contribute to vaccine failure in the elderly.[53]

Although memory cells are generated from naïve T cells in the elderly and show persistence in vivo, they exhibit impaired cytokine secretion and proliferation upon recall responses.[60] Memory $CD4^+$ T cells generated in young mice were shown to be functional in their host as they aged, while those generated in aged mice were non-functional.[60] Moreover, young $CD4^+$ T cells transferred to aged, immunized hosts maintained their capacity to induce a robust humoral response.[59] Similar results were also shown with old $CD8^+$ T cells,[61] suggesting that changes to T cell function are intrinsic and dependent on the age of the host. This implies age at primary vaccination is a more important determinant of proper memory T cell function, rather than age at recall response. A recent study demonstrated that following primary exposure to West Nile virus, antigen-specific $CD8^+$ T cells from elderly donors maintained production of antiviral cytokines, granzyme B, and perforin for up to 2 years. These results were comparable to younger adults.[51] In contrast, older primates have weak antiviral

$CD8^+$ T cell responses to West Nile virus.[62] However instead of using live virus, the Lelic et al study[51] employed synthetic immunodominant peptides for re-stimulation that did not require further processing by APCs. Since antigen presentation is also compromised with age, employing peptide-based booster vaccines may be an alternative strategy to elicit a strong $CD8^+$ T cell response in the elderly.

Even when the elderly mount a robust primary immune response, they may be less able to maintain antigen-specific memory cells. Chronic infections (ie, Herpes simplex virus, cytomegalovirus [CMV], and Epstein Barr virus) provide constant antigenic stimulation and lead to an expansion of terminally differentiated effector $CD8^+$ T cells, reducing space in the T cell repertoire for other antigen-specific T cells, including those generated by vaccination. This has been demonstrated in the elderly who are chronically infected with CMV and whose peripheral memory T cell repertoires are dominated by CMV-specific effector T cells. This prevents the expansion of T cell clones with other specificities due to limited space in the T cell repertoire. Not only do CMV effector cells impair the response to co-resident Epstein Barr virus infection,[63] but their increased numbers in the elderly have been correlated with decreased humoral responses following influenza vaccination.[64,65] Additionally, CMV memory T cells produce increased levels of interferon gamma (IFN-γ), contributing to chronic age-associated inflammation.[66,67]

As we age, levels of pro-inflammatory cytokines in the circulation and tissues increase. This state of chronic, low-grade, systemic inflammation is often called "inflamm-aging".[22] Although it is unclear why we become more inflamed with age, epidemiological data clearly demonstrate that the effects of age-associated inflammation are far-reaching. Age-associated inflammation seems to correlate with poor health in general, as higher than average levels of age-associated inflammation correlate with the development of chronic inflammatory disease, frailty, and general ill health.[68,69] Having higher than age-average levels of these cytokines increases susceptibility to infectious disease (eg, pulmonary pneumonia and influenza)[70,71] and is predictive of decreased vaccine responsiveness.[67]

Vaccine responsiveness does not decrease in a linear fashion with age, and often correlates more strongly with general health. Conversely, a robust vaccine response is a predictor of immune competence and good health.[72] In general, vaccine responsiveness correlates with frailty, defined as declining physical and mental function and reduced ability to resist environmental stressors.[73,74] Frailty is strongly associated with inflamm-aging, likely because immune competence is a mandatory requirement for overall health (eg, TNF, C-reactive protein [CRP], and IL-6).[75–79] In order to disentangle which elements of decreasing vaccine responsiveness are due to age rather than ill health, protocols have been developed to study only the healthiest older adults. The most commonly used is the SENIEUR protocol,[80] which excludes anyone taking immune-modulating medication, those with chronic disease (ie, atherosclerosis, Crohn's disease, etc), or abnormal values for common clinical measures (ie, leukocyte counts, urea, and glucose). Although this excludes the vast majority of older adults, it allows comparisons of the most immune-competent ("SENIEURs") to those with the normal allotment of age-associated changes in health ("non-SENIEURs"). A recent study evaluated influenza vaccine responses in the elderly during an epidemic season. Serum levels of IL-6 were measured before and 1 and 6 months after immunization.[67] The healthy elderly, or SENIEURs, had consistently low levels of IL-6 throughout the study, while the frail elderly, or non-SENIEURs, had significantly higher levels. The serum IL-6 levels correlated inversely with a protective vaccine response, measured by anti-hemagglutinin (HA) titer. The SENIEURs, who had low levels of IL-6, responded following their first immunization, while the non-SENIEURs with high IL-6 levels were permanent non-responders.[67]

These findings emphasize the importance of assessing immune competence in the target vaccine population prior to immunization. Currently, the elderly are uniformly treated with regard to vaccination. By developing an indicative marker of immune competence and vaccine non-response (ie, elevated serum cytokines), we can more efficiently administer vaccines to those with a higher likelihood of responding, while pursuing alternative vaccination strategies in the remaining at-risk population.

Adapting vaccination for the aging immune system

Increasing dose

In pre-clinical studies, data suggest that increasing the dose of HA antigen in influenza vaccines would increase antibody titers in the elderly.[81] A double-blinded, randomized, multicenter trial comparing the standard dose (15 μg HA per strain) to a high dose (60 μg per strain) of Fluzone® (Sanofi, Bridgewater, NJ, USA) was conducted in adults 65 years or older. The group receiving the high dose had antibody levels that were 12%–25% higher than the standard dose group for the three viral strains (H1N1, H3N2, and B).[82] In another study where trivalent split influenza vaccine (Sanofi) dose was doubled (from 15 μg to 30 μg) in the frail elderly, antibody

responses were also increased.[83] Although increasing HA antigen dose induces more antibodies, which are presumed to be a correlate of protection, studies that definitively demonstrate protection against infection are lacking.[14] The relationship between increased dose and increased immunogenicity does not appear to be universal to all vaccines. Increasing the amount of live attenuated Varicella zoster virus (1×, 2.7×, and 13× standard dose) in the elderly did not increase the vaccine-specific antibody or cell-mediated response.[84] This may be because it may be necessary to increase the amount of viral antigen content per virion, rather than the total number of virions, to enhance immunogenicity and cell-mediated immunity.[85]

Adjusting vaccine schedules

Older adults may mount efficient T cell responses in response to vaccination, but they are less able to maintain memory responses.[86] Following pneumococcal polysaccharide vaccine (PPV), not only do the elderly have decreased antibody potency against all serotypes, but there is a steady decline in serotype-specific antibody titers returning to pre-vaccination levels within 5–10 years.[87,88] In contrast, immune responses generated in youth are long-lasting and protective. An elegant example of this occurred during the H1N1 pandemic when influenza infections in even the oldest and most frail elderly were much lower than expected. Upon investigation, it was found that these individuals had protective antibodies that were generated many decades earlier in response to circulating strains that they were exposed to in youth.[89] This illustrates that memory responses that are generated in youth are long-lasting and protective well into old age. Consequently, one of the most effective ways to protect the elderly from infections may be to vaccinate them in youth. A robust primary immune response can also lead to more efficient responses to boosters, since it has been shown that pre-vaccination antibody titers dictate the magnitude of booster titers.[90] Live attenuated vaccines appear to be more efficient at providing increased protection over decades and following booster vaccinations, compared to inactivated vaccines.[91]

Utilization of alternative routes of immunization

Historically, the preferred approach to vaccine administration has been via percutaneous injection, which includes subcutaneous and intramuscular methods of immunization. However, recent advances in vaccinology and immunotherapeutics have suggested that alternate routes of vaccination may provide superior immunogenicity and protection in elderly populations.[92] In a large South African study by Holland et al, over 1,100 volunteers over the age of 60 received a trivalent, inactivated influenza vaccine via either intradermal microinjection or intramuscular administration. It was concluded that the intradermal route of vaccination elicited immune responses that were superior, as subjects had higher rates of seroconversion than those who received the conventional intramuscular administration.[93] Through exploitation of the skin immune system, intradermal vaccination directly delivers antigen to dermal DCs, which efficiently migrate and present antigen to T cells in draining lymph nodes, thereby naturally augmenting the primary immune response.[93] In theory, intradermal antigen delivery should allow for a reduction of the antigen dose required to obtain optimal protective responses in the elderly. Two separate studies have shown that using a 2.5-fold decrease in antigen dose, as compared to full-dose vaccines, achieves a suitable response via the intradermal route of vaccination.[94,95] Despite age-associated changes in skin integrity and physiology, and decreases in Langerhans and DCs, intradermal immunization can elicit protective immune responses in the elderly.[96]

Mucosal vaccination may also be a viable alternative, especially for infections that originate in the upper respiratory tract, such as influenza and pneumonia.[97] The abundance of APCs in mucosal tissues such as the nasopharynx and gastrointestinal tract facilitate antigen responses.[98] The nasopharyngeal- and gut-associated lymphoid tissues are reservoirs of immune cells that induce effective antibody production, especially IgA, upon encountering antigen in the context of the appropriate adjuvant.[99,100] Unlike conventional immunization, antigenic exposure at mucosal sites activates antigen-specific T cells and IgA^+ B cells, which subsequently transit to the lymph, enter the circulation, and seed mucosal sites, primarily the mucosa of origin.[99,101,102] Upon arrival, mucosal lymphocytes differentiate into effector or memory cells. The anatomic affinity of such cells is determined by surface site-specific integrins (homing receptors) and complementary mucosal tissue-specific receptors.[103,104] Nasal administration generates both mucosal IgA and peripheral IgG responses. IgA antibodies are particularly effective at binding and neutralizing viruses; therefore, mucosal vaccinations should be particularly protective against respiratory infections such as influenza. Currently, only one commercially available mucosal vaccine exists. Intranasal administration of FluMist® has been shown to elicit robust protective responses in adults aged <49 years.[105] However, studies involving patients aged >50 years have yet to be conducted; therefore, safe and efficacious use of FluMist in the elderly has not been established. While

mucosal immunizations demonstrate great potential, there is currently limited research on the development of mucosal vaccines that specifically target the elderly population and overcome the age-associated immune barriers to successful and effective vaccination.

Novel adjuvants that improve immunogenicity

In contrast to young people, the elderly often do not mount any detectable primary immune response to protein antigens, regardless of dose. In many cases, the commonly used alum adjuvant does not sufficiently increase the immune stimulatory activity of antigens in the elderly.[106,107] Use of more potent adjuvants may overcome this limitation. One promising candidate is the oil-in-water emulsion adjuvant, MF59™.[108] Previously, oil emulsion-based adjuvants were associated with side effects such as inflammatory reactions, granulomas, and ulcers at the injection site.[109] Replacement of mineral oil used in other emulsions with squalene in MF59, however, has limited side effects.[110] A murine study demonstrated that old mice immunized with an MF59-adjuvanted vaccine produced antibody titers to levels equivalent in young mice.[106] A similar study demonstrated that MF59 reduced to dose of antigen required, and upon secondary challenge with a wild virus, decreased total viral load and provided significant protection in both young and old mice.[107] To evaluate MF59 efficacy in humans, multiple clinical studies involving several MF59-adjuvanted vaccines have been performed. Results have demonstrated enhanced immunogenicity in all age groups, while maintaining a high level of safety and tolerability. Being the first adjuvant licensed for human other than alum, MF59 is now part of an influenza vaccine (Fluad®) designed for the elderly and is readily available worldwide.[108,110] Though adjuvant activity of MF59 is only partially understood, studies have shown that it induces monocyte recruitment and macrophage trafficking, promotes differentiation of monocytes into DCs, and fosters enhanced antigen uptake by macrophages and DCs.[111–113] Increased utilization of MF59 in vaccine development, specifically for the elderly population, may serve as a practical solution to enhance immunogenicity.

Other potential immunostimulatory adjuvants, which may enhance immunogenicity in the elderly, include the lipopolysaccharide derivative 3-deacetylated monophosphoryl lipid A, the saponin-derived lipid, QS21, oligodeoxynucleotides containing CpG motifs, and cytokines.[114] QS21, which is a derivative of the lipid saponin from the bark of the *Quillaja saponaria* tree, is being tested as an adjuvant in a pneumococcal polysaccharide vaccine (Phase II).[114] Recently, utilization of toll-like receptor (TLR) agonists as vaccine adjuvants in the elderly has delivered promising results in mouse studies.[98,115] By targeting evolutionary conserved receptors that recognize pathogens (eg, TLRs or nucleotide-binding oligomerization domain proteins), it is postulated that adjuvants might overcome the age-associated functional decline of innate immune response and induce production of pro-inflammatory cytokines.[116] Using TLR agonists as vaccine adjuvants is a method currently in the very early stages of clinical development. In older adults, TLR4 agonists have been shown to improve T cell response to influenza vaccination.[98] Additionally, HA-flagellin (TLR5 ligand) fusion proteins (VAX128) were shown to be well-tolerated and safe in aged individuals.[117] Recently, the use of cytokines in conjunction with vaccines has been explored. IL-7 is important to T cell survival, and therefore may be useful in maintaining a pool of naïve T cells in the elderly, thus allowing more efficient responses to novel antigens. While to date no studies have been performed in humans, experiments in aged macaques have had promising results, with 50% of animals demonstrating increased thymic output and restored influenza vaccination response.[118] Thus, in older adults, IL-7 could potentially be used to amplify vaccine responsiveness. Another potential cytokine candidate would be IL-2, which is well known to increase the number of peripheral T cells in addition to their responsiveness to antigen. Administration of a liposome-formulated vaccine and IL-2 induced significantly higher seroprotection and seroconversion rates against viral antigens as compared to other aged subjects receiving non-adjuvanted vaccine.[119] Furthermore, combination of coupled adjuvant systems (eg, microparticles which contain both antigen and DNA of a cytokine) may allow for a more targeted immune response in the elderly.[120]

Reversing immunosenescence

In the near term, novel vaccination strategies will involve working within the confines of the aging immune system; however, in the long term, a number of ambitious strategies are being pursued to correct some of the underlying defects in the aging immune system. Nutritional interventions have been demonstrated to increase vaccine responses in older adults and experimental animals. For example, decreasing specific lipid intake (eg, conjugated linoleic acids) appears to increase vaccine success rates in the elderly.[121] Vitamin E supplementation improves signaling between antigen-presenting cells and T cells, especially in $CD4^+$-naïve T cells in aged mice.[122] Caloric restriction seems to improve many aspects of immune function. It appears to delay T cell immunosenescence in non-human primates by maintaining both naïve T cell number and functionality, and reduces age-associated inflammation.[123]

Although caloric restriction is unlikely to ever be a viable strategy, it may be possible to target the major signaling molecule that is altered, mammalian target of rapamycin (mTOR). A recent study demonstrated that administration of an mTOR inhibitor in elderly volunteers increased their response to influenza vaccination by approximately 20%. Further, treatment reduced the percentage of $CD8^+$ and $CD4^+$ T cells that had low surface expression of co-stimulatory molecules. Therefore, mTOR inhibition during vaccination may be a potential strategy.[124] An alternate strategy would be cytokine intervention to improve thymic health in the elderly. There has been evidence that administration of IL-7 can reverse thymic atrophy and can rescue reduced naïve T cell population in old animals.[125] Other factors including IL-2, IL-10, and thymic stromal lymphopoietin have stimulatory effects on thymopoiesis.[126,127] Restoring thymic health and naïve T cell populations by modulating these cytokines may be a candidate therapy to increase primary vaccine-specific responses in the elderly.

Optimizing herd immunity

Counterintuitively, one of the best ways to reduce vaccine-preventable infections in the elderly may be targeting vaccinations, not to the elderly themselves, but to those who live, work, and care for them. Selective vaccination of children, adolescents, and healthcare workers (HCWs) reduces transmission of infections and protects unimmunized and immunocompromised individuals, such as the elderly, through herd immunity.[128–130] The add-on effects of vaccinating children to protect older adults were apparent after the pneumococcal vaccine was introduced. Not only did the total hospital admissions for IPD in older adults decrease, but the "holiday spikes" that once occurred over the winter holiday season when children were presumed to come in contact with their grandparents, disappeared.[131] Children and adolescents are major vectors for transmission of infectious diseases because of their high infection rates, prolonged viral shedding with high viral load, and frequent association with other susceptible hosts.[132] A recent study demonstrated that mass influenza vaccination of children (ages 3–6 and ages 7–17 years) with inactivated influenza vaccine lessened influenza-associated morbidity by 2- to 3.4-fold in unvaccinated, community-dwelling elderly.[133]

Establishing herd immunity is best achieved through vaccination of the youth and of HCWs, especially those working in close contact with individuals aged ≥65 years.[134] In a systematic review inclusive of 18 trials assessing the impact of HCW immunization on vulnerable populations, it was concluded that vaccination of HCWs against influenza provides significant indirect protection to the high-risk individuals.[130] Additionally, further evidence suggests that HCW vaccination is associated with substantial decreases in patient mortality.[129,130] Immunity through further implementation of vaccination programs that preferentially immunize HCWs and children shows promise in protecting our vulnerable elderly.

Conclusion

Approximately one-third of deaths in the elderly (aged >65 years) occur due to infectious disease.[135] Acquiring infections such as bacterial pneumonia in midlife or late in life often exacerbate or accelerate subclinical or existing chronic inflammatory conditions and can be the harbinger of declining health and decreased quality of life.[7,8] Therefore, the economic and social costs of infectious disease do not only include the cost of acute care, but also long-term health consequences. Prevention through vaccination would have an enormous impact on reducing the cost of care and improving the quality of life of the elderly. In the immediate term, we need to pursue the use of high-dose vaccines, optimized vaccine schedules, alternate routes of immunization, and novel adjuvants. In the longer term, we may be able to reverse some of the fundamental defects in the aging immune response, which would both increase vaccination responsiveness but also leave the elderly less vulnerable to infectious disease, should they become infected. It is imperative that we expand our understanding of the biological and molecular mechanisms underlying immunosenescence in order to provide older adults with the many years of healthy, independent living that they deserve.

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