THE ROLE OF MONOCYTES, MACROPHAGES AND THE MICROBIOTA IN AGE-ASSOCIATED INFLAMMATION DURING THE STEADY STATE AND ANTI-BACTERIAL IMMUNITY

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By

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Abstract

Inflammaging is a hallmark of human aging. Defined as low-grade, chronic inflammation, it is characterized by heightened proinflammatory cytokines in the blood and tissues and predicts morbidity and mortality. Despite this, the etiology of inflammaging and its role in infection have remained elusive, an issue this thesis addressed. First, we provided a comprehensive overview of an intranasal *Streptococcus pneumoniae* colonization model (Chapter 2). We described in detail the colonization technique, and demonstrated how to isolate and phenotype recruited cells, quantify bacterial load and measure production of immune mediators in the nasopharynx. Since both myeloid cell recruitment and tumour necrosis factor (TNF) production were increased following *S. pneumoniae* colonization with age, we investigated whether TNF directly augmented monocyte frequency (Chapter 3). TNF increased CCR2 expression on monocytes in old mice, leading to their enhanced egress from the bone marrow, resulting in enrichment of this population in the circulation. Monocyte numbers directly influenced plasma IL-6 levels, and this negatively impacted anti-bacterial responses, as monocyte blockade improved pneumococcal clearance in old mice. Lastly, to better understand the fundamental source of inflammaging, we studied the impact of the host microbiome on its development. This work was rooted in Elie Metchnikoff's early predictions that leakage of intestinal bacterial products could dysregulate macrophage function, resulting in inflammation that would progress aging (Chapter 4). We showed that old

mice had increased intestinal permeability, aberrant expression of cellular junction genes and increased microbial translocation from the gut to the blood. Germ-free mice lived longer than their conventionally colonized counterparts, and were protected from the development of inflammaging and defective macrophage function. Together, these studies resolve a major disparity in the field by demonstrating that systemic TNF production is initiated by increased levels of circulating bacterial products, driving functional defects in myeloid cells, which ultimately impairs anti-bacterial immunity.

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Studying for my PhD has been both uniquely challenging and truly rewarding. For the best four years, immunology has permeated every aspect of my life: I have lived and breathed macrophages, fallen asleep counting mice and spent my commutes plotting out experiments. It will be surreal to leave the world of science and embark on my new adventures in the legal world, and there will be many times I'll dearly miss the bench. And above all, I am so grateful to have had this opportunity: it has taught me a unique viewpoint of the world, one that will benefit me at all stages of my life. That said, it could not have been possible without the many people who have helped me throughout the stages of my doctoral studies.

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interactions, and to Jake Szamozi, whose magic with bioinformatics has allowed us to have a comprehensive overview of microbial communities. I'd also like to show gratitude to the staff at the Central Animal Facility, who care for our mice day in and day out. A special mention goes towards April, our very own mouse whisperer, who has cared for our precious old mouse colony as if they were her own; also, a huge thanks to Tammy, whose patience, help and understanding was a true asset when I was carrying out my pneumococcal colonization experiments. To our more far-flung collaborators: Dr. Jeffery Weiser at the University of Pennsylvania and Dr. Lester Kobzik at Harvard, to whom I am indebted for help with techniques beyond my skillset. And of course to Dr. Vickie Galea: teaching Physiotherapy Anatomy & Physiology, and interacting with students so keen to learn, has been one of my favourite and most rewarding experiences at McMaster. I will truly miss it.

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List of Abbreviations

The following abbreviations were used in addition to those abbreviations commonly accepted for units of measure and quantities.

Ab antibody

APC antigen-presenting cell

BAL bronchoalveolar lavage

BMDM bone marrow-derived macrophage

BSA bovine serum albumin

CCR C-C chemokine receptor

CD cluster of differentiation

CFU colony forming units

cGVHD chronic graft-versus-host disease

DC dendritic cell

DNA deoxyribonucleic Acid

ELISA enzyme-linked immunosorbent assay

FBS fetal bovine serum

GF germ-free

HIV human immunodeficiency virus

IFN interferon

Ig immunoglobulin

IL interleukin

i.n. intranasal

i.p. intraperitoneal

i.v. intravenous

KO knockout

LPS lipopolysaccharide

Ly lymphocyte antigen

M-CSF macrophage colony-stimulating factor

MCP monocyte chemotactic protein

MDP muramyl dipeptide

MHC major histocompatibility complex

mo month-old

NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells

NO nitric oxide

NOD nucleotide-binding oligo-merization domain-containing protein

r recombinant

PBMC peripheral blood mononuclear cell

PBS phosphate-buffered saline

Declaration of Academic Achievement

In accordance with the *Guide for the Preparation of Theses at McMaster University*, the research documented herein is presented as a "sandwich doctoral thesis". The articles presented in Chapters 2, 3 and 4 are three independent although conceptually related bodies of work that, as of September 2014, have been submitted for publication or are in press in peer-review journals. The work conducted in each manuscript required a collaborative effort with several colleagues, resulting in multiple authors. As such, my contributions to each article are highlighted below.

Chapter 2. Puchta A, Verschoor CP, Thurn T, Bowdish DME. Characterization of inflammatory responses during intranasal colonization with *Streptococcus pneumoniae*. *J Vis Exp.* 2014 Jan 17;(83):e50490.

This work was conducted over the period of September 2010 – November 2013. I am the primary author of this manuscript. I performed all experiments and analyzed and interpreted all the data, with exceptions as follows: the qPCR data in Figures 10-12 was generated by Dr. C.P. Verschoor and T, Thurn; Dr. C.P. Verschoor analyzed all the qPCR data. Experiments were conceived and designed by Dr. D. Bowdish and myself. Dr. D. Bowdish provided guidance and editorial input at every stage of the manuscript writing process, and helped revise the paper and respond to comments from the reviewers. The video portion of this manuscript was designed by me, including all production, shot direction, script writing, acting and post-production

editorial work. Video itself was shot by Wayne Bowdish, to whom I am indebted for his patience during the recording process.

Chapter 3. **Puchta A**, Naidoo A, Loukov D, Verschoor CP, Nguyen P, Kobzik K, Larché MJ, Xing Z, Bowdish DME. TNF-dependent increases in Ly6C^{high} monocytes occur with age and impair bacterial clearance. Submitted in June 2014 to *J Exp Med*.

This work was conducted over the period of September 2010 – June 2014. I am the primary author of this manuscript. I performed all experiments and analyzed and interpreted all the data, with exceptions as follows: the epigenetic analysis in Figure 1F was performed by P. Nguyen; the bone-marrow chimeras used in Figure 3A-B were generated by A. Naidoo; the qPCR data in Figure 5G was generated and analyzed by Dr. C. Verschoor and the qPCR data in Figure 6A was generated by D. Loukov. Experimental assistance was provided by graduate students (A. Naidoo and D. Loukov). Dr. L. Kobzik provided thoughtful discussions and helped review the manuscript. Dr. M. Larché, a clinical collaborator, provided the adalimumab used in the TNF blocking experiments. Dr. Z. Xing provided the TNF KO mice, provided invaluable insight and critically appraised the manuscript. Experiments were conceived and designed by Dr. D. Bowdish and myself. Dr. D. Bowdish provided guidance and editorial input at every stage of the manuscript writing process.

Chapter 4. **Puchta A**, Naidoo A, Verschoor CPV, Loukov D, Lee K, Jury J, Szamozi JC, Verdú E, Surette MG and Bowdish DME. Metchnikoff's theory of aging: the intestinal microbiota drives age-associated inflammation and impaired macrophage function. Submitted in September 2014 to *Science*.

This work was conducted over the period of January 2011 – July 2014. I am the primary author of this manuscript. I performed all experiments and analyzed and interpreted all the data, with exceptions as follows: A. Naidoo performed the MDP experiments in Figure 2E and analyzed the corresponding data. J. Jury performed the Ussing chamber assay and analyzed the data collected therein – this data is presented in Figure 2F and Figure 4F-G. The tissue used for Figure 2G was processed for DNA analysis by K. Lee, while the gene ontology analysis was conducted by Dr. C. Verschoor. The fecal sample sequencing and subsequent microbiome analysis was conducted by J. Szamozi, with data interpretation by Dr. D. Bowdish. Experimental assistance was provided by graduate students (A. Naidoo and D. Loukov). Dr. E. Verdú provided the young and old germ-free mice used in this work, helped analyze the gut histology and aided in design of the recolonization experiments. Dr. M. Surette provided critical insight into microbial sequencing techniques and host-microbiome interactions. Experiments were conceived and designed by Dr. D. Bowdish and myself. Dr. D. Bowdish provided guidance and editorial input at every stage of the manuscript writing process.

In addition to these three manuscripts, some of the work/concepts presented in this thesis were contributed to a book chapter. This is included as Appendix I.

Appendix I. Verschoor CP, **Puchta A**, Bowdish DME. The macrophage. *Methods Mol Biol*. 2012;844:139-56.

This article was researched and written over the period of February 2011 – November 2011. Article structure was designed by Dr. C. Verschoor, who wrote and prepared the manuscript with input from Dr. D. Bowdish. I contributed by evaluating the published literature and writing the section pertaining to murine macrophages. Figure 1 was generated by me; Figures 2 and 3 by Dr. C. Verschoor.

Chapter 1. Introduction

Inflammation and Age

Inflammation is a basic immunological response mediated by host cells via their production of immunomodulatory compounds, primarily cytokines, in response to pathogens, toxins, environmental allergens and tissue damage. Inflammation can be either acute or chronic(*1*). Initiation of the inflammatory cascade shifts physiological responses away from the basal norm, with the purpose of eliminating the causative agent, removing damaged tissue and initiating healing pathways(*2*). Many of the compounds produced to remove the causative agent are also capable of damaging bystander host tissue. Despite this, if inflammation is self-limiting, as is the case with acute inflammation, the response is largely a protective one, with the benefits outweighing the consequence of any short-term peripheral damage to the host. In contrast, chronic inflammation maintains altered physiological responses, prolonging the exposure of host tissue to harmful compounds(*3*). Systemic chronic inflammation can cause the pathogenesis of a wide variety of diseases, including type 2 diabetes, cardiovascular disease, obesity and some forms of cancer(*4*). Additionally, ample evidence demonstrates that normal aging in some species, such as humans and mice, is accompanied by a state of low-grade, systemic chronic inflammation, termed “inflammaging”. Investigation into how this state arises and how it affects morbidity and mortality in the elderly has become a major focus of aging research.

Acute Inflammatory Responses

The origin of inflammatory responses is perhaps best understood in the broad context of normal, acute inflammatory responses. Acute inflammation is a result of the coordinated delivery of leukocytes and plasma proteins to the site of infection or injury via blood vessels(*5*). Acute inflammation induced by an infectious agent is widely studied and well-understood; as such, it will be used in the below example.

During an infectious challenge, the initial inflammatory responses are mounted by innate immune cells, including tissue-resident macrophages, which produce cell-signalling molecules including (but not limited to) pro-inflammatory cytokines (which can activate leukocytes and trigger the systemic release of acute-phase proteins) and chemokines (which act as a chemotactic stimuli to recruit additional immune cells to the site of infection or injury)(*6*). The immediate result of these mediators is to establish a local inflammatory milieu. Neutrophils and recruited monocytes, two types of innate immune cells, are the first responders in many models of acute inflammation(*7*, *8*). These cells extravasate from the vasculature that they are normally restricted to, passing selectively through an activated endothelium, at the exclusion of erythrocytes(*9*).

Once they arrive, recruited neutrophils and monocytes attempt to kill the pathogenic agent via numerous mechanisms, including the production of reactive oxygen species, nitric oxide and anti-microbial peptides. They also induce autocrine and paracrine inflammation by producing additional cytokines. Although this response is essential for control of the invader, many of these molecules do not discriminate between intended target cells and bystander host cells, and thus their release may result in collateral damage to proximal host tissue(*10*).

Controlled inflammatory responses following pathogenic challenge are, in the case of many types of infection, indispensable for host survival. However, even during infections, inflammation must be balanced. Too little inflammation often proves to be ineffective in pathogenic control, but excessive inflammation may result in toxicity for the host, such as is the case with septic shock. Ideally, an acute inflammatory response will lead to sufficient inflammation to result in clearance of the infectious agent, followed by a resolution phase (and, if necessary, healing of host tissue)(*11*). Much of this process is carried out by resident and recruited macrophages, which switch from a proinflammatory phenotype to an anti-inflammatory phenotype, prompting their involvement in removal of dead cells, tissue remodelling and repair(*12*).

If the acute inflammatory response fails to eliminate the pathogen, the inflammatory process persists and undergoes further adaptation. Recruited neutrophils and monocytes are followed by additional waves of innate immune cells, as well as adaptive immune cells (T cells, B cells and ensuing antibody responses). If the combined efforts of these cells are incapable of removing the pathogen, a chronic inflammatory state develops.

Chronic Inflammation

In his seminal paper, "*Origin and physiological roles of inflammation*", Ruslan Medzhitov describes chronic inflammation as a state which arises consequent to the inability of a host's immune response to return to basal levels(*3*). In the case of acute infection, a successful acute inflammatory response can remove the inflammatory trigger, and return the system to basal conditions. In contrast, if the initiating trigger persists, then the resultant inflammation also persists, leading to a self-promoting inflammatory state that shifts the basal state more towards inflammation. In the absence of an exogenous trigger, Medzhitov argues that an inflammatory response will initiate whenever there is some level of tissue stress, damage and/or malfunction, with varying levels of magnitude dependent on the nature and degree of tissue stress. In such scenarios, tissues will start to transition from a basal state to low-grade inflammation (defined as existing between basal

levels and full-blown inflammation). If homeostasis cannot be restored and the tissue stress persists, there will be continued recruitment of immune cells that will gradually amplify the inflammatory response(*13*). The production of inflammatory mediators by these leukocytes can promote a mounting state of chronic inflammation, and subsequent collateral damage and tissue degeneration. Although this scenario describes the development of a generalized chronic proinflammatory state, it nonetheless provides a useful framework for conceptualizing specific chronic inflammatory states like inflammaging, the central topic of this thesis.

Inflammaging

The term "inflammaging" was first coined by Franceschi et al. in 2000(*14*) and refers to a low-grade, chronic proinflammatory state that occurs with age. A proinflammatory phenotype in aged humans and other mammals, such as mice, is evident in several ways: 1) levels of what are normally acute-phase inflammatory cytokines, including TNF, IL-1β, IL-6, IL-8 and C reactive protein, in the circulation are increased (*15*, *16*); 2) increased tissue expression of clusters of genes associated with inflammation (*17*–*19*); and 3) decreased inhibition of the transcription factor NF-κB (a master regulator of inflammatory responses)(*20*–*22*).

Elevations of proinflammatory cytokines in the serum, particularly TNF and IL-6, are strong independent risk factors for morbidity and mortality in older people

(>65yrd)(*23*). Increased systemic inflammation is correlated with several age-related diseases, including arthritis, dementia and cognitive impairment(*24*–*26*). In Alzheimer's disease, for example, autopsy studies have demonstrated that the brains of Alzheimer's disease patients have increased levels of proinflammatory cytokines as compared to an age-matched cohort without the disease(*27*). Similarly, hospitalization for cardiovascular diseases (including stroke, myocardial infarction and atherosclerosis) correlates with increased serum cytokines in the elderly; as do poorer outcomes during the course of the disease(*28*–*30*). In one study examining aged patients with atherosclerosis, IL-6 was lower in survivors as compared to those who died(*31*). Inflammaging is also a risk factor for the development of frailty (a marker of poor outcomes in the elderly described as a multifactorial condition that embodies sarcopenia, anemia, hormone imbalance, and compromised immune function)(*32*, *33*) and frailty-associated conditions, including osteoporosis(*34*) and loss of muscle mass(*35*). Most importantly, inflammaging is an indicator of overall mortality in elderly populations. For example, in a ten year study of an older cohort (65-100 years old), all-cause mortality was positively correlated with serum IL-6 and soluble TNF receptor(*36*). Consistent with this, a second study showed that in 5-year study of elderly adults (65 years and up), IL-6 levels in the serum were associated with a twofold greater risk of death (independent of age, sex, body mass index, and history of smoking, diabetes, and cardiovascular disease)(*37*).

Inflammaging is generally believed to be the consequence of the collective lifetime exposure to antigenic load that a host experiences(*38*). As the host progresses into old age, the cumulative effects of antigenic exposure (posited to include bacterial and viral sources) may initiate a vicious cycle where the basal state becomes shifted towards a chronic proinflammatory state(*39*). Ultimately, the identity of the antigenic factor that initiates inflammaging, remains unknown. Furthermore, although a clear link has been established between inflammaging and disease in the elderly, the main cellular players and molecular mediators that are involved remain to be elucidated. Understanding inflammaging, its roots and its repercussions, is a central question investigated in both Chapters 3 and 4 of this thesis.

Mononuclear Phagocytes

The mononuclear phagocyte system is a subgroup of leukocytes that consist of non-granulocytic, myeloid cells involved primarily in homeostasis and tissue repair in the steady state, as well as immune host responses during infection and tissue injury(*40*). These cells include blood-borne monocytes, specialized antigen-presenting dendritic cells (DCs) and tissue-resident macrophages(*41*, *42*). Monocytes and macrophages play an important role in immune defence against infectious agents, and in this capacity, they are primarily involved in the

phagocytosis of invading pathogens(*43*). Pathogen recognition by macrophages can be mediated by Complement receptor, which recognizes the opsonins that bind to microbes, as well as other pattern-recognition receptors, molecules that recognize conserved components of bacteria and viruses(*44*). Appendix I includes a book chapter with an extended section dedicated to the functional diversity and developmental background of macrophages(*45*).

Murine Monocytes

In mice, circulating monocytes constitute 1.5% of the total peripheral leukocyte blood pool during the steady state(*46*). Monocytes are defined as cells that express CD11b and the M-CSF receptor, CD115, and may also express the F4/80 antigen, particularly if they are in the process of differentiating into macrophages(*47*). Monocytes develop from myeloid precursors in the bone marrow (BM), prior to intravasation into the bloodstream(*48*). BM-resident multipotent hematopoietic stem cells can differentiate into progenitor cells that become restricted to either a lymphoid lineage or a myeloid lineage. Common lymphoid progenitor cells give rise to T, B and natural killer cells(*49*), while clonogenic common myeloid progenitor cells differentiate into either erythrocyte progenitors, or granulocyte/macrophage progenitors, with monocytes arising from the latter(*50*). In the differentiation process, monocyte commitment is influenced

primarily by the presence of the growth factor macrophage colony stimulating factor (M-CSF)(*41*).

Monocyte Subsets

Heterogeneous monocyte subsets have been identified across several mammalian species. In mice in particular, monocytes can subdivided based on their expression of the Ly6C antigen into separate $Ly6C^{low}$ and $Ly6C^{high}$ populations(*51*, *52*). Morphologically, the $Ly6C^{high}$ population is larger and more granulocytic than its $Ly6C^{low}$ counterpart(*47*, *53*) and expresses lower levels of chemokine surface receptor CX3CR1(*54*). In humans, monocytes exist as three distinct subtypes, differentiated by expression of CD14 and CD16. $CD14^{++}/CD16^{-}$ and $CD14^{++}/CD16^{+}$ are the phenotypical and functional equivalents of $Ly6C^{high}$ monocytes, while $CD14^{+}/CD16^{++}$ are phenotypically and functionally related to $Ly6C^{high}$ monocytes(*47*).

In mice, the two subpopulations of monocytes have a clear functional delineation. Adoptive transfer experiments have shown that $Ly6C^{high}$ monocytes are recruited to inflamed tissue where they undergo activation and act primarily in a proinflammatory capacity(*55*). In response to acute infection or injury, $Ly6C^{high}$ monocyte numbers in the blood compartment expand rapidly, likely in preparation for their accumulation to the localized site of injury(*46*). As the extent of injury/infection wanes, the circulating numbers of these cells decrease

accordingly(*46*, *56*). Ly6C^{high} monocytes also preferentially accumulate in the circulation during states of chronic inflammation, such as those induced during atherosclerosis, diabetes, cardiovascular disease and cancer(*57*–*59*). Once recruited to peripheral tissues in response to bacterial infection, Ly6C^{high} monocytes fulfill their proinflammatory role via the expression of molecules such as tumour necrosis factor alpha (TNF) and interleukin-6 (IL-6)(*55*, *60*, *61*). Chapter 3 offers original data that demonstrates Ly6C^{high} monocytes are significantly increased in age in the blood and bone marrow compartments, where they contribute to age-associated inflammation.

Ly6C^{low} monocytes, on the other hand, have been shown to participate in tissue maintenance during homeostasis, homing selectively to resting tissues where they can differentiate into resident macrophages(*53*). A recent study also described the functional role that these cells play in patrolling the bloodstream. A large fraction of Ly6C^{low} cells can crawl for long ranges along the endothelial layer of the vasculature, a process that allows them to survey the blood vessel wall for signs of damage(*62*, *63*). Additionally, reports suggest that Ly6C^{low} monocytes are actively recruited to sites of inflammation alongside their Ly6C^{high} monocyte counterparts, and may even precede their arrival in the early stages of the immune response(*62*). Although traditionally, monocytes have been thought of as transitional cells that act

as an intermediate between precursor cells and terminally differentiated tissue macrophages, these studies highlight their function as effector cells.

Of importance to monocyte mobilization, and the work presented in Chapter 3, is C-C chemokine receptor type 2 (CCR2), particular with regards to its involvement with Ly6C^{high} monocyte trafficking and how it impacts this process with age. Studies have shown that BM-derived Ly6C^{high} monocytes rely on the MCP-1 receptor, CCR2, to mediate egress from the bone marrow(*64*, *65*). Mice lacking this receptor have severely depleted circulating levels of all monocytes, and in particular Ly6C^{high} monocytes, while mice lacking the CXCR3 receptor (which is expressed in abundance on Ly6C^{low} but not Ly6C^{high} monocytes) show little modification of circulating monocyte numbers. As such, deployment of monocytes into the circulation from the bone marrow relies on CCR2 but is independent of CX3CR1. However, both these chemokines have been shown to be involved in cell homing to distal tissue sites during acute infection once monocytes are blood-borne(*64*).

Macrophage Populations and Function

Among their primary functions, macrophages can act in an effector cell capacity, producing key inflammatory mediators in response to antigenic stimuli, and are accordingly equipped with a broad-range of pattern-recognition receptors (PRRs). These PRRs are implicated in the production of an array of inflammatory and immunosuppressive cytokines, as well as the uptake of cellular debris and

pathogenic material(*66*). Additionally, macrophages are professional phagocytes involved in the recycling and clearance of erythrocytes during the steady state(*67*). They also play a key role in removal of apoptotic cells and the cellular debris generated in areas of tissue remodelling, and in this sense they can act as housekeeping cells. These processes are thought to occur independent of immune effector cell signalling, as it has been show that phagocytic receptors fail to induce transcription of cytokine-associated genes, and/or actively induce inhibitory cytokines(*68*).

Necrotic debris generated during acute trauma, infection or chronic stress is similarly cleared by macrophages, although uptake of this material dramatically alters a macrophage's expression profile and leads to its activation(*69*). Endogenous signals within the debris itself can act as stimuli to up-regulate the production of proinflammatory cytokines and chemokines. This process is mediated by PRRs, most of which signal through the adaptor molecule myeloid differentiation primary-response gene 88 (MyD88)(*70*, *71*).

Macrophages are prodigiously plastic cells that can rapidly shift their functional program in response to the cues generated after injury or infection(*72*). In this capacity, macrophages are highly influenced by signals derived from the microenvironment itself, although innate and adaptive immune responses can similarly influence a macrophage's phenotype(*73*). Two main phenotypic

classifications have been adopted in the macrophage literature, describing two extremes of macrophage expression profiles. M1 macrophages, or classically activated macrophages, designate the effector macrophages that arise during cell-mediated immune responses to pathogens and possess increased enhanced microbicidal activity(*74*). The M2 macrophage designation encompasses "alternatively activated macrophages" or so-called anti-inflammatory macrophages, which are involved primarily in wound-healing and regulation(*75*). It should be noted that these designations do not necessarily capture the nuances that exist between macrophage populations. Many macrophages, such as those discussed by Edwards *et al*.(*76*), lie in a more intermediate position on the M1/M2 spectrum, sharing overlapping characteristics and biological functionality with either extreme.

The role of classically activated macrophages in host defence has been well documented(*77*). These cells undergo a series of biochemical, morphological and functional modifications in response to cytokines such as interferon-gamma (IFN-γ) and TNF(*78*, *79*), or pathogen components such as lipopolysaccharide (LPS; a TR4 agonist), muramyl dipeptide (MDP; a NOD2 agonist), bacterial flagellin (a TLR5 agonist) and double-stranded RNA (a TLR3 agonist)(*80*–*83*). Classical activation is characterized by the synthesis of proinflammatory cytokines, including TNF, IL-1β, IL-6, IL-8 and IL-12(*84*). The production of mediators such as reactive oxygen

species (ROS) and nitric oxide (NO), which are microbicides (*85*) are also up-regulated in these cells.

Monocyte/Macrophage Differentiation

Identification of mechanisms of differentiation of tissue resident macrophages is an area of intense investigation and contention in the current literature. Although classically, the extravasation and differentiation of blood-borne precursors such as monocytes was considered the sole source of resident tissue macrophages, two additional mechanisms involved in replenishing macrophage numbers have recently been identified. These include the self-proliferation of cells in the resident compartment, and homing/proliferation of dedicated bone marrow derived precursors to resident tissues. It is not clear whether these mechanisms operate in a sequential manner, although it is likely none of them are mutually exclusive(*40*).

The inflammatory state of a tissue influences the route of macrophage differentiation employed. Studies have shown that during the steady state, the majority of adult resident tissue macrophages, including alveolar macrophages(*86*–*88*), splenic macrophages(*89*) and liver Kupffer cells(*90*), are maintained through local self-renewal, independent of circulating blood monocyte populations. In contrast, during times of inflammation, circulating monocytic precursors, and in particular the Ly6C^{high} subset, path to inflamed tissues and make substantial

contributions to the macrophage population in the respective tissue compartment(*91*–*93*).

Experimental evidence has shown that Ly6C^{high} monocytes are recruited to sites of injury in a murine model of skeletal muscle damage, where they differentiate into proliferating F4/80 expressing macrophages, and switch to an anti-inflammatory profile to support myogenesis and tissue repair mechanisms(*93*). In addition, Nahrendorf *et al.* reported that monocytes arriving to the injured heart tissue of mice had different functions that depended on the time course of injury. Ly6C^{high} monocytes that were recruited directly after initial injury acted in a phagocytic and proinflammatory capacity. The ensuing resolving phase was characterized by the arrival of Ly6C^{low} monocytes, which had attenuated inflammatory function and instead promoted tissue healing(*92*). In the lung, Landsman *et al.* demonstrated that resident alveolar macrophages originate from blood-borne monocyte precursors after cellular ablation, and that only Ly6C^{low}, but not Ly6C^{high} monocytes were capable of reconstituting this cellular population(*94*). Thus, monocyte functions and fates may differ based on an array of variables, including tissue compartment, localized status of infection/injury, as well as temporal factors.

Immunosenescence

Immunosenescence refers to the phenomena of decreased functional capacity that occurs in many leukocyte populations with advanced age. Age-specific defects have been observed in both the innate and adaptive arms of immunity, and are hypothesized to result in the susceptibility of elderly individuals to infection, as well as the increased incidence of chronic diseases with age(*95*). Thus, in order to develop appropriate preventative and therapeutic measures for the elderly in the context of infection and age-associated chronic disorders, it is necessary to understand the cellular and molecular basis of immunosenescence, and how these changes may alter immune responsiveness(*96*, *97*). Furthermore, immunosenescence fits into the greater paradigm of inflammaging, as it is generally believed that the exposure of immune cells to a chronic, age-associated inflammatory milieu is one of the main factors that promotes their functional deterioration(*98*, *99*). Understanding the basis for this relationship could provide new avenues for maintaining normal immune cell function with age.

Immunosenescence in Adaptive and Innate Immunity

In the adaptive arm of immunity, immunosenescence results in a remodelling of the T cell network, characterized by an inverted CD4/CD8 T-cell ratio alongside a diminution in naïve T cells and accumulation of differentiated memory cells in the

CD8+ T cell compartment(*100*). Total T cell numbers in the circulation are also decreased, which may be the result of bone marrow hematopoietic stem cells displaying a myeloid differentiation bias with age(*101*). Another key consequence of immunosenescence for adaptive immunity is poor responsiveness to new pathogens and the reduced efficacy of vaccine-induced protection against infection(*102*, *103*). Although generally, the production of antibodies at mucosal sites and in the serum is not affected with age, the efficacy of most antibodies is compromised, possibly due to defects in antigen processing and presentation and decreased affinity maturation leading to less effective binding(*104*). Finally, B- and T-cell effector responses are impaired resulting in reduced signalling, proliferation and cytokine secretion in response to an antigen(*105*).

Immunosenescence as it occurs in the innate arm of immunity has not been studied as extensively as its adaptive counterpart, and thus, in general, it is less characterized. Nonetheless, it has been demonstrated that there is no decrease in circulating neutrophil numbers and the ability to generate a robust neutrophilia in response to infection in the elderly is maintained(*106*) (with some evidence that neutrophil numbers may, in fact, be increased(*107*)). Despite this, neutrophil responsiveness to granulocyte-colony stimulating factor, a key neutrophil chemokine, is impaired. Whether this affects neutrophil infiltration to sites of infection remains controversial(*108*). Most aspects of microbicidal function in

neutrophils are compromised with age, including phagocytosis and reactive oxygen species production(*109*). Furthermore, neutrophils from elderly individuals have a shorter life-span and are more apoptotic than those from younger individuals, and thus have a reduced period of anti-microbial activity(*110*). There is a poor understanding currently of how neutrophil cytokine production changes with age, although phenotypically, neutrophils from elderly individuals are characterized by heighted expression of activation markers(*111*). In fact, in a viral infection model, activation of neutrophils from old mice was rapidly increased as compared to young mice(*112*). This was attributed to heightened IL-17 production by natural killer (NK) cells (lymphocytic members of the innate immune system that mediate cytotoxic anti-viral responses), leading to increased neutrophil apoptosis and ultimately resulted in higher mortality with age. In contrast, production of other cytokines (IL-8 and RANTES) by NK cells is reduced with age(*113*). Finally, although NK cells with age show an increase in absolute numbers, their anti-viral cytotoxic responses are decreased(*106*).

Immunosenescent changes in dendritic cells (DC), the major antigen-presenting innate immune cells, have also been detected(*114*). In aged donors, DCs were found to demonstrate no changes in total number or TLR-dependent cytokine production. DC migration and chemotaxis, however, were impaired with age; as were phago- and pinocytosis, thus decreasing their ability to present antigen(*114*).

Monocyte/macrophage Immunosenescence

Understanding immunosenescence as it affects both monocyte and macrophage populations is the major research focus of Chapters 3 and 4. Phagocytosis by macrophages isolated from the experimental wounds of aged mice has been shown to be impaired, resulting in a delay in removal of cellular debris from the site of injury(*115*). Alveolar macrophages have also been demonstrated to have a reduced capacity for phagocytosis(*116*). Several reports also suggests that a decline in the adherence, opsonisation and phagocytosis by peritoneal murine macrophages occurs with age(*117*, *118*). Interestingly, a recent study demonstrated that impaired phagocytosis could be induced in macrophages from young mice injected into the peritoneum of old mice, suggesting that age-related defects in phagocytosis may be induced by extrinsic factors in tissue microenvironment, such as increased levels of cytokines(*119*). The production of reactive oxide species, nitric oxide and inducible nitric oxide synthase at both the mRNA and protein level was diminished in aged peritoneal and splenic macrophages derived from mice in response to LPS(*120*) and IFN-γ(*121*). Similarly, alveolar macrophages from both aged mice and rats were found to have decreased nitric oxide production(*23*). Despite evidence of decreased phagocytosis and decreased NO and ROS production, it is unknown whether the ultimate purpose of these functions (microbial killing) is affected in macrophages with age. This is a question addressed in Chapter 4.

Paradoxically, recent evidence suggests that while anti-microbial functions may be diminished with age, proinflammatory capacity of monocytes and macrophages is, in fact, increased. For example, in human monocytes, age contributes to a phenotype marked by a functional shift towards a proinflammatory phenotype and reduced anti-bacterial function(*109*). Across several independent studies, circulating monocytes derived from healthy elderly donors have been demonstrated to constitutively produce more IL-6, IL-1β and TNF basally and with LPS stimulation(*122–124*). In a cross-sectional analysis of healthy women divided into four age-groups (22-31, 32-41, 42-51, and 52-63), an age-dependent increase in the ability of blood monocytes to produce TNF and IL-6 was observed, and it correlated positively with increases of total serum IL-6(*125*). Although increased cytokine production is apparent even in healthy agers, in a cohort of Alzheimer's patients, overproduction of proinflammatory cytokines by monocytes correlated positively with disease(*126*).

Although most of the above studies have focused on monocytes, recently Bouchlaka *et al*. examined the impact of age on the degree of cytokine production by macrophages, and found that, consistent with findings in monocytes, BM-derived macrophages from young and aged mice produced significantly higher TNF and IL-6 compared with young mice following LPS stimulation(*127*). Finally, the ability of macrophages to resolve inflammation via their production of suppressive cytokines

may also be attenuated with age. In a mouse model, alveolar macrophages displayed impaired production of IL-10, which was correlated with an accentuated inflammatory response following pulmonary challenge with carrageenan polysaccharides(*128*). As a whole, these studies suggest that aging contributes to the increased proinflammatory status of macrophages. In the studies conducted by Bouchlaka *et al.*, it was also demonstrated that macrophage depletion led to significant decreases in systemic TNF and IL-6, providing experimental evidence for the hypothesis that macrophages are a central cell type involved in the promotion of inflammaging.

Finally, there is experimental evidence that monocyte/macrophage differentiation may also be altered due to aging. With age, hematopoietic stem cells (HSC) have been shown to be increased in the bone marrow compartment(*129*). In these cells, self-renewal was increased, as was the capacity to generate committed myeloid progenitors, while the capacity to generate lymphoid progenitors was diminished(*130*). Furthermore, aged HSCs had an up-regulation of genes involved in specifying myeloid fate and function. These changes at the level of the HSC may have downstream effects, as skewing the lineage potential of HSCs from lymphopoiesis towards myelopoiesis with old age seems to result in an increase of circulating monocytes, and a decrease in circulating lymphoid cells(*101*). The consequences of

increased myelopoiesis and mobilization of monocytes to the blood with age are explored in Chapter 3.

Streptococcus pneumoniae

Streptococcus pneumoniae is a diplococcus, gram-positive, alpha-haemolytic, anaerobic member of the Streptococcus family that is the major causative agent of pulmonary pneumonia(*131*). *S. pneumoniae* possesses a polysaccharide capsule that protects the bacterium against host immune effector cells(*132*). 92 different strains (serotypes) are known, which differ in virulence, prevalence, and extent of drug resistance(*132*).

S. pneumoniae in the elderly

S. pneumoniae can cause a broad range of diseases, including pneumonia, meningitis, sepsis, otis media and sinusitis. The burden of disease lies primarily in the very young (<2 years) and the very old, with incidence increasing at around age 55(*133*). In the elderly, invasive pneumococcal disease manifests predominantly as pneumonia, leading to serious health problems in this population. In the United Kingdom, the annual incidence of pneumonia is approximately 75 cases for every 1000 people over 75 years of age, while the comparative incidence in young adults (18-39) is only 6 cases per 1000 people(*134*). The case fatality rate for individuals 75 years and older is 39%(*135*). Residents in long-term care facilities are especially

vulnerable, not only because of their advanced age and underlying co-morbidities, but also because they live in close mutual proximity and have extensive contact with a range of caregivers(*136*). The exact immunologic basis for the increased susceptibility of the elderly to invasive *S. pneumoniae* infection is unknown, but it is likely that immunosenescence, the down-regulation of immune function that occurs with age, plays a key role.

In Canada, the National Advisory Committee on Immunization recommends a one-time pneumococcal vaccination, consisting of a 23-valent vaccine directed against the polysaccharide coat of 23 capsular serotypes of *S. pneumoniae*, for all people over the age of 65 years(*137*). A recent meta-analysis conducted on the efficacy of this vaccine suggested that the vaccine is ineffective in preventing pneumonia infection in the elderly(*138*). Furthermore, the authors found no evidence of protection against the risk of death from pneumococcal infection and all-cause mortality for individuals of any age group(*138*). This led them to conclude that the vaccine does not appear to be effective in preventing pneumonia, even in populations for whom it is currently recommended, highlighting the need for novel prevention strategies.

Carriage of *S. pneumoniae* in the nasopharynx has been shown to be as high as 50% in infants, but declines progressively with age, and ranges from 1 – 10% of the healthy adult population (18-39 years)(*139*, *140*). In young, healthy adults,

carriage is typically transitional and asymptomatic, with individuals becoming colonized in periodic waves. Furthermore, colonization is seemingly not restricted to one particular subset of the population. In one study of nasopharyngeal carriage, 36% of all adult participants were positive at least once for colonization over the 10-month study period(*141*).

After a strain of *S. pneumoniae* colonizes the nasopharynx, clearance typically occurs 4-8 weeks after the original period of acquisition, although this carriage length is highly variable, and both host and pathogen factors may modulate this time course(*142*).

The Role of Monocytes and Macrophages in *S. pneumoniae* Colonization

Understanding the mechanisms behind the clearance of *S. pneumoniae* colonization in the elderly is important not only in the context of the affected individual themselves, but also against the scale of the community as a whole. These factors are likely to affect the frequency of horizontal transmission of the pathogen within the population, and thereby influence the overall incidence of pneumococcal disease(*143*). Furthermore, identification of the immune defects that lead to increased and/or prolonged nasopharyngeal colonization in the elderly may provide potential targets for therapeutic intervention, as colonization at this level is likely a preceding step to more invasive disease. Chapter 2 of this thesis provides an overview of the *S. pneumoniae* intranasal colonization model as developed in mice,

to allow for the understanding of bacterial clearance kinetics, immune cell recruitment and inflammatory responses. Additionally, Chapter 2 provides a comprehensive overview of a model system of study that allows for the study of nasopharyngeal colonization, and resultant secondary infection, in a manner that is comparable to physiological *S. pneumoniae* infection in humans.

In experimental mouse carriage studies, clearance is temporally unassociated with the antibody response(*143*). Similarly in humans, nasopharyngeal colonization generates only minor amounts of antibodies directed against pneumococcal capsular polysaccharides(*144*). A recent set of studies conducted by the Weiser group identified the importance of innate immune responses, but not humoral adaptive immune responses in clearance of *S. pneumoniae* from the nasopharynxes of genetically modified mice. In these experiments, μMT mice (which are unable to produce specific antibodies) and TLR4 knockout mice intranasally colonized with *S. pneumoniae* were able to clear colonization with a similar efficacy to parental C57BL/6 mice(*145*). In contrast, TLR2 knockout mice, and major immunohistocompatability complex-II (MHC-II) deficient mice (which are functionally T-helper cell deficient and show decreased CD4+ T-cell counts) were unable to clear colonization(*143*). Furthermore, clearance occurred independent of levels of complement component 3, inducible nitric oxide synthase, IL4 and IL-12(*143*).

In a follow-up study, this group linked the role of CD4+ T-cell function in the clearance of a particular *S. pneumoniae* strain, P1121, from the nasopharynx to recruitment and retention of monocytes and macrophages in this area(*146*). Tracking the cellular response in wild-type mice inoculated with *S. pneumoniae* demonstrated that although an influx of neutrophils was recruited early-on and peaked at day 3 post-inoculation, this acute inflammatory response was insufficient to clear the organism from the nasopharyngeal mucosal surface(*147*). Monocytes and macrophages, but not neutrophils, isolated from the nasopharynx were shown to associate with the colonizing pneumococcus(*146*). Furthermore, monocyte/macrophage numbers (which peaked at day 7) correlated with the steady decline in the density of colonizing *S. pneumoniae*, and mice that had their monocyte/macrophage populations depleted with clodronate could not clear colonization efficiently. CD4+ T cell depletion was associated with poor monocyte/macrophage recruitment to this compartment, thereby delaying the time to clearance.

Th17 cells, a subset of CD4+ T cells, were shown to be particularly important in eliciting the monocyte/macrophage response, via their generation of IL-17A, a cytokine responsible for governing neutrophil and macrophage characterized inflammation during infection in the respiratory tract(*148*, *149*). This occurred in a manner mediated by MCP-1, a chemokine generated in response to IL-17A(*150*),

although these effects were likely additive to the endogenous potential of IL-17 to exert direct chemotactic effects on blood monocytes itself(*151*). As IL-6 transcription has been shown to be up-regulated in the nasopharynx in response to pneumococcal colonization early on, and is known to contribute to the induction of IL-17 expressing cells(*152*), IL-6 may also play a contributing role.

Previous reports have demonstrated that specific antibody coating of the target pneumococcus does not mediate macrophage recognition of the pathogen(*153*). Although complement components may play a role in mediating pathogen uptake in an opsonin-mediated manner by macrophages in other tissues(*154*), clearance from the nasopharynx of strain P1211 has been shown to occur independent of levels of complement component 3(*146*). In addition, surface components of macrophages, such as the macrophage receptor with collagenous structure (MARCO), have been demonstrated by our research group to be crucial for the recognition and clearance of *S. pneumoniae*(*155*).

Although the importance of monocytes and macrophages in host defense during nasopharyngeal *S. pneumoniae* colonization has been demonstrated, very little is known about the mechanisms underlying the recognition and phagocytosis of bacterial pathogens by these cells. Furthermore, the attenuation/alteration of these responses in the aged host organism has had limited study. Chapter 3 provides novel insights to this end.

Inflammaging and Pneumonia

In addition to ample evidence that inflammaging correlates with chronic disorders in the elderly, emergent studies indicate acute susceptibility to pneumonia, as well as outcomes as a result of pneumonia may be influenced by inflammaging. A longitudinal analysis conducted over 6.5 years of a cohort of 70- to 79-yr-old healthy, community-dwelling elderly individuals found that pre-infection systemic levels of TNF and IL-6 were associated with higher risk of pneumonia requiring hospitalization(*156*). A study of patients hospitalized with pneumonia found that IL-6 and TNF levels correlated with disease severity (based on APACHE II scores) upon admittance, and that levels of IL-6 on day 3 and day 5 post-admittance were decreased in survivors as compared to patients who died(*157*). Elderly individuals who are hospitalized for chronic inflammatory conditions are also at increased risk for the development of pneumonia(*158*). Mouse models support these findings; for example, infusing young mice with TNF systemically increases their susceptibility to *Streptococcus pneumoniae* (*116*). Although mechanistic understanding of the reasons as to why higher systemic inflammation leads to increased susceptibility to pneumococcal infection is lacking, one recent study suggests that age-associated levels of TNF are capable of altering macrophage function(*159*). The authors demonstrated that TNF had a negative effect on phagocytic capacity in macrophages, which ultimately impaired control of *S.*

pneumoniae lung infection in old mice. In this thesis, we provide findings that demonstrate TNF and monocytes govern impaired bacterial clearance from the nasopharynx with age in Chapter 3. We also demonstrate that TNF can negatively modulate bacterial killing of *S. pneumoniae* in aged macrophages in Chapter 4.

The Intestinal Microbiota

The human intestinal microbiome, which represents an evolutionarily adapted ecosystem, is extensive, and has been estimated to include 10^{14}microbes(*160*). Two bacterial phyla, the *Firmicutes* and the *Bacteroidetes*, comprise an overwhelming majority of the bacteria of the gut microbiome in humans, representing over 99% of its inhabitants(*161*). This dominance pattern is conserved in mice(*162*). The complex microbial community residing in the gut is essential for metabolic functions(*163*), epithelial maintenance(*164*), mucosal immune defenses(*165*) and the development of mature, full-spectrum systemic immunity(*166*). The symbiotic relationship between the host and intestinal microbes can be considered to exist in a homeostatic state, which is continuously being adapted and fine-tuned(*167*). Understanding the gut microbiome, particularly in the context of the aged host, is the research focus of Chapter 4.

The study of the microbiota can be traced back to the early 1900s, when the microbiologist and father of macrophage biology, Elie Metchnikoff, proposed that

the gut microbiota may play a role in aging(*168*). Metchnikoff observed that the longest lived, healthiest people in the world, including the Hunzas of Kashmir, Georgians of Eastern Europe and Bulgarian peasants, regularly consumed fermented food rich in bacteria. Metchnikoff focused much of his study on the Bulgarians, with their exceptionally long average life spans, which he attributed to their daily consumption of fermented milk products. Metchnikoff postulated that with age, the toxins produced by the microorganisms in the gut were responsible for the aging process. Dysbiosis (a microbial imbalance) promoted exposure of the body to bacterial by-products, which he termed "toxins". The crossing of these by-products from the gut to the blood allowed them the opportunity to activate phagocytes, leading to the destruction of body tissues, which ultimately advanced the aging process(*169*). Metchnikoff believed that by exercising strict control over diet and nutrition, one could influence the nature of gut microbiota, limit production of their toxic by-products and thus delay aging(*170*). In Chapter 4 of this thesis, we provide novel data that provides experimental evidence for Metchnikoff's early theory that the gut microbiota itself is responsible for advancing aging via passage of microbial by-products from the intestines to the circulation, resulting in inflammaging and tissue damage.

Gut Microbiota Composition with Age

Symbionts in the microbiota promote a constant state of mild immune activation that contributes to the physiological inflammatory tone in the gut, which helps preserve tolerance for the microbial community while at the same time cultivating its membership(*171*). It has been proposed that, under pathological conditions, the normal relationship between gut microbes and gut immune cells can become disrupted, tipping the scales from a mild inflammatory tone to overt inflammation. Decreased representation of bacterial species important for modulation of inflammation permits for more pathogenic microbes to have an increased representation in the microbial community and can ultimate increase intestinal inflammation(*172*). Additionally, because many pathobionts (bacteria present at low quantities in the gut, which can outgrow and cause infection) can induce rapid and overt inflammation in the gut, outgrowth of these bacteria may further contribute to inflammation in a self-sustaining loop(*173*). Thus, understanding what alterations microbial communities in the gut undertake with age is important for understanding how they may, in turn, influence inflammation with age.

Initial exposure to the microbiome occurs *in utero*, with the community reaching maturity by the age of three(*174*). Although the gut microbiome is generally stable in adults, several shifts in its composition have been noted to occur

with age. The ratio of the two dominant bacterial phyla, the *Firmicutes* and the *Bacteroidetes*, is globally altered with age, decreasing from 10.9 in healthy adults (25 to 45 years) to 0.6 in the elderly (70 to 90 years)(*175*). Studies investigating the quantity of specific bacterial genera have shown that there is relative decrease in beneficial anaerobes like *Bifidobacteria* and *Lactobacillus*, and an increase in *Enterobacteria*(*176*, *177*). Other studies have identified that in addition to this, species diversity in genera such as *Bacteroides, Prevotella* and*, Bifidobacteria* is also decreased, which is paralleled with increases in diversity of more pathogenic genera, including, notably *Clostridium*(*178*, *179*).

Although the above studies were conducted in healthy elderly individuals, studies examining microbial changes in elderly individuals with frailty found that frailty correlated negatively with the abundance of gut *Lactobacilli*, *Bacteroides* and *Prevotella* and positively with *Enterobacteria*(*180*). Hospitalization in elderly individuals was similarly associated with decreases in *Bacteroides* and *Prevotella* and increases in *Enterobacteria*(*181*). Another study found correlations between dysbiosis and health status, as well as prevalence of co-morbidities, in elderly subjects(*182*). This data suggests that age-associated dysbiosis of the gut microbiota occurs along a continuum of both age and health status, from the healthy elderly on one end, and the so-called “unhealthy agers” on the other.

Inflammaging and the Microbiota

The consequences of age-associated shifts in the microbiota are largely unknown, although it has been demonstrated that symbiotic bacteria identified to be decreased with age (*Bacteriodes*, *Bifidobacteria* and *Lactobacillus*) possess immunosuppressive properties and are important for maintaining immune tolerance in the gut(*183*, *184*). Conversely, pathobionts that are increased with age have been shown to promote overt gut inflammation(*185*, *186*). Thus, it can be speculated that an optimal composition of microbes is essential for maintaining gut health, while dysbiosis may be maladaptive for the host, shifting the balance between protective symbionts and opportunistic pathobionts in favour of the latter(*187*).

Beyond Metchnikoff's early hypothesis and observations, there is emerging evidence to suggest that the human microbiome can impact immune phenotypes and inflammatory status not just locally in the intestines, but systemically as well(*166*). Based on this, it may be hypothesized that age-associated immunomodulation, and thus the establishment of inflammaging, is mediated by alterations in the intestinal microbiota. One study demonstrated that the amount of *Bifidobacterium* species was negatively correlated with serum levels of TNF in elderly subjects(*188*). Furthermore, circulating TNF levels in these individuals were

down-regulated following direct oral administration of *Bifidobacterium*. A second study demonstrated that centenarians had a gut microbiota with an increased representation of pathobionts, which was positively correlated with an increase in proinflammatory cytokines in the blood, including notably IL-6 and IL-8(*189*). In accordance with this, a third study found that changes in gut microbiota in unhealthy agers (long-stay nursing home elderly and hospitalized elderly) correlated with increased levels of serum IL-6 and TNF, as compared to healthy community-dwelling elderly(*182*). In spite of these recent findings, a direct involvement of the gut microbiota in the development of inflammaging has not been identified, particularly since it is not clear whether age-related changes in the gut microbiota are a cause of increased inflammation, or a consequence of it. In Chapter 4, we address this deficiency by providing data that demonstrates that the development of inflammaging is dependent on the presence of a gut microbiome.

In healthy adults, the intestinal epithelium provides a barrier between the gut microbes and the rest of the host. However, even in these individuals, a low-level of translocation of bacteria and their products occurs constitutively(*173*). Thus, increased representation of gut bacteria that have the capacity to more strongly activate the immune system upon translocation (such as occurs with age) could result in systemic activation of the immune system. At the same time, even microbes normally considered symbionts may lead to systemic immune activation if the

epithelial barrier in the gut becomes compromised. This could allow for leakage of bacteria and their products beyond physiological norms, resulting in increased antigenic load. In fact, failing epithelial integrity and large-scale microbial translocation have been implicated in the pathogenesis of numerous disorders, including chronic host-graft disease, liver disease and human immunodeficiency virus(*190–193*). Interestingly, younger people with controlled HIV infection, who show elevated levels of serum cytokines that correlated with increased microbial translocation, also have been found to suffer from so-called "premature aging". This state of premature aging is characterized by immunosenescent cells and increased incidence of age-associated cardiovascular and neurologic disorders(*194*), and provides support for the notion that the microbiota can act as a source of accelerated aging. Consistent with this observation, Chapter 4 provides novel data that demonstrates that loss of epithelial barrier function in the gut occurs with age, is linked to increased translocation of bacterial products into the circulation, which results in systemic inflammation.

Central aim and hypotheses

The fundamental goal of inflammation is to protect the host against pathogens, even at the cost of tissue damage at the host's expense. Nonetheless, it is crucial that inflammatory processes be tightly regulated to prevent unnecessary damage to the host. However, the aging process is well characterized to be accompanied by a chronic state of systemic inflammation that is correlated with increased incidence of chronic disease, as well as, paradoxically, infection. Despite the established incidence of inflammaging in the elderly population, the answer to the questions *"what are the consequences of inflammaging for host immunity?"* and "*what are the initiating and propagating drivers of inflammaging?"* remain elusive. This thesis investigates the ontogeny of inflammaging, providing novel insights into key molecular, cellular and microbial factors influencing its development. It also explores the consequences of inflammaging, linking a systemic state of heightened inflammation to alterations in monocyte/macrophage frequency and function, which ultimately impair anti-bacterial responses in the aged host.

To investigate the role of inflammaging and immunosenescence on monocytes/macrophages in infection, we first established a model appropriate for the study of infectious disease in aged animals. Due to its high disease burden and unique mortality profile in the elderly, we chose *Streptococcus pneumoniae* as our model

pathogen of bacterial infection. To employ a model that was as clinically relevant as possible, we developed a nasopharyngeal model of colonization, which recapitulates the necessity for preliminary intranasal colonization as a prerequisite to secondary invasion to the lungs or bloodstream in human infection by *S. pneumoniae*, the technical details of which are described in Chapter 2. Much work remains to be done characterizing the nature of the immune response with regards to the involvement of specific cell types and inflammatory mediators elicited in this type of model, and virtually little is known about how these responses are altered with age. To this end, in Chapter 2 of this thesis, we hypothesized that the kinetics of immune cell trafficking and local nasopharyngeal cytokine/chemokine production changed dynamically throughout the course of *S. pneumoniae* colonization in response to bacterial load, and that strains of differing virulence could influence the length of colonization and degree of invasive disease. Three clinical isolate strains were used to assess carriage lengths and incidence of invasion to the blood and lungs. Moreover, the evolution of immune responses was characterized at key time points throughout colonization, uncovering the relationship between colonization status, immune cell recruitment and the production of local immune mediators.

As mentioned earlier, inflammaging is strongly linked to pathogenesis during many chronic disorders, as well as heightened acquisition of, and poorer outcomes during infectious disease. In many diseases of chronic inflammation, Ly6C^{high}

inflammatory monocytes are heightened and closely involved in pathogenesis. Based on these two observations, in Chapter 3, we hypothesized that Ly6C^{high} monocytes are increased in the aged host during the steady state as a result of inflammaging and can contribute to pathology following exposure to *S. pneumoniae.* We found that increased TNF production with age increased numbers of Ly6C^{high} monocytes in the blood via its up-regulation of CCR2. We also demonstrated that these cells could then further contribute to inflammaging via their production of IL-6 and additional TNF. Our data showed that this increase could be reversed by use of anti-TNF, which may have important therapeutic implications for bacterial immunity with age, as we also found that Ly6C^{high} monocyte trafficking to the nasopharynx during *S. pneumoniae* colonization impaired bacterial clearance in old mice.

Although inflammaging has emerged as a central concept in the aging and immunity fields, very little is understood about its origins. Using insights from Metchnikoff's, "*The Prolongation of Life: Optimistic Studies*", in Chapter 4 we hypothesized that translocation of the intestinal microbiota is able to provide a source of antigenic stimulation for systemic immune cells, thereby establishing a state of chronic, low-grade inflammation that promotes immune dysfunction and ultimately the aging process. We showed that, with age, increased systemic hyper-inflammatory responses, tissue damage and defects in macrophage function are apparent. TNF KO mice (protected from the development of inflammaging) and germ-free (GF) mice

(lacking an intestinal microbiota), were protected from these age-associated changes. Combined with evidence that age results in disrupted barrier function in the intestinal epithelium and increased circulating microbial products, this work provides experimental evidence for the theory that the gut microbiota plays a crucial role in the origin of inflammaging.

Collectively, the work in this thesis offers novel mechanistic insights into how inflammaging is initiated and promoted, thus providing a framework for the development of new intervention strategies that could protect the elderly from infectious disease and promote healthy aging.

Chapter 2.

Characterization of inflammatory responses during intranasal colonization with *Streptococcus pneumoniae*

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Characterization of inflammatory responses during intranasal colonization with Streptococcus pneumoniae.

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Short Abstract:

Colonization of the murine nasopharynx with *Streptococcus pneumoniae* and the subsequent extraction of adherent or recruited cells is described. This technique involves flushing the nasopharynx and collection of the fluid through the nares and is adaptable for various readouts, including differential cell quantification and analysis of mRNA expression *in situ*.

Long Abstract:

Nasopharyngeal colonization by *Streptococcus pneumoniae* is an essential prerequisite to invasion to the lungs or bloodstream[1]. This organism is capable of colonizing the mucosal surface of the nasopharynx, where it can reside, multiply and eventually overcome host defences to invade to other tissues of the host. Establishment of an infection in the normally lower respiratory tract results in pneumonia. Alternatively, the bacteria can disseminate into the bloodstream causing bacteraemia, which is associated with high mortality rates[2], or else lead directly to the development of pneumococcal meningitis. Understanding the kinetics of, and immune responses to, nasopharyngeal colonization is an important aspect of *S. pneumoniae* infection models.

Our mouse model of intranasal colonization is an adaptation from human models[3] and has been used by multiple research groups in the study of host-pathogen responses in the nasopharynx[4,5,6,7]. In the first part of the model, we use a clinical isolate of *S. pneumoniae* to establish a self-limiting bacterial colonization that is similar to carriage events in human adults.

The procedure detailed herein involves preparation of a bacterial inoculum, followed by the establishment of a colonization event through delivery of the inoculum via an intranasal route of administration. Resident macrophages are the predominant cell type in the nasopharynx during the steady state. Typically, there are few lymphocytes present in uninfected mice[8], however mucosal colonization will lead to low- to high-grade inflammation (depending on the virulence of the bacterial species and strain) that will result in an immune response and the subsequent recruitment of host immune cells. These cells can be isolated by a lavage of the tracheal contents through the nares, and correlated to the density of colonization bacteria to better understand the kinetics of the infection.

Protocol:

Before you begin: all steps are done in a Biohazard Level 2 (BSL2) Biological Safety Cabinet (BSC) unless otherwise stated. Please ensure that you have obtained the appropriate Biohazard Approval for use of infectious bacterial pathogens as per institutional guidelines prior to initiation of the experiments. Additionally, please ensure that you have all the materials and reagents necessary to conduct the procedure prepared beforehand. Mice used in these experiments have included female C57BL/6 mice from Jackson Laboratories, Charles River or Taconic and were 10-14 weeks of age (although we have not found any gender-dependent significant differences in kinetics of nasal colonization clearance or infection). All mice used in these experiments were bred and maintained under specific-pathogen free conditions, and

were free of common viruses, (LCMV, MNV,MPV, reovirus ECTV and other) bacteria(e.g. *H. pylori)* and parasites (e.g. pinworm, ectoparasites)by fecal sample testing as well as frequent anatomical assessment of sentinel mice co-housed within their facility rooms. When conducting these experiments, we recommend using control mice no younger than 10-12 weeks of age and no older than 6 months of age. Mice younger or older than this age range are more susceptible to longer nasopharyngeal carriage duration and increased likelihood of disseminating infection. Mouse background is another important consideration that may impact the outcomes of a colonization experiment, as several groups have demonstrated that mice of different genetic backgrounds have different susceptibilities to the *S. pneumoniae* D39 (serotype 2) strain[9,10]. *S. pneumoniae* is not a naturally occurring murine pathogen and its only natural reservoir is the human nasopharynx. Transmission occurs via respiratory droplets, and as mice do not produce respiratory secretions, individual mice cannot transmit the bacterium to other mice, so there is no concern for mouse-to-mouse transmission[11].

1) Preparation of *S. pneumoniae* culture

1.1) Inoculate 5 ml of tryptic soy agar for the suspension growth of *Streptococcus pneumoniae.*

1.2) Culture under static conditions at 37 °C in 5% CO_2 until the bacterial inoculum reaches log phase growth with a corresponding liquid density of 10^8 CFU/ml as determined by an odometer set to 600 nm. The exact reading corresponding to this CFU will differ depending on the specific selected bacterial strain; for most strains of *S. pneumoniae* this corresponds to an OD_{600} range of 0.45-0.55. Typically, *S. pneumoniae* strains in liquid culture will grow to this density within 1.5-2.5 hours under the recommended conditions, with no need for sub-culturing. The culture should not be allowed to

overgrow (beyond an OD reading of 0.75) as this represents the point at which the bacteria are no longer in log phase growth and are undergoing extensive autolysis.

1.3) Each mouse will be inoculated with approximately 10^7 bacteria. Therefore, for every 9 mice to be colonized, pipette 1 ml of inoculum into an Eppendorf tube and spin at 15000 g for 1 minute. A whitish pellet should be visible. Remove the supernatant, being careful not to disturb the pellet and resuspend the bacteria in 100 µl of phosphate buffered saline (PBS), thus increasing the concentration to 10^9 CFU/ml. At this stage, the bacteria should remain viable, but will not readily replicate.

1.4) If using multiple aliquots, combine into one tube to control for slight inter-sample variations in bacterial density.

1.5) Keep bacteria on ice until ready for inoculation, for a maximum of 1 hour.

1.6) To obtain an exact bacterial count, perform logwise serial dilution series starting with neat bacterial inoculum. Dilute serially 10-fold, adding 10 µl of to 90 µl of sterile PBS.

1.7) Plate out 3 drops of 10 µl samples of dilutions 10^{-5} – 10^{-9}, plus a PBS-only contamination control, onto separately labeled sections of tryptic soy agar (TSA) plate supplemented with 5% sheep's blood (Figure 2). Ensure that pipette tips are changed for each step diluting from a higher CFU concentration to a lower CFU concentration to avoid carrying over excess bacteria and increasing variability of results. Human blood agar (HBA) plates may also be used in lieu of TSA. Since many strains of *S. pneumoniae* are resistant to neomycin (from 5 µg/ml – 20 µg/ml), this antibiotic may also be added to the agar medium of choice during the plate preparation phase. This facilitates enumeration as it eliminates non-resistant bacteria. The antibiotic susceptibility of each strain must be tested in advance to determine the optimal concentration of antibiotic to use for each bacterial strain.

1.8) Allow to dry for 15-30 minutes uncovered, then cover plates and place upside down in bacterial incubator set to 37°C and 5% CO_2. Grow up bacterial colonies on plate for 24 hours.

1.9) Determine number of colony forming units and their corresponding concentration. Based on determinations of the OD_{600} value, concentration should be within the range of 1 - 4 x 10^9 CFU/ml. *S. pneumoniae* colonies should appear as small, circular colonies a yellowish-beige in colour, with a small depression at the center giving them a donut-like appearance (Figure 3).

2) Murine intranasal colonization

2.1) Restrain mice by placing them into a mouse restrainer apparatus (a modified 50 ml Falcon tube with the tip cut off to create an aperture) securing them by the base of their body with thumb so that their noses just emerge out of tapered end of the restrainer apparatus (Figure 4). Use of this apparatus allows for immobilization of the mouse's head and segregation of its nares in a manner that minimizes movement as well as impedes attempts from the animal to masticate the pipette tip, allowing for complete delivery of the inoculum. Alternatively, mice can be immobilized via scuffing at the neck and manual restraint. We do not recommend anesthesia of the animals prior to intranasal inoculation. Administrating the inoculum to animals under anesthesia results in some of the inoculum spreading to the lungs[12],[13].

2.2) Using a P10 or P20 pipette, inoculate each mouse by depositing 10 µl of the prepared culture, distributing it evenly between both nares (allow inoculum to drip into nose by pulsing the inoculation gradually, taking time for mice to inhale inoculum). To achieve complete delivery of the inoculum, pause administration at any point the mouse begins to move its nose excessively. The entire inoculum may not be injected into the nares as the mice may expel some through the nose during exhalation; however, as the expelled amount tends to be minuscule, and the inoculum contains an extremely high amount of bacteria, this does not significantly affect the colonizing bacterial load. Additionally, the surface area available for colonization in the nasopharyngeal mucosa is limited and consequently we and others have found that the recommended 10^7 dose is sufficient to obtain consistent levels of bacteria in all mice, resulting in minimal variability in initial amounts of colonizing bacteria[14],[15].

2.3) Weigh mice if utilizing weight indicators as part of your end-point monitoring. Monitor mice every 12-24 hours for clinical symptoms, including lethargy, ruffled fur and weight loss. Mice typically will not show symptoms of illness until 3-5 days post-colonization, and these will be preceded by weight loss which may average around 5% of total body weight a day. As the mice become increasingly ill they will assume hunched postures and show decreased activity and decreased responsiveness to stimulation, including handling. At this stage, illness is typically indicative of sepsis and/or pneumonia and will likely be terminal, although mice may be treated with 1 ml of subcutaneous saline daily to improve outcomes. Surviving mice should start showing improvement after day 7 post-colonization, as evidenced by stabilization of weight followed by weight gain, although different strains of *S. pneumoniae* may induce illness more quickly and result

in progression of clinical symptoms along a different time-line. Please see Figure 5 for a representative result of weight tracked in mice colonized with the P1547 strain.

3) Nasal lavage sample collection

Before beginning: prepare cannulated needles using 1 ml syringes capped with 26 3/8 gauge beveled needles. Cut 2.5 cm pieces of PE20 polyethylene tubing with an inner diameter 0.38mm, ensuring that each end has a beveled tip. Using forceps, slide a 2.5 cm long piece of PE20 polyethylene tubing (inner diameter 0.38mm) on to the needle tip, avoiding puncturing the tubing side. Cannulated needles can be kept in 70% ethanol until needed.

3.1) Euthanize experimental mice. As cervical dislocation can potentially damage the trachea, this method of euthanasia must be avoided. Our preferred method is isofluorane anesthesia followed by exsanguination, however, ensure you follow institutional guidelines when selecting mode of euthanasia.

3.2) Using 70% aqueous ethanol, sterilize the superoanterior fur of the animal, particularly the neck, taking care to prevent ethanol from accessing the nares.

3.3) Make a single longitudinal cut along the midline of the neck of the animal, and two horizontal cuts on either end, creating an opening to envision the trachea.

3.4) Carefully peel back skin to either side, revealing neck tissue beneath.

3.5) Trachea should be visible, surrounded by longitudinal muscles on either side. Carefully snip these to provide a clear view of the trachea itself, taking care not to sever the surrounding vasculature.

3.6) If the vasculature was cut and blood is present, prior to proceeding, allow bleeding to stop, and then cleanse the area several times by dispensing sterile PBS and using sterile gauze to gently soak up excess moisture in the area.

3.7) Once trachea is properly exposed, make a transverse, semilunar cut in the trachea about half-way up (Figure 6).

3.8) Draw up 1000 µl of sterilized PBS into previously prepared cannulated needle.

3.9) Insert cannula into the trachea towards the nose, keeping the beveled edge pointing downwards for ease of insertion (Figure 7). Once needle is in place, rotate it 180 degrees, and gently probe upwards until you feel light resistance.

3.10) Place Eppendorf designated for sample collection just beneath the nose of the mouse.

3.11) Test correct placement of needle by dispensing a minimal (~20 µl) amount of PBS lavage fluid – drop of fluid should form around the nares of the mouse; if this is the case, proceed to step 3.13).

3.12) If test PBS emerges directly out the mouth of the animal, pull the cannula back, and reposition by again gently probing forward until very slight resistance is felt – take care not to push cannula too far past this resistance, as you will move it past the nasal palate and through to the oral cavity.

3.13) Dispense contents of needle rapidly to help displace and collect maximal amount of cells – contents should flow out through the nares of the mouse and into collection tube. Place sample immediately on ice.

3.14) To collect samples for RNA analysis, repeat steps 3.8) – 3.13) using a cannulated needle containing 500 µl of RNA lysis buffer on the same mouse. This will allow the collection of lysate sample from the remaining cell populations, largely composed of the nasopharyngeal mucosal epithelium, as non-adherent cells should have been removed following the initial PBS lavage. Please note that the RNA lysis buffer will denude the epithelium and destroy surrounding tissue, so care must be taken to avoid contact with organs such as the lungs, if retention of these tissues is desired. Once collected, place sample in RNA lysis buffer directly on dry ice to snap-freeze. Once in lysis buffer, samples can be stored as per the manufacturer's instructions, and are stable typically at 70°C for several months.

4) Determination of bacterial load in the nasopharynx

4.1) Quantitate bacteria by preparing a serial dilution series for each murine nasal lavage sample. In general, bacterial load can be expected to be between 0 – 10^4 CFU, therefore conduct three 10-fold serial dilutions. Add 10 µl of the neat nasal lavage

sample (10^0 CFU/ml) to the first tube to a concentration of 10^{-1} CFU/ml. Vortex thoroughly.

4.2) Divide bacteriological plate into quadrants, and label quadrants each with a member of the dilution series (10^0 CFU/ml - 10^{-3} CFU/ml). Plate out 3 drops of 10 µl samples of the 3 dilutions and the neat sample on tryptic soy agar plates supplemented with 5% sheep's blood, as in Figure 2.

4.3) Allow to dry for 15-30 minutes uncovered, then cover plates and place upside down in bacterial incubator with optimal conditions for bacterial growth (typically 37°C and 5% CO_2).

4.4) Grow up bacterial colonies on plate for 18-24 hours.

4.5) Determine number of colonizing bacteria by averaging the colonies formed on plate for each dilution (Figure 3).Figure 8 demonstrates bacterial density during different time points, as determined by culture of nasal lavages, in mice colonized with 3 different strains of *S. pneumoniae* for up to 21 days.

5) Preparation of samples for flow cytometry

Before beginning: Prepare mix of antibodies. For quantification of leukocyte populations, we recommend the following mix at the specified dilutions: PE-Ly6G (clone 1A8, 1 µg/ml), FITCLy6C (clone AL-21,1 µg/ml), eFluor 450-CD45 (clone 30-F11, 2.67 µg/ml), APC-F4/80 (clone
PM8 RUO, 0.67 µg/ml), PerCP-Cy5.5-CD11c (clone N418 RUO, 0.5 µg/ml), PE-Cy7-CD11b
(clone M1/70, 0.33 µg/ml), Alexa Fluor 700-CD3 (clone 1782, 4 µg/ml), eFluor 605NC-CD4 (clone GK1.5, 6.67 µg/ml). Please note that this mix is 2x concentration (see step 5.5). All antibodies should be diluted in FACs Wash buffer (0.5% fetal calf serum, 2mM EDTA, 0.1% sodium azide in PBS) which should also be prepared beforehand. In a mixture of isotype matched control antibodies, ideally from the same supplier as the labeled antibodies and at the same concentrations as the specific antibodies, should be prepared. The samples treated with the isotype control antibodies will function as the negative control. Any fluorescence observed in the samples treated with the isotype control antibodies should be considered background.

5.1) Pre-chill a centrifuge capable of spinning 1.5 ml Eppendorf tubes to 4°C.

5.2) Centrifuge nasal lavage samples at 2000xg for 10 minutes at 4°C. Carefully pipette out supernatant and reserve. Note: due to the small amount of cells within the nasopharynx, the cell pellet will not be visible unless there is undesired red blood cell contamination, which will bright red. If this is seen, sample should be discarded.

5.3) Re-suspend sample in 50 µl of FcγRIIb/CD16-2 (2.4G2) antibody (which binds Fc receptors and reduces non-specific antibody binding) in FACs Wash Buffer at a concentration of 4 µg/ml.

5.4) Incubate sample on ice for 30 minutes.

5.5) Add 50 µl of pre-prepared 2x concentrated fluorescent antibody mix to sample. Set aside a representative sample from each experimental group to act as an isotype control. Add the isotype antibody mix to this sample in lieu of stain mix.

5.6) Incubate sample on ice for 1 hour.

5.7) Centrifuge samples at 2000xg for 10 minutes at 4°C. Discard supernatant and re-suspend in 200 µl of PBS.

5.8) Repeat step 5.7.

5.9) After the second wash, centrifuge samples again at 2000xg for 10 minutes at 4°C.

5.10) Re-suspend in either in PBS (if running sample immediately) or 2% paraformaldehyde (if running samples 1-3 days post-staining).

5.11) When conducting flow cytometry collect the maximum amount of events per sample or until the entire sample has been aspirated. In uninfected, healthy, young mice, this will be as little as 1000-2000 total events; during a bacterial colonization event, this number may increase more than 2-to-5-fold depending on disease status in the animal and factors such as age and genetic background. Figure 9 shows representative flow cytometry results
collected with a 3-laser Becton Dickenson LSRII flow cytometer using a Forward Scatter of 450 and Side Scatter of 300, although we recommend optimizing parameters for the specific flow cytometer you intend to use prior to sample collection. Note: if a sample contains even trace amounts of blood contamination, the total events collected will be significantly higher than expected and the sample should be discounted from analysis.

6) Quantitative PCR (qPCR) analysis of nasal lavages

6.1) Thaw cell lysates from step 3.14 room temperature.

6.2) Follow the recommended protocol provided with the preferred RNA extraction of choice.

6.3) After completing RNA extraction procedure as instructed, quantify the amount of RNA using a spectrophotometer or electrophoresis based method (Figure 10). We routinely obtain between 975 and 3250 ng total RNA per sample with a 260/280nm ratio of >1.7 or an RNA integrity number (RIN) around, 8.1 ± 0.13.

6.4) Transcribe cDNA using the M-MULV Reverse Transcriptase according to the manufacturer's protocol with 1000 ng of RNA (maximum 13 µl).

6.5) Dilute resulting cDNA samples 8x, and aliquot equally into 4 separate tubes for long term storage at -20 or -80 °C.

6.6) To measure gene expression by qPCR, prepare 25 µl samples reactions in triplicate on ice or cold block containing: 12.5 µl of 2x qPCR master mix from qPCR kit of your choice, 0.25 µl reference dye, 2 µl of diluted cDNA (step 7.5), 1 µl of mixed forward and reverse primers (400 nM final), 9.25 µl of RNAse-DNAse free water. This protocol is an adaptation of previously published methods[16].

6.7) In general we find that a two-step qPCR amplification (95°C x 10 min followed by up to 40 cycles x [95°C x 15 s, 60°C x 1 min]) is effective (Figure 11a); however, each primer pair must be optimized. Dissociation (melting) curves must be performed following amplification to ensure that no non-specific amplification occurred. amplification (Figure 11b)

6.8) We routinely run standard curves for each gene analyzed as well as a standard calibrator (derived from lung or spleen homogenate) for each 96-well plate analyzed. Relative transcript amounts are obtained by first normalizing raw cycle threshold (Ct) values by the reference dye and transforming the resultant values through the respective standard curve. These relative amounts are subsequently normalized to the standard calibrator and a housekeeping gene, as applicable.

Representative Results:

Figure 1 represents an overview schematic summarizing the main steps of the protocol. Figures 2-3 provide visualization of the microbiological methodology inherent to the protocols described herein. Figure 4 represents proper positioning of a mouse to perform an intranasal colonization, while figure 5 depicts typically changes in weight of mice colonized with *S. pneumoniae* strain P1547. Figures 6-7 represent specific stages of the nasal lavage portion of the process, for assisted visualization of these two techniques. Figures 8-11 consist of representative results of analyses conducted on samples collected from the nasopharynx of a mouse following nasal lavage. Specifically, Figure 8 is a representative result of bacterial load in the nasopharynx, as determined through culturing of nasal lavages obtained from mice colonized either with *S. pneumoniae* strain P1121, P1547 or P1542. Figure 9 represents cell phenotyping of isolated nasopharyngeal immune cells using flow cytometric techniques. Figures 10-11 display representative results pertaining to expressional analysis of nasopharyngeal mRNA via quantitative PCR.

Discussion:

In this study we presented detailed methods for the intranasal colonization of mice using a clinical isolate strain of *Streptococcus pneumoniae* and the subsequent isolation and characterization of the immune cells recruited to nasopharynx in response to this bacteria. We demonstrated how a bacterial inoculum can be cultured in nutrient-rich media and used to

establish a colonization event in mice restricted initially to the nasopharynx via delivery through the nares while the animals are unanesthetized. We then showed how responding immune cell types that are localized to nasopharynx can be isolated following tracheal exposure, incision and a nasal lavage through the use of a cannulated needle. Nasal lavage samples can be collected in PBS to isolate intact, lightly adherent cells; the RNA from more tightly adherent cells and surrounding epithelial mucosal layer can be isolated by applying a secondary wash consisting of RNA lysis buffer. The former of these samples can then be used to phenotype the specific cells recruited in the context of the colonization via flow cytometry techniques, while the latter can be applied to Q-PCR analysis, to determine the effector functions of these recruited cells by looking at the transcriptional expression of immune regulators of interest. Nasal lavage samples can additionally be used to determine the kinetics of clearance of a bacterial colonization event comparing different experimental groups to address specific research questions.

Utilization of this method of intranasal colonization allows for the establishment of a colonization event that is initially limited to the nasopharynx of the animal. Any subsequent dissemination of the bacteria to the blood or organs therefore occurs secondary to breaches in the immune defences localized within the nasopharyngeal mucosa. The stepwise progression achieved through this model reflects more accurately the process of pneumoccocal invasion in humans, allowing one to study the dynamics between the colonizing bacteria and the host nasal mucosa – and perhaps better understand shifts in bacterial pathogenicity and/or host immunity that allow for the development of disseminating disease. This is in contrast to models

that forgo the establishment of an initial colonization event and elect to study invasive disease in isolation through direct delivery of the bacterial inoculum to the lungs via intratrachael instillation, to the blood via vascular injection or to the peritoneum via peritoneal injection.

Conducting a PBS nasal lavage following a colonization event allows for isolation of non- or mildly-adherent cells recruited to the nasopharynx, as well as any mucosally-associated bacteria. It should be noted, however, that this technique is limited as it will not release cells or bacteria that have travelled between or beneath the epithelium, nor will it allow for the harvesting of cells or bacteria that have localized to the nasal-associated lymphoid tissue (NALT), a lymphoid organ that has been reported to be a potential site of infection following a pneumococcal colonization[17,18]. If further study of the NALT is desired, we recommend microdissection and removal of this tissue wholesale for study following PBS nasal lavage; as these two techniques are not mutually exclusive, they may be conducted on the same animal. However, due to the lytic and destructive nature of the RNA harvesting step (the secondary lavage using RNA lysis buffer), this step should be omitted if intending to harvest the NALT. Although the nasal lavage is a less technically challenging procedure, for groups wishing to obtain a more comprehensive assessment of bacterial load that includes not only mucosally-associated bacteria, but also those that have invaded the nasopharyngeal tissue, we suggest harvesting the nasopharyngeal tissue following removal of the upper skull bone of colonized mice and dissection of the tissue within the nasal conchae, as described by others[19].

The nature of an elicited immune response is dependent on the interaction between host and pathogen. Over 90 serotypes of *S. pneumoniae* have been characterized to date, all with

differing levels of pathogenicity and virulence factor expression, resulting in differential prevalence in the human population[20,21,22,23]. Similarly, in mice, it has been reported that the extent of, and kinetics associated with, the immune response elicited in response to a nasopharyngeal colonization is dependent on the colonizing strain itself[24]. Thus, selection of an appropriate strain to utilize for the establishment of a nasopharyngeal colonization is not a trivial matter, nor is selection of mouse genetic background. Figure 8 provides sample data that depicts the kinetics of clearance of a nasopharyngeal colonization from 3 different *S. pneumoniae* strains following intranasal colonization of female mice on a C57BL/6 background. Table 1 provides an overview of the degree of virulence and length of colonization time expected (when utilized on the C57BL/6 background) with 4 *S. pneumoniae* clinical isolate strains described in the literature and known to be capable of establishing a nasopharyngeal coloninization[25]: the avirulent P1121 (serotype 23F)[26,27], the low-virulence P1542 (serotype 4)[28], the mid-virulence P1547 (serotype 6A)[29,30,31] and the widely-used, well-characterized, highly virulent D39 (serotype 2)[32,33 34,35,36]. If the experimental goal is to strictly study an asymptomatic nasal colonization event with no accompanying bacterial dissemination to other tissues, we recommend use of the avirulent P1121 strain, which is characterized as a potent colonizer, as longer colonization events (up to 28 days prior to observed clearance) are a hallmark of this strain. Typically mice colonized with P1121 will run no risk of invasive disease and will display no clinical indicators of illness (with the exception of temporary weight loss). The remainder of the strains should be employed depending on desired degree of virulence and associated mortality, with virulence taken to mean not the degree of infection that

develops within an individual mouse, but rather the proportion of mice that display clinical signs of illness. It should also be noted that typically, degree of virulence correlates inversely with length of colonization duration, with more virulent strains colonizing for a shorter period of time. All 3 of the described virulent strains lead to mortality in mice due to, most commonly, sepsis, with fulminant pneumonia, or concurrent pneumonia and sepsis developing in a subset of mice. The differences in localization of invasive bacteria may be strain specific, as it has been previously reported that certain strains show tropisms for particular organs[37]. In a small percentage of animals, spontaneous meningitis may also develop following colonization. Determination of cause of death, as well as degree of invasiveness, can be accomplished via collection of associated tissues (lungs, spleen and/or brain) from animals at endpoint. Homogenization of these tissues and subsequent plating can indicate presence of invasive bacteria and corresponding titres.

An example of a bacterial culture density quantification is shown in Figure 3. If the culture is too concentrated, colonies grow too densely to be individually counted, however colonies derived from single cells can be distinguished if a logwise dilution series is plated. Plating three technical replicates per dilution minimizes variability. Please note that when quantifying bacteria retrieved from a nasal colonization event, one may encounter co-cultured contaminants, representing other bacterial species concurrently isolated from the murine nasopharynx. If the bacterial strain of interest has any known antibiotic resistances (for example, many strains of *S. pneumoniae* are resistant to gentamycin or neomycin up to 5 µg/ml), one can minimize the

incidence of contaminants by supplementing the growth media with the antibiotic at an appropriate concentration, thereby limiting contaminant growth.

Flow cytometry can be used to analyze cell surface markers on nasal lavage samples. For example, for the analysis of cell types recruited in the context of an infection, a mix of antibodies specific for the gross differentiation of leukocytes, including macrophages ($F4/80^+$), neutrophils ($CD11b^+$ and $Ly6G^+$) and T-cells ($CD3^+$ and $CD4^+$ or $CD8^+$), can be used as previously published[38]. Furthermore, these analyses can be combined with flow cytometric analysis conducted on different tissues or blood, to better understand immune cell trafficking during the course of an infection. Due to the limiting number of cells (typically numbering in the low thousands) that can be isolated from the nasopharynx, identifying rare subsets is typically challenging, although researchers wishing to accomplish this should consider pooling samples from multiple mice to achieve desired cell counts. Furthermore, because a finite number of cells can be extracted from this region, we recommend analyzing this data with regards to total cell numbers.

Although protein expression levels are typically low in the nasopharynx limiting the possibility of assaying protein production, it is possible to analyze the production of host molecules in response to the colonizing bacteria at the RNA level. To accomplish this, nasal lavages can be conducted using RNA lysis buffer in lieu of PBS, which allows for analysis of gene expression. For qPCR amplification detection, it is important to run a corresponding dissociation curve (Figure 11) to ensure the correct and desired product was detected. This is due to the fact that the assay will detect any double stranded DNA including primer dimers, contaminating DNA, and PCR product from mis-annealed primer.

We hope the methods described here will encourage you to apply an intranasal colonization model to study host responses to pathogens important in the context of this understudied region. For certain human pathogens, such as *S. pneumoniae,* a preceding nasopharyngeal colonization event acts as an important precursor to ensuing bacterial dissemination and the fatal sequelae that may follow, including propagation into the lungs, which may lead to pneumonia, or else to the blood, and resultant bacteremia and septic shock. Thus, by studying bacterial colonization in this region, we may understand better how to control it, and prevent more serious pathology from occurring altogether.

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Figures:

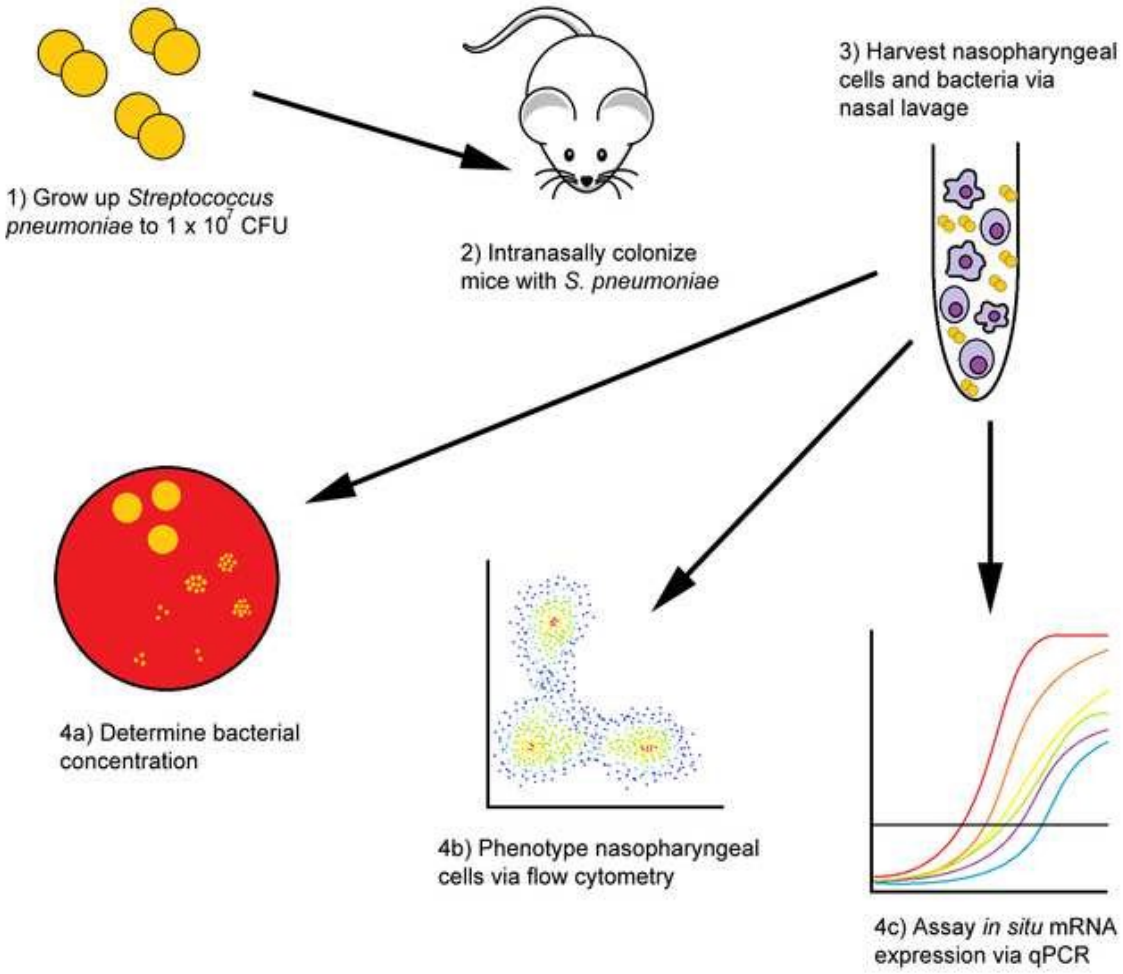


Figure 1: Flow chart of the intranasal inoculation and nasal lavage cell isolation procedures using a mouse model. First, the bacteria are prepared for inoculation, and then given to murine subjects intranasally. After the desired length of time elapses, mice are euthanized via terminal bleed, and their nasopharyngeal cells are isolated via two nasal lavage steps: a PBS wash step followed by a secondary wash in RNA lysis buffer. The cells from the preliminary PBS wash are isolated and analyzed using flow cytometry techniques, while RNA isolated from the second sample can be used to investigate the relative abundances of molecules of interest at the transcriptional level.

Figure 2: To determine bacterial concentration, 10 µl drops are plated in triplicate on a plate divided into sections representing a different serial dilution. These drops are then allowed to dry and the plates are incubated overnight at 37°C, 5% CO_2.

Figure 3: Concentration of *Streptoccous pneumoniae* isolated from the nasopharynx of a representative animal. Each discrete colony represents one colony forming unit, each collection of colonies represents one 10 µl drop (plated in triplicates) and each quadrant on the plate represents a separate serial dilution. Bacterial concentration is determined in CFU/ml by averaging the number of countable, fully formed colonies within, and then between, qualifying quadrants.

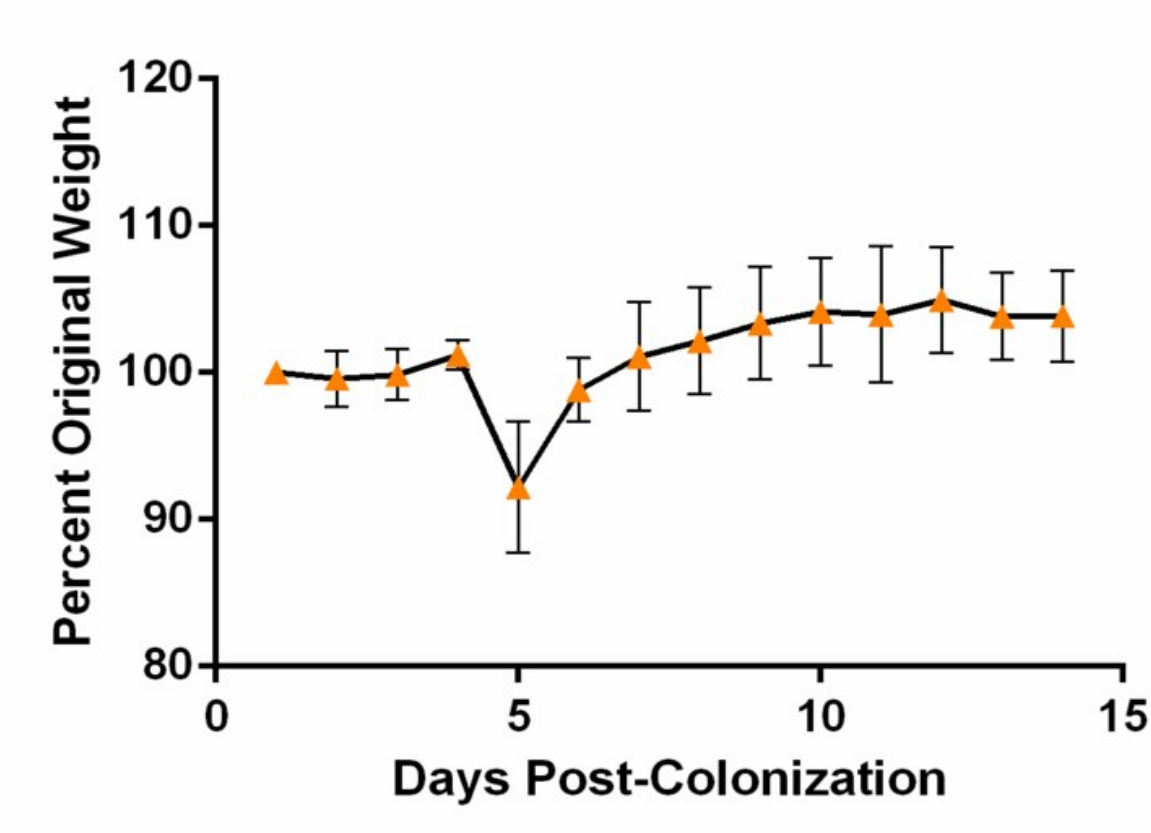


Figure 4: The movement of any mouse to be inoculated must be minimized, particularly at the neck, to allow for proper delivery of bacterial inoculum. To accomplish this, subject mouse is restrained in a modified restriction apparatus consisting of a 50 ml Falcon tube with an aperture at its tapered end. The mouse is then positioned so that its nose emerges from the aperture, where it can be accessed by the researcher, allowing for intranasal inoculation to be performed.

Figure 5. Weight of mice colonized with strain P1547 from a minimum of 2 representative experiments tracked daily following initial inoculation (n=6) to depict typical changes in weight expected following nasopharyngeal colonization. Weight is shown as a percent change of initial weight. Please note the expected sharp initial weight loss seen between days 3-5, followed by stabilization and gradual increase in weight in surviving mice.

Figure 6. Upon tracheal exposure, flanking longitudinal muscles are removed carefully prior to tracheal incision in a manner that does not severe the surrounding blood vessels. A small, semilunar incision is then made halfway up the trachea using fine surgical scissors. It is important to cut through the diameter of the trachea only partially, leaving it intact posteriorly.

Figure 7: Insertion of the cannulated needle into the tracheal aperture upwards towards the nose. Once cannula is in place, probe gently until resistance is met, then flush contents out through the nares.

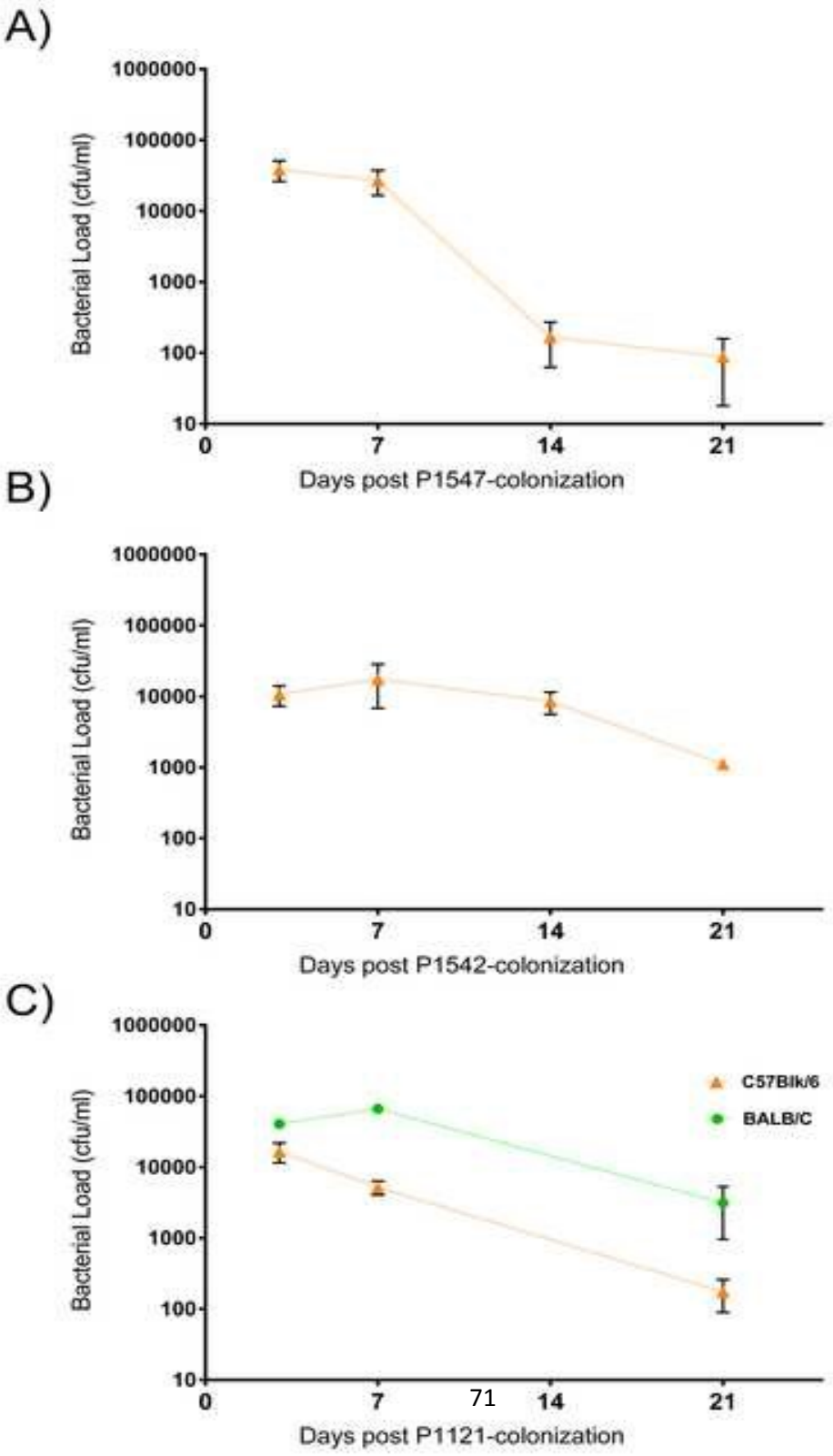

A)
Bacterial Load (cfu/ml)
1000000
100000
10000
1000
100
10
0
7
14
21
Days post P1547-colonization
B)
Bacterial Load (cfu/ml)
1000000
100000
10000
1000
100
10
0
7
14
21
Days post P1542-colonization
C)
Bacterial Load (cfu/ml)
1000000
100000
10000
1000
100
10
C57Blk/6
BALB/C
0
7
14
21
Days post P1121-colonization

Figure 8. A representative series of bacterial load isolated from the nasopharynx using the nasal lavage procedure described following colonization of C57BL/6 mice (triangles) with *S. pneumoniae* strain P1547 (A), P1542 (B) or P1121 (C). A comparative colonization of BALB/C mice (circles) following P1121 colonization is also displayed in panel C). Different time points are shown throughout the course of colonization, including days 3, 7, 14 and 21. Generally, a high initial load is expected at day 3, with little diminishment at day 7. Clearance is typically initiated by day 14, with full or near-full clearance evidenced by day 21 following colonization with most strains.

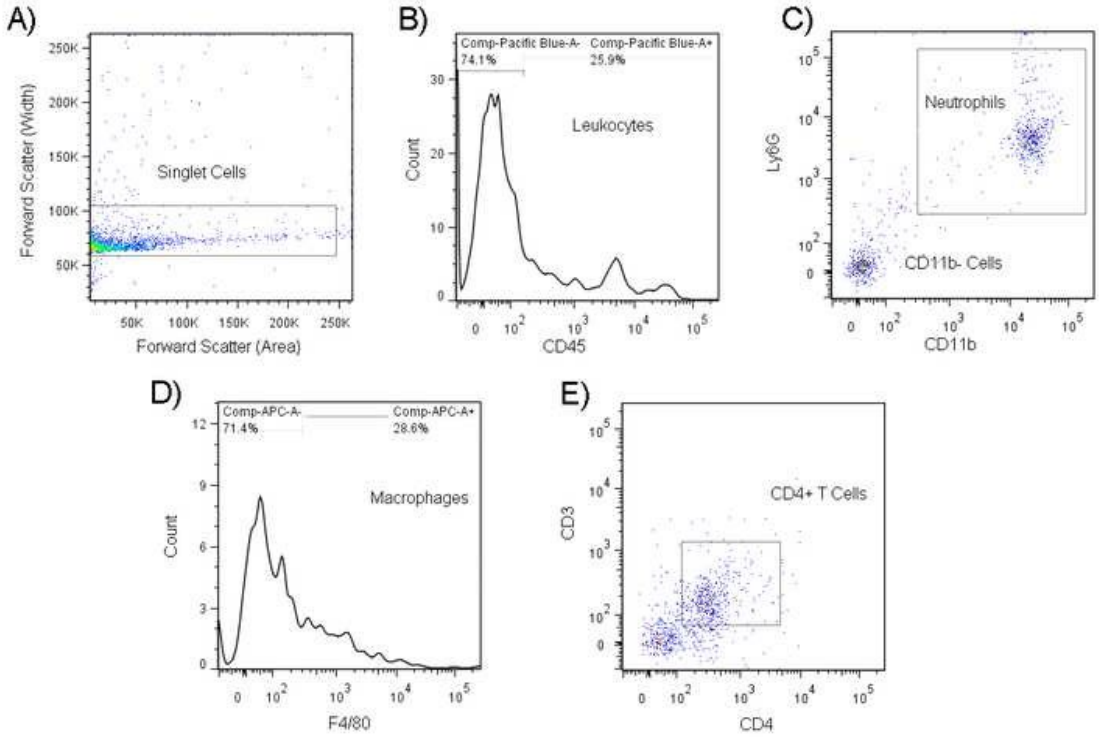
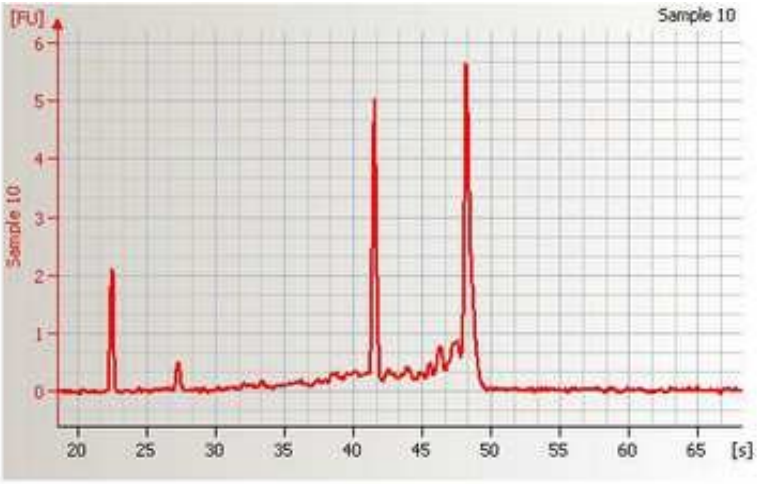
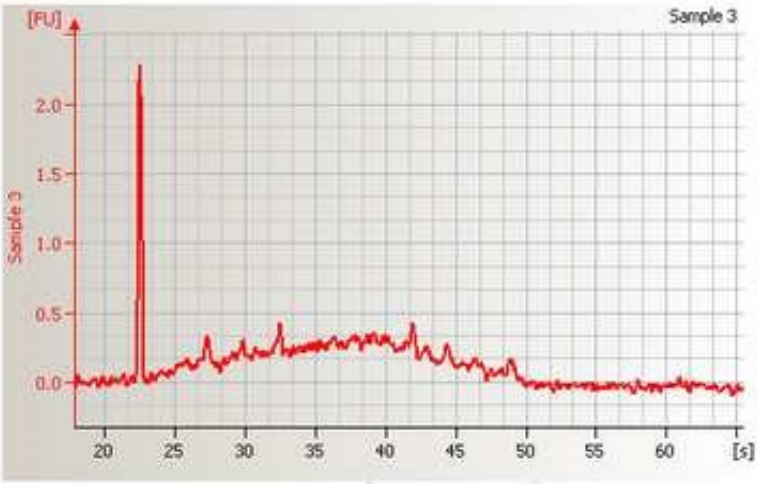


Figure 9: Representative histogram (A) and dot plot (B) of total cells isolated from murine nasal lavages as analyzed by flow cytometry. The differential expression of markers on cell populations allows for the identification of leukocyte subsets through the use of flourescent antibodies directed against these proteins. As shown here, leukocyte populations are selected by first gating on singlet cells using a Forward Scatter (area) versus Forward Scatter (width) gate (Panel A), and then enriching for CD45+ cells within that subset (Panel B). This population can be further subdivided into specific cell types by gating for CD11b and Ly6G double positive neutrophils (C). Analysis of the CD11b- population can be conducted to reveal F4/80+, CD11bmacrophages (D) or CD11b-, CD3 and CD4 double positive CD4 T cells (E). Cell populations can be phenotyped as long as they express either one, or a combination of several unique surface receptors that can be used to distinguish them from other cell types

A)


Sample 10
[FU]
Sample 10
0
1
2
3
4
5
6
20
25
30
35
40
45
50
55
60
65
[s]

B)


Sample 3
[FU]
Sample 3
0.0
0.5
1.0
1.5
2.0
20
25
30
35
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[s]

C)

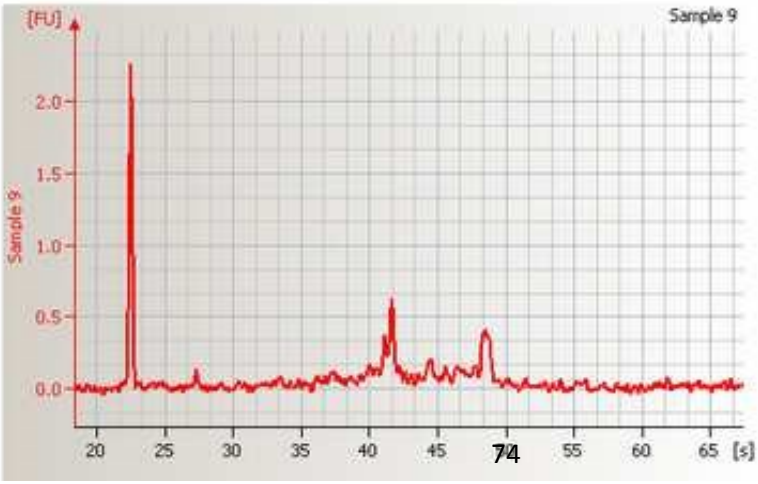
Sample 9
[FU]
Sample 9
0.0
0.5
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Figure 10: Representative electropherogram following electrophoresis automatic sequencing of a sample isolated from murine nasal lavages. The resulting electropherogram shows the quantitation data and the characteristic signature of a high quality total RNA sample derived from the nasopharyngeal region. When conducting analyses of total RNA, the areas under the RNA peaks for the two major ribosomal RNA, 18S and 28S, are used to calculate their corresponding ratio. Significant changes in the ratios of peaks attributable to 18S and 28S are typically indicative of degraded RNA. The degree of degradation can be summarized by RNA integrity number (RIN); the RIN for this representative sample is 8.1. An example of highly degraded RNA is shown in B) and C), and the subsequent RIN is 1.9 and 4.6, respectively.

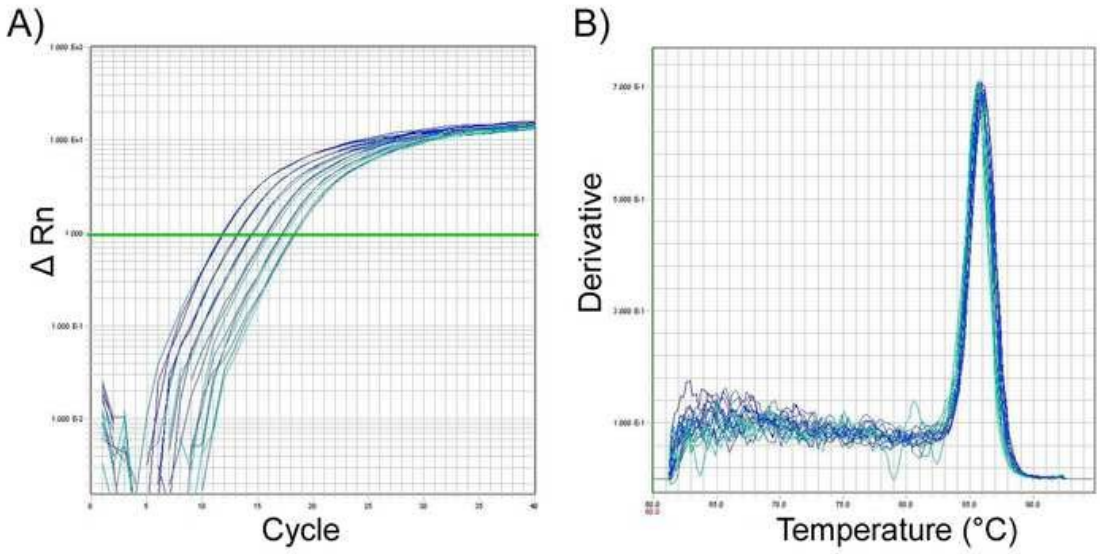

Figure 11: Amplification plot (A) and dissociation (melting) curve (B) from qPCR analysis of nasal wash cell lysates, providing an example of how these two readouts should typically look following an efficient and correctly detected amplification of mRNA products isolated from the murine nasopharynx. Represented is a standard curve for the housekeeping gene 18S. The results displayed in (A) show the desired PCR product following amplification using primers against GAPDH. The line represents the cycle threshold (Ct). The point at which the amplification plots corresponding to different samples cross this threshold allows for comparison across samples, with lower values corresponding to higher amounts of RNA of interest contained therein. The plot in (B) shows that the maximum melting temperature of the qPCR product is 85 C° and that there are no contaminating products present in this reaction, which would show up as an additional peak separate from the desired product peak.

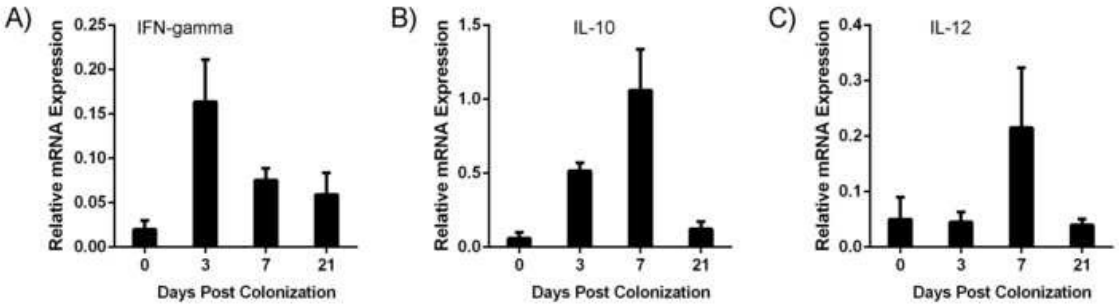

Figure 12: Production of elevated levels of pro-inflammatory cytokines in response to *S. pneumoniae* during the course of colonization. RNA was isolated from the nasopharynx of mice during the course of colonization. In general, peak cytokine production of proinflammatory cytokines (IFN-gamma) occurs earlier during colonization, whereas anti-inflammatory IL-10 production peaks at day 7. By day 21, coinciding with clearance, most cytokines have returned to basal levels.

Tables:

Strain Name	Serotype	Virulence	Mortality in Mice	Expected Colonization Duration
P1121	23F	Asymptomatic	0%	21-28 days
P1542	4	Low	0-20%	21-28 days
P1547	6A	Mid	20-50%	14-21 days
D39	2	High	70-100%	14-21 days

Table 1. A tabular overview of 4 commonly employed *S. pneumoniae* clinical isolate strains, their corresponding serotype number, associated degree of virulence, expected proportion of in invasiveness within a colonized subset of mice and typical duration of a nasopharyngeal colonization.

Video: The video that accompanies this manuscript can be accessed at:

https://www.dropbox.com/s/f16hebnaeckvpcy/JoveVideo.mp4

Chapter 3.

TNF-dependent increases in Ly6C^{high} monocytes occur with age and impair bacterial clearance

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TNF-dependent increases in Ly6C^{high} monocytes occur with age and impair bacterial clearance

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Running Title: TNF drives increases in Ly6C^{high} monocytes with age

Character count: 32841

Abstract

Monocyte phenotype and output changes with age but whether this is caused by, or contributes to age-associated inflammation or impaired anti-bacterial immunity is not known. We found that old mice (18-22 mo) had increased numbers of Ly6C^{high} monocytes in the blood and bone marrow that expressed lower levels of the maturity marker F4/80, but produced more IL-6 constitutively and upon stimulation. Ly6C^{high} monocytes from old mice had increased CCR2 expression, which promoted premature egress from the bone marrow during the steady state and when challenged with *Streptococcus pneumoniae*. TNF drove age-related changes in monocyte numbers as old TNF KO mice and ablation of TNF in old wildtype mice reduced CCR2 expression, lowered numbers of circulating Ly6C^{high} monocytes and decreased serum IL-6. Although Ly6C^{high} monocyte recruitment and TNF levels in the blood and nasopharynx were higher in old mice during *S. pneumoniae* colonization, bacterial clearance was impaired. In fact, in old mice elevated TNF and excessive monocyte recruitment contributed to impaired anti-pneumococcal immunity, as bacterial clearance was improved upon either ablation of TNF or reduction of Ly6C^{high} monocytes. Thus, with age TNF impairs inflammatory monocyte development, egress and function, which contributes to systemic inflammation and is ultimately detrimental to antibacterial immunity.

Introduction

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(Geissmann et al., 2003; Gordon and Taylor, 2005). In humans, the equivalents are CD14^{++}CD16$^{-/+}$ and CD14^{+}CD16^{++} monocytes respectively(Geissmann et al., 2003; Cros et al., 2010). Ly6C^{high} monocyte output from the bone marrow to the blood increases in a CCR2-dependent manner during early inflammatory responses(Barbalat et al., 2009; Dunay et al., 2008), and they become the dominant monocyte subtype in the blood, preferentially homing to sites of inflammation(Serbina et al., 2008). Ly6C^{high} monocytes produce high levels of TNF(Barbalat et al., 2009; Dunay et al., 2008; Kim et al., 2011); thus, they are often called "inflammatory monocytes".

Monocyte phenotype and function change with age. In the elderly, numbers of circulating CD14^{++}CD16^{+} and CD14^{++}CD16^{-} monocytes, (equivalent to Ly6C^{high} murine monocytes(Ziegler-Heitbrock et al., 2010)), are significantly higher(Seidler et al., 2010). CD14^{++}CD16^{+} monocytes derived from elderly individuals are more senescent (i.e. have shorter telomeres) than other monocyte subsets and have enhanced pro-inflammatory cytokine production (IL-6, TNF, IL-1β, IL-12p70) and higher levels of some chemokine receptors (e.g. CCR2, CCR5, CCR7, CX3CR1)(Alvarez-Rodríguez et al., 2012; Merino et al., 2011). 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(Lutgens et al., 2010; Tacke et al., 2007; King et al., 2009; Martinez et al., 2011; Rivollier et al., 2012; Ren et al., 2012; van den Brand et al., 2013). During chronic inflammatory conditions, the number of circulating Ly6C^{high} monocytes increase progressively(Swirski et al., 2007) and their ablation is an effective strategy for decreasing pathology(Leuschner et al., 2011; Ren et

al., 2012; van den Brand et al., 2013; Swirski et al., 2006). Whether Ly6C^{high} monocytes contribute to chronic age-associated inflammation and increased susceptibility to infection is not well understood 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 (IL1 β) and 6 (IL-6) in the serum and tissues, a phenomenon that has been termed "inflamm-aging"[reviewed in [(Franceschi et al., 2007; Franceschi, 2007)]]. It has been posited that a lifetime of antigenic challenge results in progressive increases in the production of inflammatory mediators by activated immune cells. This age-associated, systemic state of chronic, low-grade inflammation (defined as "para-inflammation" by Medzhitov(Medzhitov, 2008)) is well-documented although its cellular source has yet to be definitively identified. Adipose tissue,(Starr et al., 2009) CD4^{+} T cells or macrophages(Franceschi et al., 2007; Bonafè et al., 2012; Bouchlaka et al., 2013) have all been proposed to contribute. Increases in serum cytokines (particularly IL-6 and TNF) are generally thought to be a pathological consequence of aging, as they correlate with risk of classical "diseases of age" (i.e. dementia,(Forlenza et al., 2009) stroke,(Whiteley et al., 2009) cardiovascular disease(Cesari et al., 2003)) as well as frailty(Li et al., 2011; Giovannini et al., 2011) and all-cause mortality.(Varadhan et al., 2014; Tuomisto et al., 2006) Conversely, lower than average levels of age-associated inflammation predict good health in age(Harris et al., 1999). Furthermore, most chronic inflammatory conditions are characterized by increased numbers of CD14^{++}CD16^{+} and/or CD14^{++}CD16^{-} monocytes(Torres et al., 2014; Qu et al., 2009; Leng et al., 2004; Rogacev et al., 2012; Berg et al., 2012; Heine et al., 2008; Kaito et al., 2013). Herein, we asked whether inflammatory

Ly6C^{high} monocytes, which are known to be critical mediators of chronic inflammation, contribute to age-associated inflammation as well.

Inflamm-aging contributes to susceptibility to infectious disease, and particularly pneumonia, which is a major cause of death in the elderly(Shivshankar, 2012). Susceptibility to pneumonia and disease severity correlate with increased levels of IL-6 and TNF before and during infection(Yende et al., 2005; Glynn et al., 1999; Antunes et al., 2002), and elderly individuals hospitalized for chronic inflammatory conditions are at increased risk for the development of pneumonia(Yende et al., 2005, 2006). When young mice are infused with TNF, they become as susceptible to experimental infection with *Streptococcus pneumoniae* as old mice(Hinojosa et al., 2009). Using a mouse model of pneumococcal colonization, we investigated whether changes in monocyte phenotype adversely affect host defense towards *S. pneumoniae.* We show with age that there is an in increase in circulating Ly6C^{high} 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. Furthermore, we identify that age-associated increases in TNF are the driving factor behind changes in monocyte phenotype, as TNF deficiency or treatment with anti-TNF antibodies results in a reduction of CCR2 on Ly6C^{high} monocytes. Decreased CCR2 expression results in decreased numbers of monocytes in the circulation and reduced production of TNF and IL-6. Finally, we demonstrate that, although TNF levels and the recruitment of Ly6C^{high} 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^{high} inflammatory monocytes as central mediators of inflamm-aging and demonstrates that TNF is a key contributor to age-associated defects in myeloid phenotype and function. These data indicate that Ly6C^{high} monocyte frequency and increased production of pro-inflammatory cytokines contributes to both age-associated inflammation and declining anti-bacterial immunity.

Results

Ly6C^{high} monocytes increase with age

It has been reported that with age the proportion of myeloid cells and cytokines in the blood is increased(Geiger et al., 2013). We quantitated circulating leukocyte populations in old (18-22 mo) mice and compared them to young (10-14 wk) mice. While absolute numbers of CD45^{+} cells remained the same in old mice, consistent with previously published data, we found that there was a decrease in total T cells, and an increase in both total monocytes and neutrophils(Cho et al., 2008) (Supplementary Figure 1A), indicating a shift towards myelopoiesis. Analysis of monocyte subsets indicated that the absolute number of both Ly6C^{high} and Ly6C^{low} monocytes was increased with age (Figure 1A). An increase in Ly6C^{high} monocyte frequency within the blood of old mice was paralleled by a similar increase in the bone marrow (Figure 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 (Supplementary Figure 1C).

Ly6C^{high} monocytes from old mice have increased CCR2 but decreased F4/80 expression

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 macrophage chemokine protein 1 (MCP-1/CCL2)(Tsou et al., 2007). Since CCR2 is required for 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 (Figure 1C). 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 (Figure 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 (Supplementary Figure 1D).

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 MCP-1 and measured Ly6C^{high} monocyte recruitment after 4 hours. We found that despite administering an equivalent dose of MCP-1, Ly6C^{high} monocyte recruitment to the

peritoneum was increased ~5-fold in old mice relative to young mice (Figure 1E). Analysis of the CCR2 promoter regions in DNA blood samples from young and old WT mice suggested that changes in CCR2 expression may due to epigenetic changes, as old mice showed hypomethylation in promoter regions 4 and 5 relative to young mice (Figure 1F).

Ly6C^{high} monocytes are potent producers of proinflammatory cytokines with age

Since we found that there was an expansion of Ly6C^{high} monocytes with age and these cells are known to be potent producers of pro-inflammatory cytokines, we postulated that Ly6C^{high} monocytes may contribute significantly to age-associated inflammation. To determine whether the increased numbers of Ly6C^{high} monocytes with age contributed to age-associated increases in IL-6 production, we targeted this cell population using carboxylated polystyrene microparticles (PS-MPs), which have been shown by others to lead to a reduction of Ly6C^{high} monocytes in the blood(Getts et al., 2014). We found that when circulating Ly6C^{high} monocytes were decreased in old mice (Figure 2A), this reduced circulating levels of IL-6 (Figure 2B).

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} monocytes was increased to levels comparable to old mice (Figure 1A) or old recipient mice who had received old donor marrow (Figure 3A). In contrast, young recipient mice that had received old donor marrow had Ly6C^{high}

monocyte numbers comparable to young mice (Figure 1A) or to young recipient mice that had received young donor bone marrow (Figure 3A). In addition, the increase in CCR2 expression observed on circulating monocytes from old mice (Figure 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(Figure 3C). These data demonstrate that increases of Ly6C^{high} 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 next sought to determine whether TNF was sufficient to drive expansion of the Ly6C^{high} monocyte subset. 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 (Figure 3C), but did have an increase in bone marrow Ly6C^{high} monocytes compared to their young counterparts (Figure 3D). Surface expression of CCR2 on Ly6C^{high} monocytes in both the blood (Figure 3E) and the bone marrow (Figure 3F) of old TNF KO mice was comparable to the levels seen in their young counterparts.

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^{high} monocytes contribute to elevated levels of circulating cytokines with age (Figure 2), old WT mice produced more IL-6 than young mice following 24 hour stimulation of whole blood with either PBS or LPS (Figure 3G). In comparison, old TNF KO mice, which did not have an increase of Ly6C^{high} monocytes in the

blood did not have an age-associated increase in IL-6 in whole blood in response to PBS or LPS (Figure 3G).

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

We wondered whether it was chronic or acute exposure to TNF that mediated age-related increases in serum IL-6 and changes in monocyte phenotype and function. We first sought to determine whether increases in circulating Ly6C^{high} monocytes were inducible after short-term, low dose administration of TNF. Young mice showed a large increase in Ly6C^{high} monocytes in the blood after 3 weeks of intraperitoneal delivery of 5 ng/g body weight of TNF (Figure 4A). This was accompanied by a significant increase in serum IL-6 in TNF-treated, but not vehicle control mice (Figure 4B). We next asked whether blocking TNF could reduce numbers of Ly6C^{high} 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 number of circulating Ly6C^{high} and Ly6C^{low} monocytes in the blood to levels similar to young mice (Figure 4C). 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 (Figure 4D) and reduced the percentage of monocytes that stained positive for IL-6 or TNF by ICS after LPS stimulation (Figure 4E). Anti-TNF treatment reduces IL-6 levels in the circulation of old mice (Figure 4F) and when blood from young and old mice treated with anti-TNF or IgG controls was stimulated with LPS, IL-6 levels were lower in old mice treated with anti-TNF compared to those that were treated with IgG(Figure 4G).

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 we investigated the trafficking of these cells following nasopharyngeal colonization of young and old mice with the bacterial pathogen, *Streptococcus 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(Davis et al., 2011). Following intranasal delivery of *S. pneumoniae*, we found that old 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 (Figure 5A). Old mice were also more susceptible to bacterial invasion to the lungs at day 3 (Figure 5B) and mortality throughout the course of colonization (Figure 5C). Although serum production of MCP-1 in old mice was comparable to that of young mice (Figure 5D), old mice had increased Ly6C^{high} monocyte numbers in the circulation during colonization (days 3, 7, 14, 21) (Figure 5E).

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 (Figure 5F), suggesting that the decreased F4/80 expression seen in the bone marrow during the steady state (Figure 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 MCP-1 in the nasopharynx during colonization (Figure 5G), and

had higher numbers of recruited Ly6C^{high} monocytes (Figure 5H) and macrophages (Figure 5I) 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(Figure 5J), similar to that seen in their counterparts in the blood(Figure 5F).

Ly6C^{high} 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 (Figure 3C), likely through increasing CCR2 expression, was increased with age during *S. pneumoniae* colonization in the nasopharynx (Figure 6A) and blood (Figure 6B). 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 (Supplementary Figure 2), we found that old TNF KOs had significantly fewer CFUs in the nasopharynx compared to their old WT counterparts at day 3 (Figure 6C). Old TNF KO mice also had decreased recruitment of Ly6C^{high} monocytes in the blood (Figure 6D), confirming that TNF can regulate mobilization of these cells during infection as well as 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 targeted this cell population using negatively-charged polystyrene microparticles (PS-MPs). Old mice were given PS-MPs every 3 days during the course of *S. pneumoniae* colonization and bacterial loads were measured at day 14. PS-MP-treated old mice had lower bacterial loads compared to old control mice (Figure 6e), but lower circulating Ly6C^{high} monocytes (Figure 6F), confirming that increased trafficking of this cell type during *S. pneumoniae* colonization adversely affects host defense.

Discussion

Macrophages have been proposed to be a central cell type involved in promoting inflamm-aging(Franceschi et al., 2000). Our data suggest that the contribution made by macrophages to inflamm-aging may occur downstream to an initial dysfunction in monocytes. Herein, we discovered that during the steady state there was an increase in IL-6 and TNF-producing Ly6C^{high} monocytes with age and that these monocytes produced more IL-6 both constitutively and following LPS stimulation. Finally, we found that we could decrease age-associated, hyper-inflammatory IL-6 production in the blood of old mice if we selectively decreased the numbers of circulating Ly6C^{high} monocytes. These data are consistent with the observation that in humans, the percentage of CD14^{++}CD16^{-} monocytes increases gradually with age and become more frequently positive for TNF(Kassel et al., 2013).

Although we demonstrated that old mice had an increase in bone marrow Ly6C^{high} monocytes and an increase of these cells in the circulation, this was not due to intrinsic defects in myeloid precursors in the bone marrow compartment. Rather, our bone marrow chimera studies indicated that the increase in circulating Ly6C^{high} monocytes that occurs with age is dependent on the microenvironment. Using TNF KO mice, we identified that TNF in the bone marrow microenvironment is the driving cause of changes in monocyte phenotype (i.e. CCR2 and F4/80 expression). The increase in circulating Ly6C^{high} monocytes, as well as age-associated hyper-inflammatory responses in the blood, were absent in old TNF KO mice. Furthermore, by treating young WT mice with a low-dose regime of TNF delivered intraperitoneally, we found that we could in fact induce a state wherein Ly6C^{high} monocytes were increased in the blood in a manner similar to old mice, demonstrating that TNF is both necessary and sufficient to increase numbers of circulating Ly6C^{high} monocytes. Thus, we have shown that Ly6C^{high} monocytes are both caused by, and contribute to, inflamm-aging, in a TNF-dependent manner.

The observation that CCR2 levels were higher on Ly6C^{high} monocytes from old mice provides a mechanism for their increased presence in the circulation. CCR2 is essential for Ly6C^{high} monocyte egress from the bone marrow in a MCP-1 dependent manner. Others have demonstrated that CCR2 ablation results in increased retention of Ly6C^{high} monocytes in the bone marrow and consequently decreased numbers of these cells in the blood(Tsou et al., 2007). Here, we found that numbers of Ly6C^{high} monocytes were increased in the bone marrow of both old WT and old TNF KO mice; however, old TNF KO mice did not have elevated CCR2 expression on these cells as compared to old WT mice, and thus did not have higher levels of Ly6C^{high} monocytes in the blood. Pharmacological ablation of TNF in old WT

mice reduced the number of circulating monocytes. Therefore, it seems that TNF plays a mechanistic role in mediating recruitment of Ly6C^{high} monocytes to the blood via regulation of CCR2, thus leading to monocyte egress, but does not directly cause age-associated increases in Ly6C^{high} monocyte generation in the bone marrow.

Our hypothesis that increased expression of CCR2 on monocytes from aged mice serves as a mechanism of increased monocyte trafficking was supported by our studies of Ly6C^{high} monocyte recruitment following intraperitoneal MCP-1 injection. We demonstrated that there was a substantial increase in the number of Ly6C^{high} monocytes recruited to the peritoneum of old mice as compared to young mice when given an equivalent dose of MCP1.

Studies in patients on anti-TNF therapy for rheumatoid arthritis validate for our observations that TNF drives the increase in circulating Ly6C^{high} monocytes and that blocking TNF reduces the number of circulating Ly6C^{high} monocytes. Anti-TNF therapy decreases the levels of circulating CD14^{++}CD16^{-} monocytes (the equivalents of Ly6C^{high} monocytes in mice) in the blood and synovial fluid and has a positive predictive value for patient prognosis(Chara et al., 2012). Although the mechanism behind this phenomenon has not been identified, rapid decreases in circulating monocytes were reported to be independent of apoptosis(Wijbrandts et al., 2008). Our data provide an insight into potential mechanisms behind these observations, since our data indicated that anti-TNF therapy reduced monocyte numbers by down-regulating CCR2 and preventing large-scale monocyte efflux from the bone marrow into the blood. This is consistent with the observation that anti-TNF therapy in humans leads to a significant decrease in CCR2 expression on peripheral blood mononuclear cells(Xia et al., 2011).

Increases in Ly6C^{high} monocytes appeared to be associated with defects in maturity. Although F4/80 levels were equivalent on blood monocytes during the steady state, they were lower in the bone marrow and 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. This may reflect the role of Ly6C^{high} monocytes as potent pro-inflammatory effectors but poorer phagocytes as compared to Ly6C^{low} monocytes(Menéndez et al., 2012).

We found that old mice had impaired bacterial clearance despite robust production of TNF, indicating that TNF is not protective in this context. This is consistent with the observation that TNF is required for control for *S. pneumoniae* bacteremia but not for survival in lung infection (Kirby et al., 2005) and that inflammation is associated with *S. pneumoniae* disease severity in the elderly (Menéndez et al., 2012). In fact, our data suggest that TNF is detrimental to clearance of *S. pneumoniae* from the nasopharynx and lungs with age, as old TNF KO mice had lower bacterial loads compared to their WT counterparts. Interestingly, old TNF KO mice recruited less circulating Ly6C^{high} monocytes during *S. pneumoniae* colonization as compared to old WT mice, suggesting that TNF can control bone marrow egress of these cells during infection as well as the steady state. Furthermore, our data indicated that this increase of Ly6C^{high} monocytes may be one of the reasons that old WT mice had poorer clearance than old TNF KO mice. When we decreased circulating Ly6C^{high} monocytes during *S. pneumoniae* in old mice using carboxylated polystyrene microparticles colonization, bacterial loads in the nasopharynx decreased.

This observation has important therapeutic significance, since many have previously assumed that host responses to bacteria are impaired with age due to poor innate cell recruitment. Counterintuitively, we have demonstrated that Ly6C^{high} monocyte recruitment was, in fact, increased during *S. pneumonaie* colonization, and that enhanced monocyte recruitment negatively affects bacterial clearance. There have been 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 (Søgaard, 2011) and (Cheng et al., 2007)]. Our data suggests that use of G-CSF, GM-CSF or other myeloid chemoattractant-based therapies in aged individuals would enhance recruitment of a population that is fundamentally immature and functionally predisposed towards TNF and IL-6 production that provides no functional benefit to the host for clearance and may even exacerbate infection. Another key finding of our study was that the effect of TNF on monocytes was reversible. Many, if not all of the major degenerative diseases of age are strongly associated with inflammation as a major risk factor [reviewed in (Burmester et al., 2009) and (Burmester et al., 2013)]. Having higher than average levels of circulating pro-inflammatory cytokines is a risk factor for poor health in old age, and individuals on anti-TNF therapy live slightly longer than their untreated counterparts, despite an increased risk in re-activation of chronic infections (e.g. tuberculosis)(Burmester et al., 2009, 2013). Thus, this study suggests that reducing levels of TNF may improve monocyte function, which will impact anti-bacterial immunity and other age-related diseases in which monocytes play a pathological role.

In conclusion, we have demonstrated that increased Ly6C^{high} monocytes contribute to age-associated increases in TNF and IL-6 during the steady state and following nasopharyngeal colonization with *S. pneumoniae*. Our data indicate that the TNF drives the exacerbated hyper-inflammatory responses that occur with age and which predispose elderly individuals to bacterial infections and age-related diseases. Furthermore, we show that TNF increases monocyte recruitment via increasing CCR2 expression. Blocking TNF may be an effective strategy in returning the numbers of Ly6C^{high} monocytes in aged hosts to baseline levels seen in young hosts, thereby reducing their contribution to increases of circulating TNF and IL-6 in the steady state. This study highlights the importance of investigating TNF and Ly6C^{high} monocytes in the future as independent risk factors, and potential targets for therapeutic intervention, in studies dealing with infections and diseases that occur in older adults.

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Methods

Animals

C57BL/6J mice were purchased from Charles River or Jackson Laboratories. 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.(Zganiacz et al., 2004) All mice were housed in pathogen-free conditions in accordance with Institutional Animal Utilization protocols approved by McMaster University's Animal Research Ethics Board as per the recommendations of the Canadian Council for Animal Care. All of the animals that were used were sex-matched to their controls and maintained in the same animal room. Continual monitoring of the health status of mice was performed.

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), IL-6 (PE) or TNF (PECy7). Samples were stained according to previously published procedures(Fung et al., 2010; Puchta et al., 2014), run on a LSRII flow cytometer (Becton Dickinson) and analyzed using FlowJo 9 software (Treestar) using gating strategies as shown in Supplementary Figure 1. Total cell counts were determined using CountBright Absolute Counting Beads (Life Technologies).

MCP-1 monocyte recruitment

100 nM of recombinant murine MCP-1 (eBioscience) was diluted in sterile saline and administered intraperitoneally to young and old WT mice in a volume of 200 µl. Recruited cells were isolated via peritoneal lavage and assayed using flow cytometry.

Measurement of cytokine production

For whole blood stimulation studies, 100 µl of whole blood was collected in heparin from young and old WT or TNF KO mice and stimulated with 100 ng/ml of LPS or PBS. Samples were incubated for 24 hours at 5% CO_2 and 37°C then centrifuged at 15000 x *g* for 5 minutes. IL-6 production was measured by ELISA as per the manufacturer's direction (eBioscience). For intracellular IL-6 and TNF staining, cells were incubated with Golgi stop (BD Pharmingen) with or without the presence of 100 ng/ml LPS for 4 hours, fixed, permeabilized, and then stained with fluorochrome-conjugated antibodies. Serum TNF and MCP-1 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.

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.(Getts et al., 2014) Monocyte depletion was confirmed by flow cytometry.

Generation of bone marrow chimeric mice

Young and old WT recipient mice were pre-conditioned for two weeks with pH=2.0 drinking water containing 100mg/L levofloxacin and 100mg/L fluconazole. Bone marrow (BM) cells were collected from spines of young and of mice. Four hours after irradiation with 10Gy X-rays, recipient mice were intravenously injected with 1×10^7 BM cells, and then the mice were kept in a specific pathogen-free environment for 4 weeks to reconstitute their BM. Four groups of chimeric mice were generated: young to young, old to old, young to old and old to young.

Administration of TNF in vivo

Murine recombinant TNF (eBioscience) diluted in sterile saline was administered intraperitoneally to young WT mice every other day for 3 weeks at a dose of 5 ng per gram of body weight in a volume of 200 µl.

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.

Murine model of intranasal colonization

S. pneumoniae P1547 (serotype 6A), a clinical strain was a kind gift from Dr. J. Weiser (University of Pennsylvania). Colonization was performed by inoculating the nares of unanesthetized mice with 10^7 CFU as previously described. Mice were weighed and monitored frequently for clinical symptoms. At selected timepoints mice were anesthetised and sacrificed by exsanguination. PBS nasal lavages were performed and used to quantitate bacterial load and leukocytic recruitment as in [(Puchta et al., 2014)]. For monocyte depletion experiments, mice were given PS-MPs every 3 days during the course of colonization.

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. Statistical significance was defined as a p value of 0.05.

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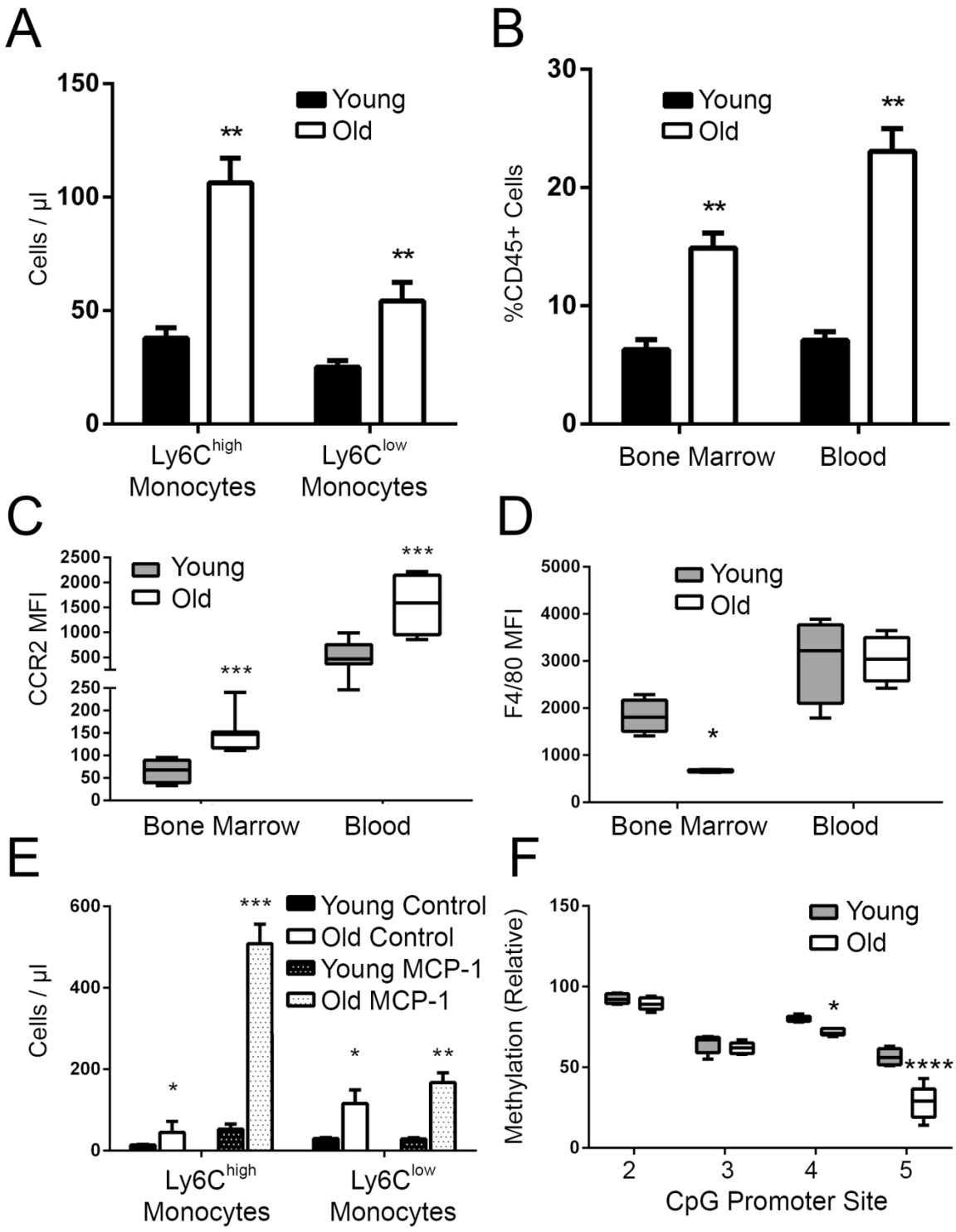

A
Cells / µl
150
100
50
0
Young
Old
**
**
Ly6C^high Monocytes
Ly6C^low Monocytes
B
%CD45+ Cells
30
20
10
0
Young
Old
**
**
Bone Marrow
Blood
C
CCR2 MFI
2500
2000
1500
1000
500
250
200
150
100
50
0
Young
Old
***
***
Bone Marrow
Blood
D
F4/80 MFI
5000
4000
3000
2000
1000
0
Young
Old
*
Bone Marrow
Blood
E
Cells / µl
600
400
200
0
Young Control
Old Control
Young MCP-1
Old MCP-1
*
***
*
**
Ly6C^high Monocytes
Ly6C^low Monocytes
F
Methylation (Relative)
150
100
50
0
Young
Old
*
****
2
3
4
5
CpG Promoter Site

Figure 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/MCP-1. The recruitment of Ly6C^{high} and Ly6C^{low} monocytes was greater in old mice (± SEM; n = 5). (F) Whisker plot of relative DNA methylation levels at four sites within the promoter region of the CCR2 gene in the blood of young and old WT mice (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-F) is representative of 2 independent experiments.

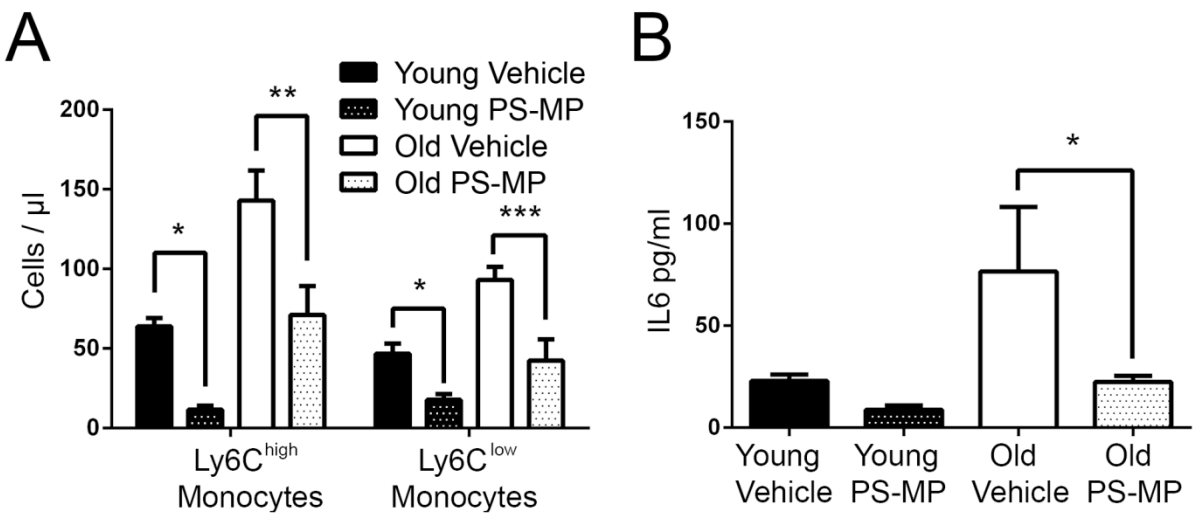


Figure 2. Ly6C^{high} monocytes contribute to elevated levels of serum IL-6 and TNF in aged mice. Young and old mice were injected with 500 nm negatively-charged polystyrene microparticles (PS-MPs) previously shown to reduce numbers of circulating Ly6C^{high} monocytes. Circulating monocyte populations (A) and IL-6 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.

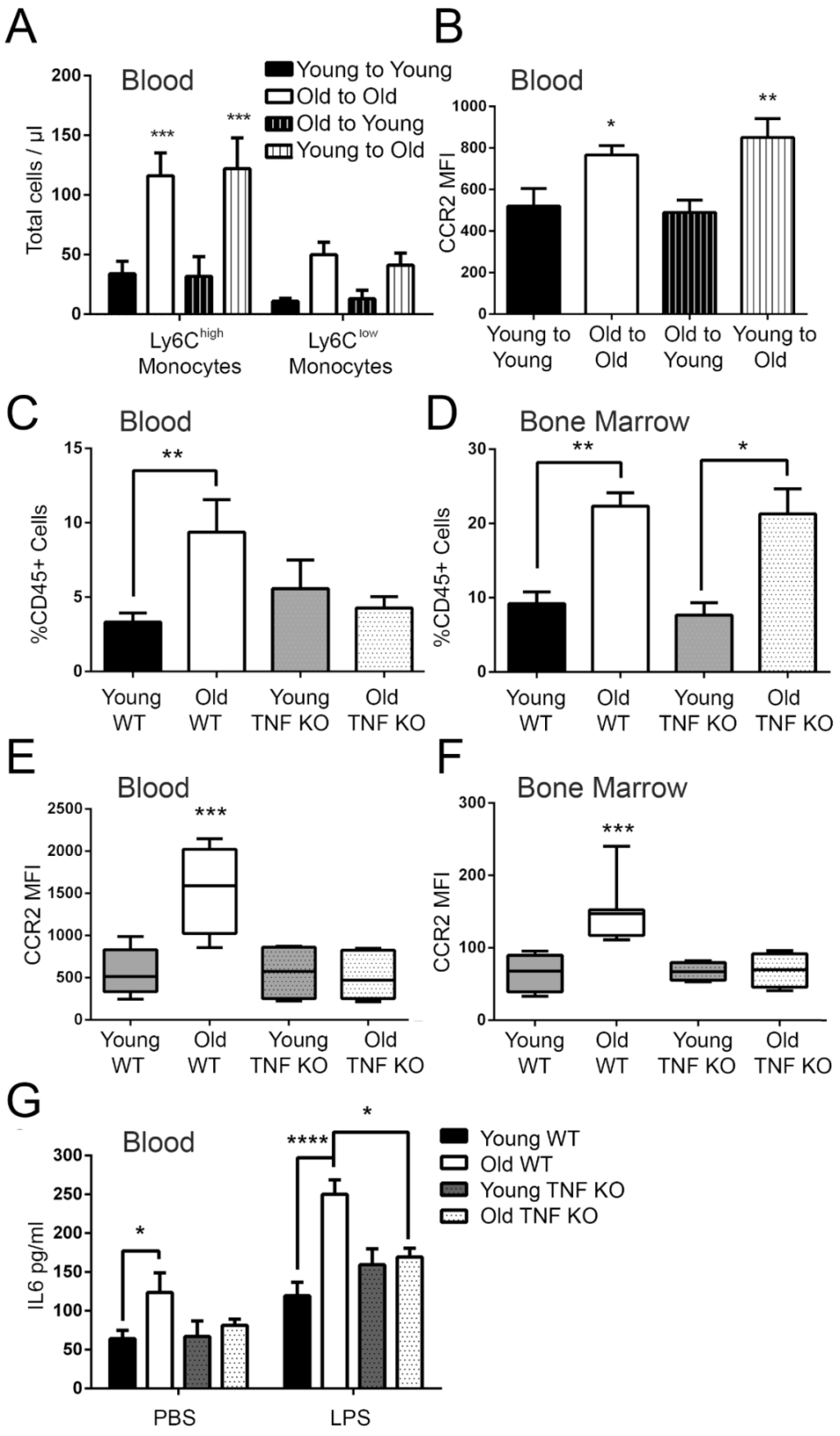

A
Blood
Young to Young
Old to Old
Old to Young
Young to Old
Total cells / µl
200
150
100
50
0
***
***
Ly6C^high Monocytes
Ly6C^low Monocytes
B
Blood
CCR2 MFI
1000
800
600
400
200
0
*
**
Young to Young
Old to Old
Old to Young
Young to Old
C
Blood
%CD45+ Cells
15
10
5
0
**
Young WT
Old WT
Young TNF KO
Old TNF KO
D
Bone Marrow
%CD45+ Cells
30
20
10
0
**
*
Young WT
Old WT
Young TNF KO
Old TNF KO
E
Blood
CCR2 MFI
2500
2000
1500
1000
500
0
***
Young WT
Old WT
Young TNF KO
Old TNF KO
F
Bone Marrow
CCR2 MFI
300
200
100
0
***
Young WT
Old WT
Young TNF KO
Old TNF KO
G
Blood
IL6 pg/ml
300
250
200
150
100
50
0
*
****
*
PBS
LPS
Young WT
Old WT
Young TNF KO
Old TNF KO

Figure 3. 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} 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} 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) IL-6 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.

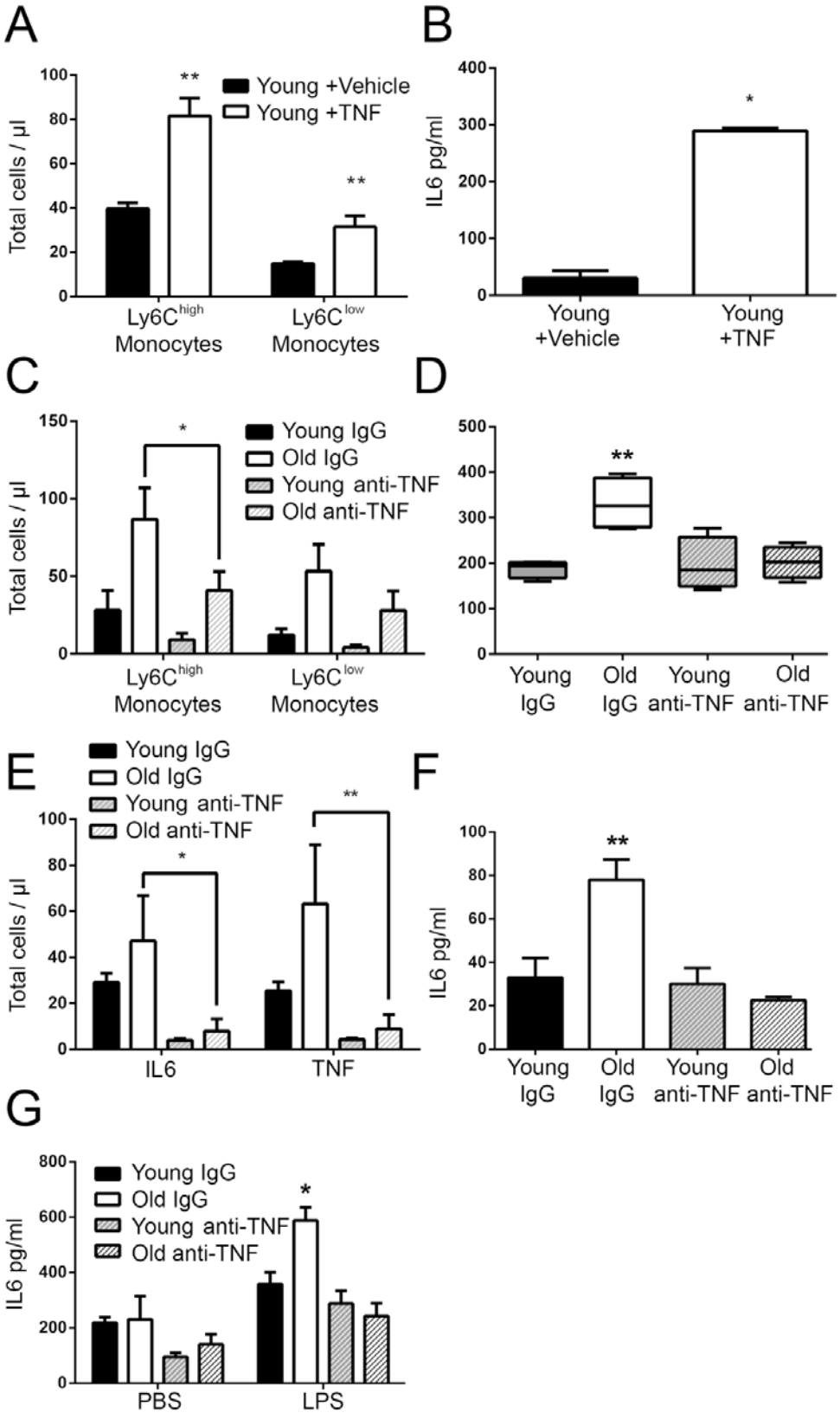

A
Total cells / µl
Young +Vehicle
Young +TNF
Ly6C high Monocytes
Ly6C low Monocytes
B
IL6 pg/ml
Young +Vehicle
Young +TNF
C
Total cells / µl
Young IgG
Old IgG
Young anti-TNF
Old anti-TNF
Ly6C high Monocytes
Ly6C low Monocytes
D
Young IgG
Old IgG
Young anti-TNF
Old anti-TNF
E
Total cells / µl
IL6
TNF
F
IL6 pg/ml
G
IL6 pg/ml
PBS
LPS

Figure 4. 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 IL-6 (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 IL-6 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 IL-6 or TNF are decreased with anti-TNF therapy(± SEM; n = of 4). (F) Serum IL-6 is reduced in old mice treated with anti-TNF but not the IgG control. (G) IL-6 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 = of 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.

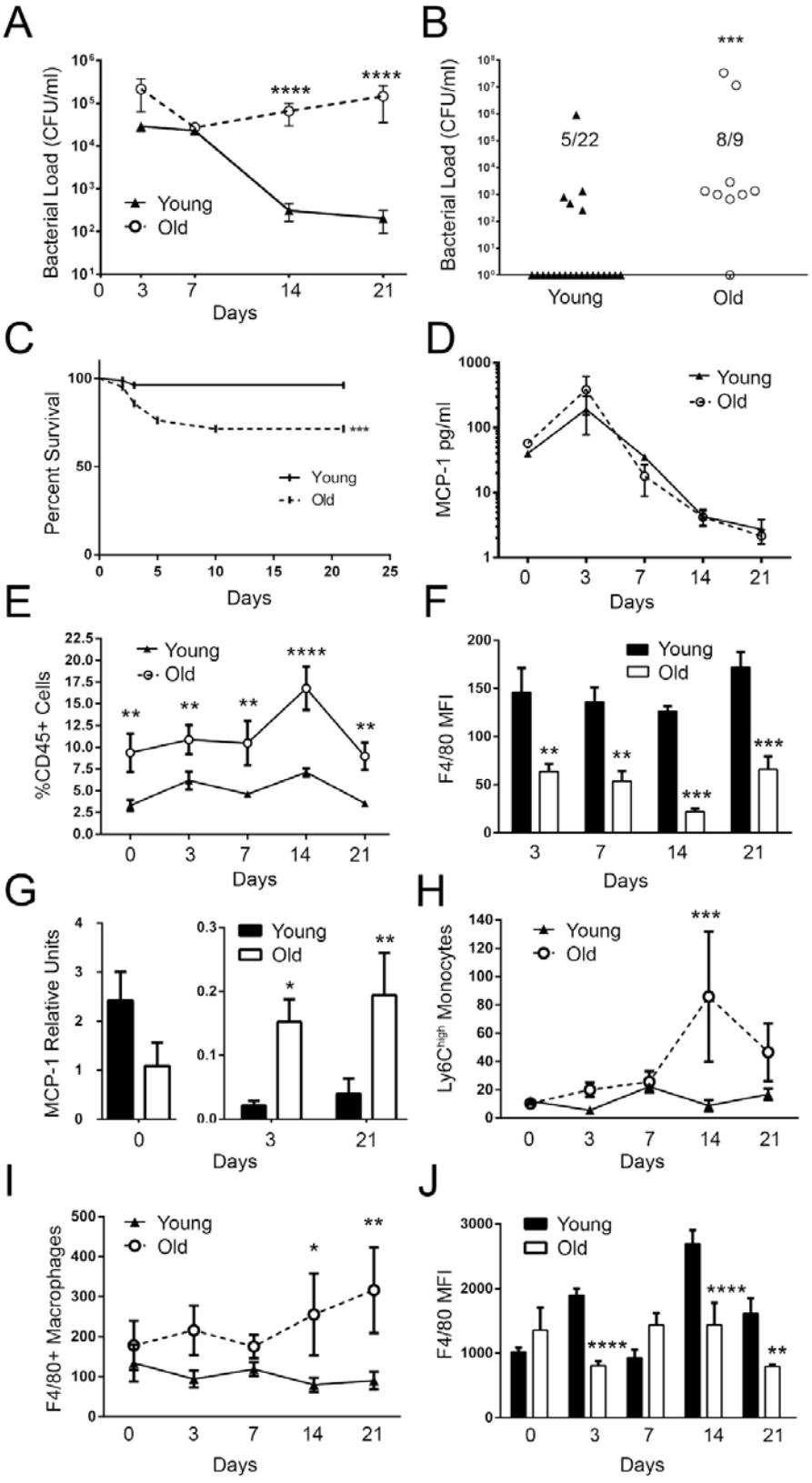

A
Bacterial Load (CFU/ml)
Young
Old
Days
B
Bacterial Load (CFU/ml)
5/22
8/9
Young
Old
C
Percent Survival
Young
Old
Days
D
MCP-1 pg/ml
Young
Old
Days
E
%CD45+ Cells
Young
Old
Days
F
F4/80 MFI
Young
Old
Days
G
MCP-1 Relative Units
Young
Old
Days
H
Ly6C^high Monocytes
Young
Old
Days
I
F4/80+ Macrophages
Young
Old
Days
J
F4/80 MFI
Young
Old
Days

Figure 5. 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 MCP-1 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) Percent of Ly6C^{high} monocytes 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 MCP-1 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 nasopharynx 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. 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.

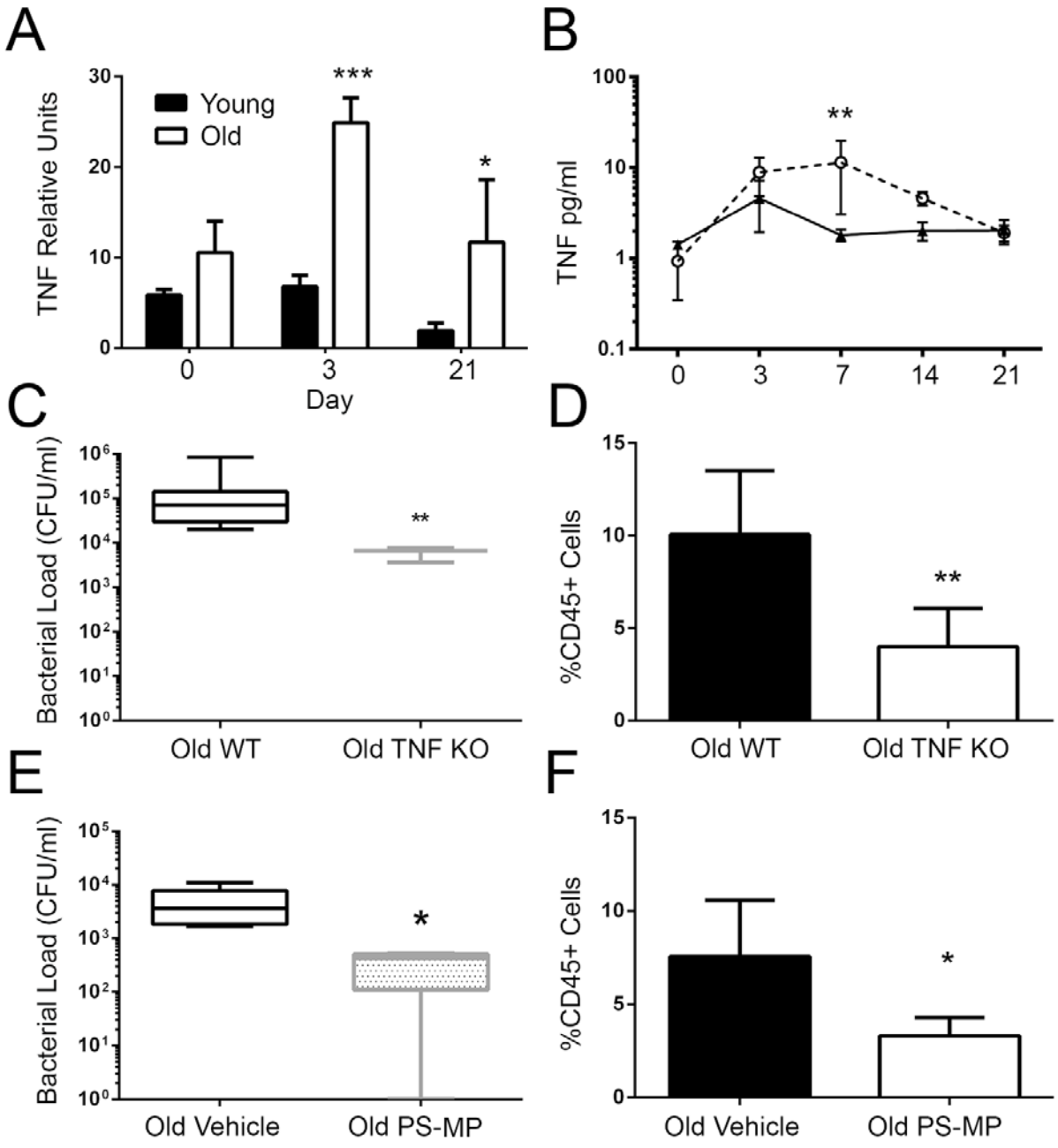
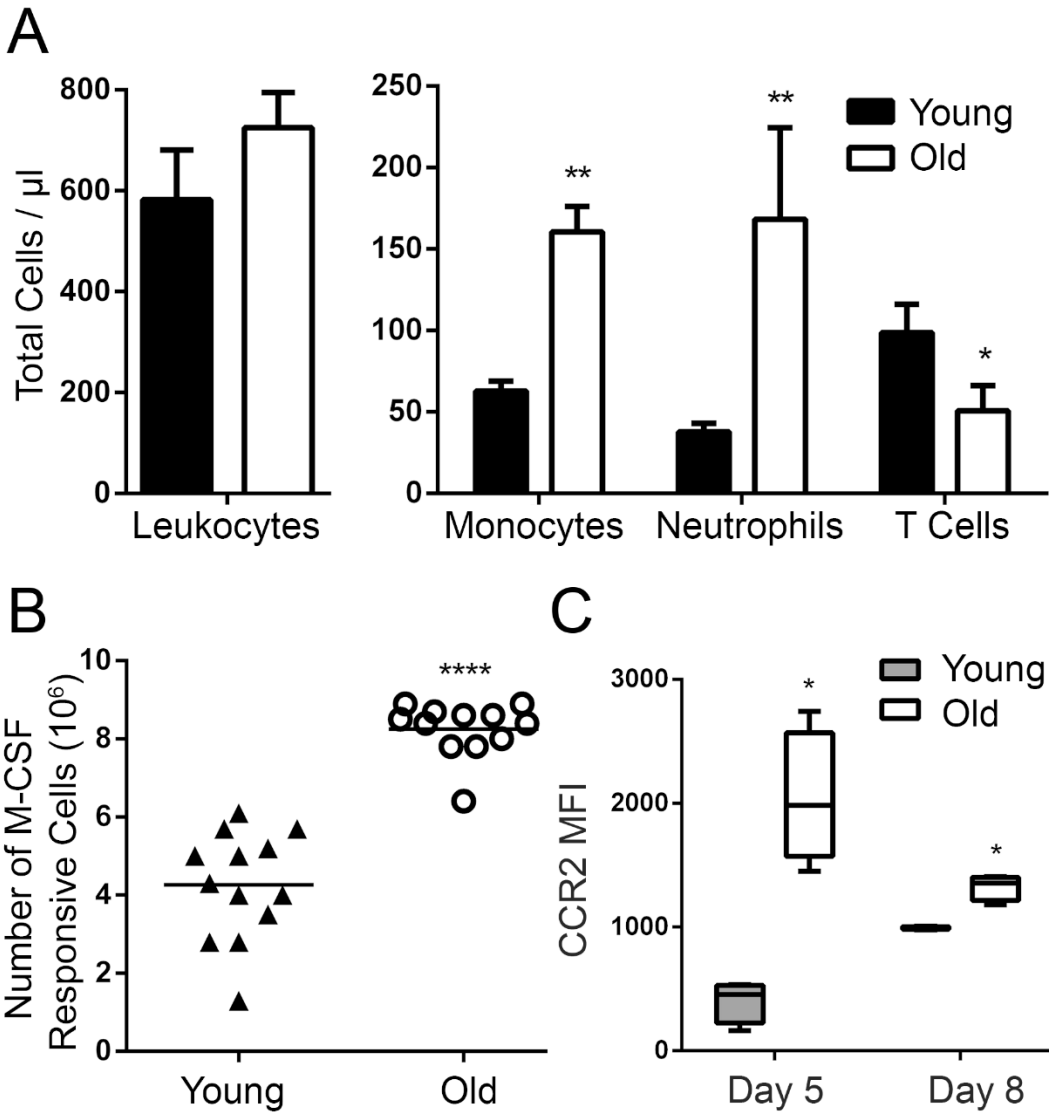
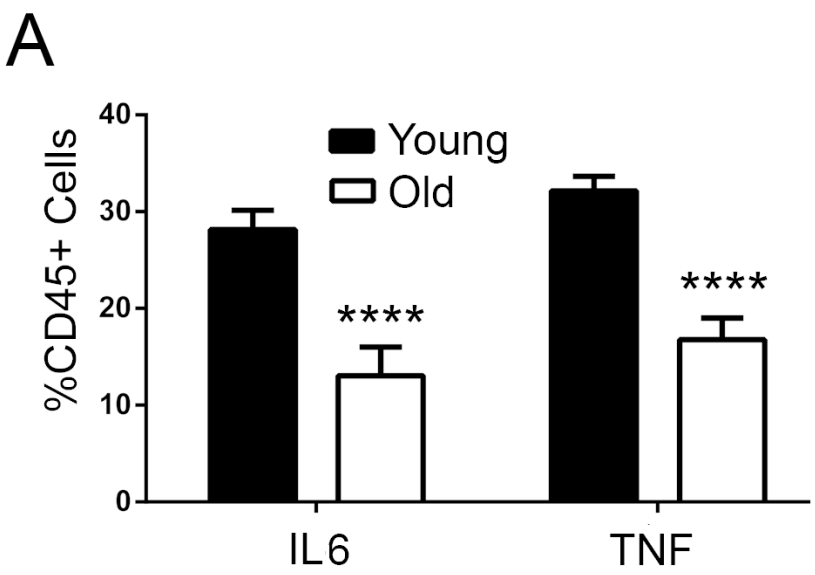

A
TNF Relative Units
30
20
10
0
Young
Old
***
*
0
3
21
Day
B
TNF pg/ml
100
10
1
0.1
**
0
3
7
14
21
C
Bacterial Load (CFU/ml)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
**
Old WT
Old TNF KO
D
%CD45+ Cells
15
10
5
0
**
Old WT
Old TNF KO
E
Bacterial Load (CFU/ml)
10^5
10^4
10^3
10^2
10^1
10^0
*
Old Vehicle
Old PS-MP
F
%CD45+ Cells
15
10
5
0
*
Old Vehicle
Old PS-MP

Figure 6. 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) nasopharynx 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 3 after colonization with *S. pneumoniae* (± SEM; *n* = 3-4). (D) Ly6C^{high} monocytes as a percent of circulating CD45+ cells in old WT and TNF KO mice on day 3 of *S. pneumoniae* colonization (± SEM; *n* = 3-4). (E-F) Old WT mice were given negatively-charged polystyrene microparticles (PS-MPs) every 3 days during the course of intranasal *S. pneumoniae* colonization. (E) CFUs in the nasopharynx and (F) proportions of Ly6C^{high} monocytes in the blood were determined on day 14 (± SEM; *n* = 4-5). 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 $p < 0.00005$. (A-F) is representative of 1 independent experiments.

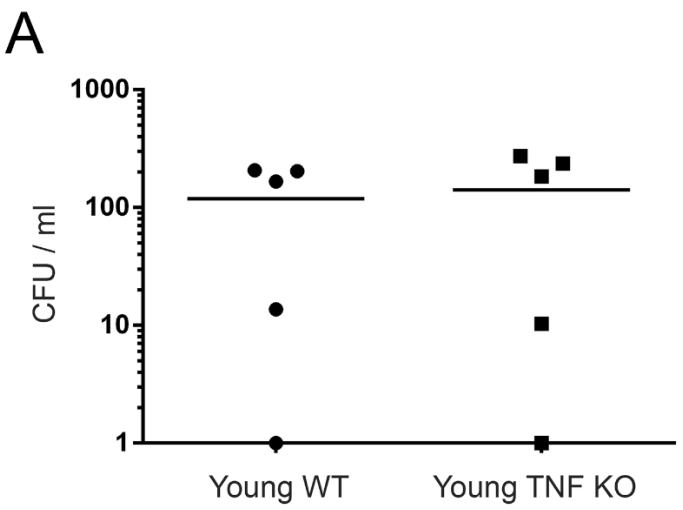
Supplementary Figures and Supplementary Figure Legends



Supplementary Figure 1. 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.



Supplementary Figure 2. T cell positivity of IL-6 and TNF is decreased with age. (A) Intracellular staining analysis was conducted for IL-6 and TNF on T cells. A small proportion of T cells were positive for these two cytokines in old mice as compared to young controls.



Supplementary Figure 3. TNF does not contribute to bacterial clearance in young mice during S. pneumoniae nasopharyngeal colonization. (a) Young WT and TNF KO mice were colonized with S. pneumoniae and bacterial loads in the nasopharynx were measured at day 21. No significant difference was found in bacterial numbers.

Chapter 4.

Metchnikoff's theory of aging: the intestinal microbiota drives age-associated inflammation and impaired macrophage function

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Title: Metchnikoff's hypothesis of aging: the intestinal microbiota drives age-associated inflammation and impaired macrophage function

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Abstract: In 1901, Elie Metchnikoff hypothesized that the intestinal microbiota or its products could contribute to tissue damage by passing through the intestinal barrier, activating macrophages and resulting in systemic inflammation. He reasoned that this was the cause of declining health with age. Here, we provide experimental evidence in support of this hypothesis. Hyperinflammatory responses and decreased anti-bacterial activity occurred in macrophages from old wild-type (WT) mice (18-22 mo), but not mice protected from age-associated inflammation via genetic ablation of tumour necrosis factor (TNF). Systemic inflammation and macrophage defects were also absent in old germ-free mice, which lived longer than their WT counterparts. With age, intestinal permeability and circulating levels of the bacterial component muramyl dipeptide increased in WT mice but not TNF KO or germ-free mice. Reintroduction of a microbial community to old germ-free mice resulted in increased proinflammatory cytokine production in the blood. Thus, with age, the translocation of microbial products from the intestinal lumen into the circulation drives systemic inflammation that ultimately dysregulates macrophage function and promotes aging.

One Sentence Summary: Translocation of microbial products from the intestines occurs with age and drives a state of chronic, low-grade inflammation that promotes macrophage dysfunction and the aging process.

Main Text:

Metchnikoff proposed that tissue destruction and senescence was a consequence of chronic systemic inflammation due to increased permeability in the colon and the escape of bacteria and their products(*1*). He believed that these bacterial products drove the activation of phagocytes, resulting in inflammation that led to the deterioration of surrounding tissues. Metchnikoff had remarkable foresight: his identification of the microbiota of the gut as a community whose composition could be altered(*2*, *3*), and his

belief that health(*4*–*6*) and behavior(*7*) could be altered by bacterial products or "toxins" have since been proven experimentally. Metchnikoff's theory that declining health with age is caused by systemic inflammation due to exposure to bacterial components from the gut has been hypothesized by others(*8*–*10*), but until now, has not been not experimentally validated.

Chronic inflammation caused by increased mucosal barrier permeability and microbial translocation (defined as the translocation of microbes and/or their products from the mucosal compartment to the circulation without overt bacteremia) has been implicated as a factor in many pathological conditions, including graft versus host disease(*11*–*13*), inflammatory bowel disease(*14*–*16*), liver disease(*17*, *18*) and HIV(*19*–*21*). In HIV, immune activation leading to chronic inflammation has been proposed to accelerate immunological aging (*22*, *23*). Aging is also characterized by a state of chronic, low-grade, systemic inflammation(*24*) and although it has been demonstrated that gut microbial composition correlates with levels of circulating cytokines and markers of health in the elderly(*25*), whether the microbiome directly promotes age-associated inflammation is not known.

Herein, we report that, in agreement with Metchnikoff's theory of aging, microbial translocation occurs with age due to increased permeability in the intestinal tract. Both TNF and the presence of a microbiota drive this permeability. Microbial products enter the bloodstream in aged mice where they trigger systemic inflammation (i.e. elevated levels of serum IL-6). Chronic exposure to inflammation alters macrophage function, rendering these cells poor bacterial killers but potent producers of inflammatory cytokines and ultimately contributing to age-associated inflammation. Although the presence of even a limited microbiome is sufficient to induce intestinal permeability and age-associated inflammation, the dysbiosis that occurs in the intestinal microbial community with age contributes to increased inflammation in the circulation.

TNF drives age-associated defects in macrophage function

We found that bone marrow derived (Figure 1A) or resident peritoneal (SFigure 1A) macrophages from old wild-type (WT) mice (18-22 mo) were impaired in their ability to kill *Streptococcus pneumoniae* as compared to those from young WT (10-14 wk) mice. Bacterial uptake was also compromised in aged macrophages, although their pattern recognition receptor expression was unaffected (SFigure 1B-C). Following internalization, bacterial lysis was observable in macrophages from young mice, but reduced or delayed in old mice (SFigure 1D). Maturation markers on macrophages from young and old mice were expressed at equal levels, indicating that differences observed with age were not due to altered differentiation or maturity (SFigure 1E-F).

Bone marrow derived macrophages from old mice produced more TNF following stimulation with LPS or *S. pneumoniae* compared to young mice (Figure 1B). Furthermore,

the production of TNF contributes to the reduction of killing of *S. pneumoniae* as killing was further reduced by the addition of exogenous TNF (10 ng/ml) to culture media (Figure 1C).

Since an acute increase in TNF impaired macrophage killing of *S. pneumoniae*, we postulated that chronic age-associated inflammation, which is characterized by a systemic increase in TNF, might be the cause of the defects we observed in macrophage anti-bacterial function. We aged TNF knockout (KO) mice and examined the capacity of their macrophages to mount anti-bacterial responses. Unlike macrophages from old WT mice, those from old TNF KO mice did not produce enhanced levels of IL-6 constitutively or following stimulation with LPS (Figure 1D) or *S. pneumoniae* (data not shown). Furthermore, macrophages from old TNF KO mice did not have the impaired pneumococcal killing observed in WT mice (Figure 1E). Thus, age-associated inflammation, and more specifically, chronic exposure to TNF, contributes to changes in macrophage function, resulting in decreased *S. pneumoniae* killing capacity and increased proinflammatory cytokine production.

Hyperinflammatory responses and tissue damage with age are dependent on chronic exposure to TNF

As macrophages from old TNF KO were protected from age-associated changes in bacterial killing and cytokine production, we wondered whether these mice were also protected from other, more global, manifestations of age-associated inflammation. Unlike old WT mice, which had increased IL-6 in the blood, plasma IL-6 levels in old TNF KO mice were similar to those in young controls (Figure 2A). Leukocyte infiltration (primarily consisting of lymphocytes) in the absence of infection was evident in the lungs of old WT mice (Figure 2B, SFigure 2A) and was accompanied by an increase of IL-6 in the lung tissue (Figure 2C). In contrast, there was no evidence of infiltrating leukocytes and no increase in IL-6 in the lungs of old TNF KO as compared to young controls (Figure 2D). When precision-*cut tissue slices* were obtained from the lungs of old and young WT mice, cultured and stimulated with *S. pneumoniae* for 4 h, old WT lungs produced more IL-6 than their young WT counterparts (SFigure 2B). This indicates that hyperinflammatory responses in the lung also occurred in old mice exposed to *S. pneumoniae,* as has been observed in the elderly(*26*, *27*).

Although our data demonstrated that the presence of TNF promotes systemic inflammation and impaired macrophage function, the cause of increased TNF production with age is unclear. Based on Metchnikoff's hypothesis that bacterial components from the gut microbiota could cause systemic inflammation, we investigated whether intestinal permeability and translocation of bacterial products occurred in aged mice.

Plasma levels of the bacterial cell wall component muramyl dipeptide (MDP) were significantly increased in old WT mice as compared to young mice (Figure 2E). To determine whether this arose due to impaired barrier function of the gut, we investigated whether there were differences in paracellular permeability in tracts of the ileum and colon of young

and old WT mice. Although there were no gross changes in intestinal architecture (SFigure 3A), we measured mucosal-to-serosal flux by using ^{51}chromium-EDTA (Cr-EDTA) and found that paracellular permeability increased in the colon in aged mice where microbial density in the gut is highest (Figure 2F). No differences in permeability between young and old mice in the ileum were found (Figure 2F). Tissue conductance, a measure of passive permeability to ions and small molecules (*28*), was also found to be increased in the colon in an age-dependent manner (SFigure 3B). Consistent with our findings that colonic barrier function declines with age, gene expression analysis of colons from young and old mice demonstrated that key genes regulating cellular junctions have significantly altered expression with age (Figure 2G).

Since TNF has previously been implicated in the induction of intestinal epithelial damage in HIV and resultant microbial translocation(*29*), we next sought to determine whether TNF could regulate intestinal permeability with age. We performed oral gavages with 3-5kDa FITC-labelled dextran on young and old mice, which allowed us to compare intestinal permeability in a non-lethal manner by measuring fluorescence of any FITC-dextran that had translocated to the plasma. Consistent with our measurements of paracellular permeability *ex vivo*, total circulating amounts of FITC-dextran were increased in old, but not young mice (Figure 2H), corroborating our findings *in vivo*. Furthermore, intestinal barrier function was found to be maintained in old TNF KO mice, and this correlated with circulating levels of MDP in these mice that were equivalent to young controls, confirming that TNF mediates age-associated changes in intestinal permeability (Figure 2E&H).

Finally, we wondered whether the induction of age-associated changes in permeability was also dependent on microbial exposure, or whether these changes were strictly host-mediated. Consequently, we investigated intestinal permeability in old germ-free (GF) mice and found no age-associated increase (Figure 2H). Thus, defects in barrier function in the gut depend on both host TNF and gut-microbial interactions.

Germ-free mice are protected from age-associated inflammation and dysregulated macrophage function

The central postulate of Metchnikoff's theory of aging is that the microbiome drives age-associated inflammation and macrophage dysfunction. Although we demonstrated that the microbiota can influence permeability with age, it remained unknown whether increased systemic inflammation followed as a *de facto* consequence as Metchnikoff had predicted. To test this, we measured systemic inflammation and macrophage function in old germ-free mice. Old GF-mice have a complete absence of any circulating microbial products, but, unlike old TNF KO mice, still possess the capacity to produce TNF, which we identified as the major driving cytokine of age-associated inflammation. As such, these mice serve as

an ideal model to determine whether the microbiota drives age-associated inflammation and downstream functional defects in macrophages.

Bone marrow derived macrophages from old GF mice did not have impaired *S. pneumoniae* killing capacity (Figure 3A) or produce more IL-6 than young GF mice either basally or after stimulation with LPS or *S. pneumoniae ex vivo* (Figure 3B). Furthermore, old GF mice did not demonstrate pockets of cellular inflammation (Figure 3C) or an increase in IL-6 in their lungs (Figure 3D). Similarly, old GF mice had plasma IL-6 levels that were similar to those from their young counterparts (Figure 3E). Levels of IL-6 were greater in the blood of old WT mice stimulated with LPS or a vehicle control; however, this did not occur in the blood of old GF mice (Figure 3F). Most strikingly, our aging GF mice lived longer than their WT counterparts (Figure 3G) and appeared healthier in late life, lacking prototypical phenotypic markers of aging otherwise observed in our old WT mice, including greying, dull, ruffled and patchy fur, reddened and swollen joints and mottled tails that occur due to breakdown of collagen (SFigure 4A-B). These data demonstrate chronic age-associated inflammation is strongly influenced by the presence of a microbiome.

The microbial community can influence the induction of age-associated inflammation

To better understand the timeline along which changes in barrier function are induced during the aging process, we performed a cross-sectional analysis of intestinal permeability in aging mice (3, 12, 15 and 18 month old). We found that permeability increased in a manner best modelled by exponential growth as a function of age, suggesting that the intestinal barrier becomes increasingly permeable later in life (Figure 4A).

Next, we sought to better understand the nature of the host-microbial relationship required to elicit age-associated inflammation and specifically whether the composition of the microbial community of the gut influenced the development of age-associated inflammation. To determine whether a low-diversity microbial community was sufficient to drive age-associated inflammation, we used mice colonized for 2 generations with the altered Schaedler flora (ASF) and allowed to diversify with time as previously described(*30*). Similar to old WT mice, old ASF-derived mice had increased intestinal permeability (Figure 4B), increased IL-6 production in whole blood following PBS or LPS stimulation (Figure 4C) and higher levels of plasma IL-6 (Figure 4D). The composition of the ASF-derived microbiome did not change significantly with age as modelled with DESeq2. No grouping by age was observed in either Bray-Curtis or weighted UniFrac distances, and the Shannon diversities did not vary with age (data not shown).

Although our data demonstrated that a microbiota of limited diversity was sufficient to elicit age-associated changes in permeability and inflammation, we next investigated whether there was an effect of microbial composition on the extent of these changes. Similar to what others have reported, we found that there was a marked shift in the composition of the microbiome with age (Supplementary Figure 5A-B)(*31*, *32*), although α-diversity, as

measured by Shannon index, was unaffected (data not shown). We found that, with age, a number of *operational taxonomic units (*OTUs) representing the order *Bacteroidales* were decreased in old mice and OTUs representing *Clostridiales* and *Erysipelotrichales* were increased in old mice (Supplementary Figure 5C and Supplementary Table 1). This is consistent with numerous studies that have demonstrated that these changes are characteristic of age-associated shifts in microbial composition in elderly humans (*31*–*35*), and correlate with health status in the elderly population (*25*, *36*). Age-associated inflammation may be a driving force in the microbial dysbiosis as TNF KO mice have less dramatic changes in their microbiomes as they age than do WT mice (Supplementary Figure 5D).

To determine whether this age-associated dysbiosis could affect intestinal permeability and age-associated inflammation, we recolonized young and old germ-free mice via oral gavage with a fecal solution sourced from either young or old mice raised under conventional specific pathogen free (SPF) conditions. After a minimum period of 12 weeks, we quantified changes in permeability and age-associated inflammation in the recolonized mice. We found that there was a slight but not statistically significant increase in plasma IL-6 in young mice that were recolonized with old microbiota and that there was a significant increase in plasma IL-6 levels in old GF mice recolonized with an old microbiota. Neither young nor old germ-free mice demonstrated increased plasma IL-6 when recolonized with microbiota from young mice (Figure 4E). These data indicate that the composition of the aging microbial community contributes to age-associated inflammation in the aged host.

To determine whether the composition of the microbial community influenced intestinal permeability, we measured both paracellular, macromolecular permeability (using ^{51}Cr-EDTA), as well as passive permeability (as indicated by tissue conductance) in the colon in our young and old recolonized mice. We found that paracellular permeability was not significantly increased, irrespective of donor or recipient age (Figure 4F). Passive ionic permeability, was increased only in old recipient mice recolonized with an old microbiota, indicating that while either variable in isolation may not influence ionic permeability, there may be a synergistic effect between the two (Figure 4G). These data indicate that there are age-related changes in passive permeability of the colon that are exacerbated in the presence of the microbial dysbiosis that occurs with age. Additionally our data indicate that chronic exposure to the gut microbiota can and does drive paracellular permeability. As such, breakdowns in barrier integrity likely contribute to passage of additional bacterial products to the circulation, thereby accelerating the induction of age-associated inflammation in chronically exposed hosts.

To determine whether the age of the recipient influenced the composition of the microbiota in our recolonized mice, we compared the composition and community structure of the fecal microbiota 12 weeks after colonization. There were no clear trends that would indicate that alpha-diversity, as measured by Shannon's index, was affected by the age of the recipient (data not shown). Due to the availability of aged mice, two separate experiments were performed in which young and old mice were recolonized with old microbiomes.

When beta diversity was visualized by weighted Unifrac or Bray-Curtis, it appeared as though the recipients clustered more closely with their donor microbiome than with the age of the donor or age of recipient(Supplementary Figure 6A-B). There was no distinct clustering of the microbial communities between mice colonized with old versus young microbiomes when visualized by either Bray-Curtiss or weighted Unifrac; however, there was a statistically significant difference between the overall microbial communities of mice recolonized with old versus young microbiomes as modelled with DESeq2. In addition, there were specific OTUs whose abundance was significantly different between the microbiomes of mice recolonized with old versus young donor microbiomes. OTUs whose expression changed by at least four-fold and had a p-value of at less than 0.05 were plotted in Supplementary Figure 6C. As in our analysis of microbiomes from young and old SPF mice collected in Supplementary Figure 5, microbiomes from mice 12 weeks post-colonization with microbiomes from old donors had fewer OTUs representing *Bacteriodales* and more representing *Clostridiales* and *Erysipelotrichales.*

Discussion

Age-associated inflammation is strongly linked with the development of chronic inflammatory disorders in the elderly, and is a strong risk factor for overall mortality in this population(*37*, *38*). Despite this, the etiological factors that lead to the development of age-associated inflammation have not been identified. This study demonstrates, for the first time, that the establishment of age-associated inflammation occurs due to the presence of a microbiota, similar to the established role of microbial translocation in the progression of disorders such as HIV.

The term “immunosenescence” describes the deleterious alterations in immune cell function that occur with age. Immunosenescence is most notably characterized by decreases in circulating lymphocytes and loss of antibacterial activity in natural killer (NK) cells, neutrophils, monocytes and macrophages(*39*). Immunosenescence is the major contributor to the susceptibility of the elderly to infectious disease in general(*39*–*41*), and pneumococcal infection in particular(*42*). Manipulation of the gut microbiome can reverse age-associated loss of function in immune cells. For example, oral supplementation with *Bifidobacterium* increased lymphocyte proportions in the circulation, improved the anti-tumoricidal activity of natural killer cells and restored phagocytosis in peripheral blood mononuclear cells and neutrophils(*43*, *44*). Interestingly, these benefits were most strongly evident in individuals 70 years of age and older, as well as those individuals who demonstrated the greatest degree of initial cellular immunosenescence. Furthermore, dysbiosis in HIV patients, which shows many parallels to that which occurs in the elderly (including decreased *Bifidobacteria* frequency and increased clusters of *Clostridium*) has been shown to shift following prebiotic administration. This led to a decrease in the overall degree of microbial translocation and ultimately improved NK and T cell function(*45*). These findings are consistent with our own data demonstrating that chronic exposure to the microbiome has detrimental consequences

for macrophage function in old age. Furthermore, they suggest that macrophage immunosenescence, like that of other immune cell populations, may be mutable.

Microbiome-driven hyperinflammation (both via the stimulation of increased proinflammatory cytokine production at the cellular level in macrophages, and the establishment of a systemic, tissue-wide inflammatory state) in the elderly may compound the deleterious effects of macrophage immunosenescence, particularly in response to infections. Age-associated inflammation has been shown to influence susceptibility to pneumococcal infection with age(*46*), and is also associated with increased disease severity(*46–48*). In fact, heightened systemic levels of TNF have been directly implicated in the impairment of *S. pneumoniae* clearance following systemic infusion in young mice(*49*). Thus, our data and that of others suggests that the consequences of long-term microbial exposure in the elderly for infection are two-pronged, involving both functional changes in anti-bacterial immunity and promotion of hyperinflammation that ultimately compromises host microbial defense.

Although the presence of even a minimal microbiota is sufficient to promote age-associated inflammation, microbial dysbiosis accentuates this phenomenon. Increases in plasma IL-6 occurred exclusively in old GF mice recolonized with an old donor microbiota, which had increased tissue conductance but no changes in paracelluar permeability to specific probes, indicating that the composition of the microbial community of old mice can influence systemic inflammation. In fact, analyses of alterations in gut microbial communities with age indicate that the *presence of beneficial commensals, such as Bifidobacteria,* negatively correlate with serum levels of TNF with age(*25*, *50*)*. Conversely,* there are increases in both the number and diversity of *Enterobacteria* and other Gram-negative, LPS-producing bacteria, and these bacteria correlate with increased proinflammatory cytokines in the blood of elderly patients, and could be further correlated with health status(*9*, *25*). The microbial communities of the elderly gut appear to be strongly influenced by diet (*25*) and dietary interventions designed to restore a robust microbiome may improve anti-bacterial immunity in the by reducing age-associated inflammation and macrophage immunosenescence.

Consistent with our findings that the gut microbiota can also influence systemic (i.e. lung) inflammation and damage, a recent study has shown that increased circulating bacterial toxins resulted in reduced tight junction gene expression and lethal pulmonary damage following fecal transplantation (*51*). The others suggest that these changes may occur following overgrowth of gut microbes and/or threshold production of bacterial products, resulting in their systemic translocation, increased inflammation, and ensuing pulmonary endothelial damage. The species that were mainly implicated in this pathogenicity were members of *Clostridia*, which others have also demonstrated have distinct abundance patterns in the aging gut microbial community(*32*). Furthermore, members of the genus are known to produce toxins with pathogenic properties that can

induce systemic inflammation(*52*) and changes in permeability in both the intestinal mucosa(*53*) and alveolar endothelium(*54*). Whether enrichment of *Clostridia* ultimately impacts age-associated induction of systemic inflammation or alterations in permeability with age remains to be determined.

Clostridiales are also believed to be key immunomodulatory species in the gut mucosa and are hypothesized to promote the pro-inflammatory loop that sustains other inflammatory disorders(*55*). This order also includes key short chain fatty acid (SCFA)-producing anaerobic species, which have been demonstrated as essential for the maintenance of intestinal epithelium barrier function(*56*) and the prevention of inflammation(*57*). Elderly humans have been found to have declined SCFA production(*58*); and others have suggested that loss of SCFA production may result in the passage of whole bacterial products that can disrupt immunological tolerance(*4*). Whether the increase of *Clostridiales* that occurs in the elderly ultimately impacts SCFA production immune tolerance, barrier function or systemic inflammation with age is not known.

Our data reveal a mechanism by which the gut flora promotes age-associated inflammation in the host. An overview of our proposed model can be found in Figure 4H. We have identified both TNF and the chronic presence of a microbiome as key regulators of permeability of the intestines in aged hosts. The increased translocation of microbial products that ensues increases inflammation in the blood and lungs, which ultimately dysregulates macrophage function. Furthermore, our data indicates that the resulting age-associated dysbiosis of the gut microbial community can differentially influence the degree of systemic inflammation in the aged host. These results represent an important advance in our understanding of how the intestinal microbiome can influence the aging process and may inform the design of therapeutics aimed at microbial manipulation to promote healthy aging.

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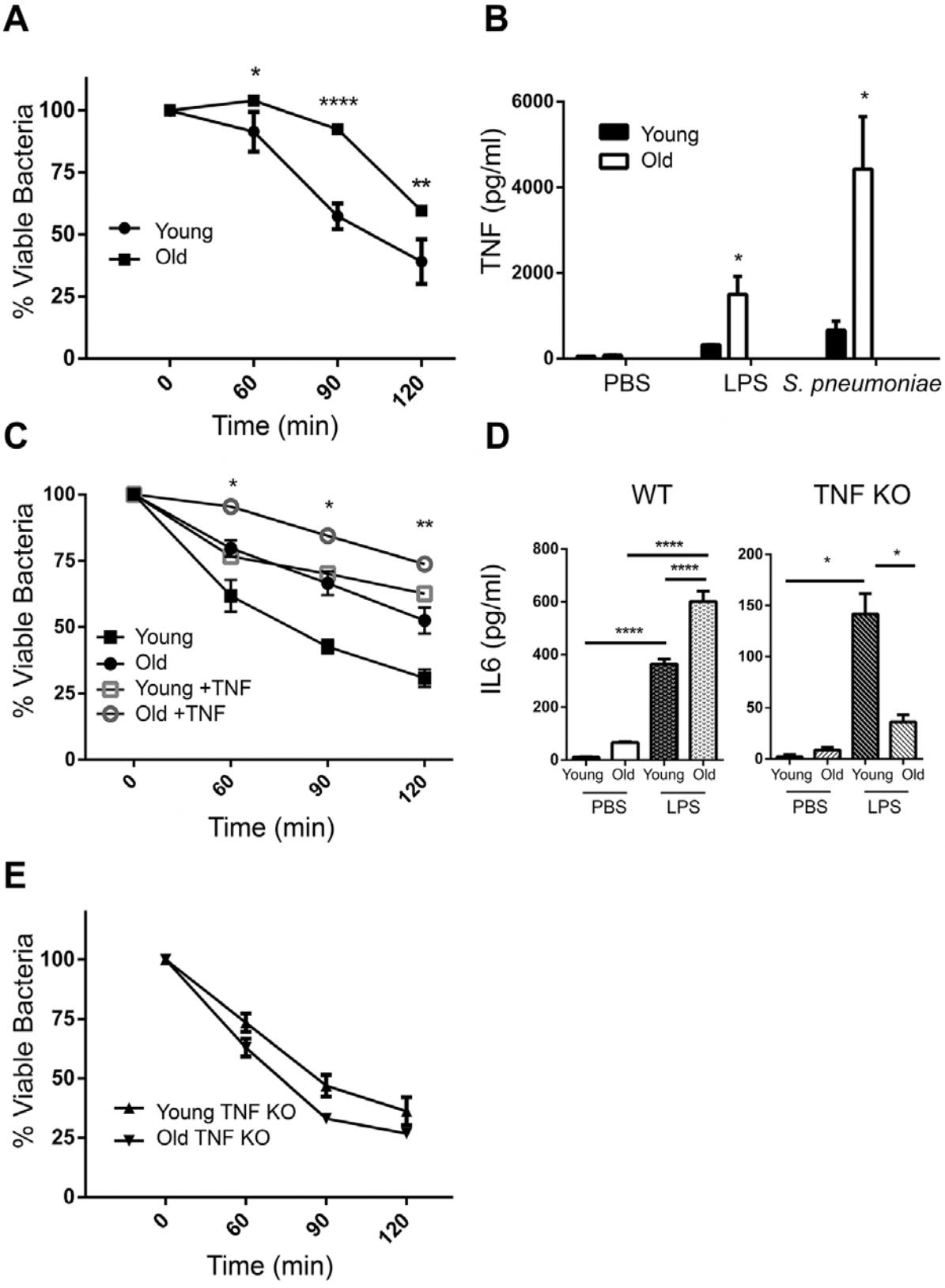

A
% Viable Bacteria
100
75
50
25
0
Young
Old
*
****
**
0
60
90
120
Time (min)
B
TNF (pg/ml)
6000
4000
2000
0
Young
Old
*
*
PBS
LPS
S. pneumoniae
C
% Viable Bacteria
Young
Old
Young +TNF
Old +TNF
*
*
**
Time (min)
D
WT
TNF KO
IL6 (pg/ml)
800
600
400
200
0
****
****
****
200
150
100
50
0
*
*
Young Old Young Old
PBS
LPS
E
% Viable Bacteria
Young TNF KO
Old TNF KO
Time (min)

Fig. 1. Macrophage function is dysregulated with age in a TNF-dependent manner. (**A**) Killing of *S. pneumoniae* by bone marrow derived macrophages from young and old C57Bl/6 mice (n = 6). (**B**) TNF production from macrophages derived from young and old mice following 24-hour stimulation with a vehicle control (PBS), LPS or *S. pneumoniae*, as measured by ELISA (n = 6). (**C**) Young and old murine bone marrow macrophage-mediated killing of *S. pneumoniae* in the presence of 10 ng/ml of exogenous TNF (n = 5). Changes in significance are shown relative to age-matched, vehicle controls. (**D**) IL-6 production in macrophages from young and old WT and TNF KO mice after 24 hour stimulation with a vehicle control, LPS or *S. pneumoniae* (n = 5 to 6); significant changes shown are relative to young WT mice. (**E**) *S. pneumoniae* killing in macrophages derived from young and old TNF KO mice (n = 5). Results represent pooled data and are shown as mean ± SEM. Statistical significance was determined using the Mann-Whitney test or two-way ANOVA with Fisher's post-test where appropriate. $p < 0.05$ is indicated by *; $p < 0.005$ by ** and $p < 0.0005$ by ***.

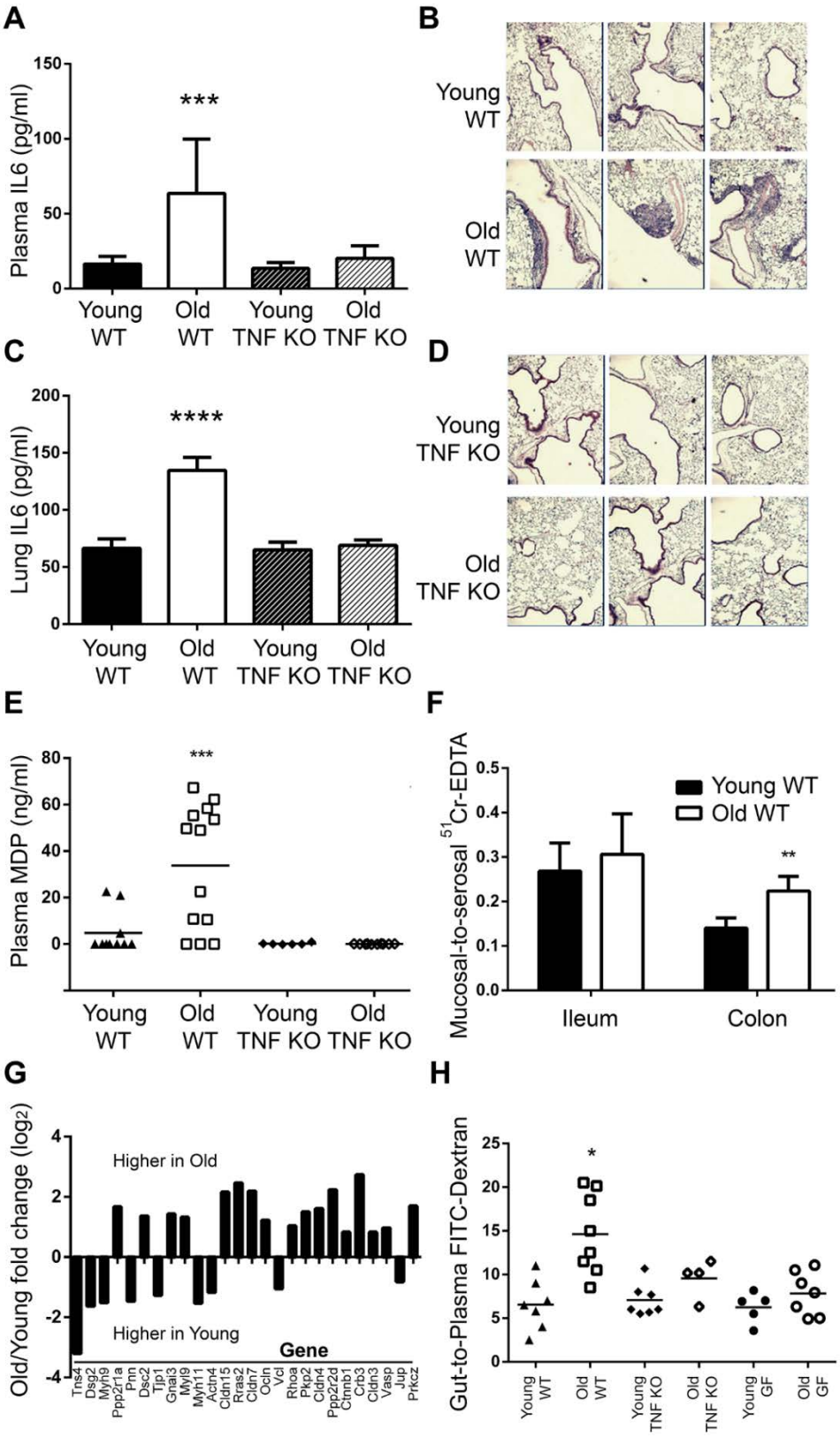

A
Plasma IL6 (pg/ml)
150
100
50
0
***
Young WT
Old WT
Young TNF KO
Old TNF KO
B
Young WT
Old WT
C
Lung IL6 (pg/ml)
200
150
100
50
0
****
Young WT
Old WT
Young TNF KO
Old TNF KO
D
Young TNF KO
Old TNF KO
E
Plasma MDP (ng/ml)
80
60
40
20
0
***
Young WT
Old WT
Young TNF KO
Old TNF KO
F
Mucosal-to-serosal 51Cr-EDTA
0.5
0.4
0.3
0.2
0.1
0.0
Young WT
Old WT
**
Ileum
Colon
G
Old/Young fold change (log2)
4
2
0
-2
-4
Higher in Old
Higher in Young
Gene
Tns4
Dsg2
Myh9
Ppp2r1a
Pnn
Dsc2
Tjp1
Gnai3
Myl9
Myh11
Actn4
Cldn15
Rras2
Cldn7
Ocln
Vcl
Rhoa
Pkp2
Cldn4
Ppp2r2d
Ctnnb1
Crb3
Cldn3
Vasp
Jup
Prkcz
H
Gut-to-Plasma FITC-Dextran
25
20
15
10
5
0
*
Young WT
Old WT
Young TNF KO
Old TNF KO
Young GF
Old GF

Fig. 2. Chronic exposure to TNF contributes to hyperinflammatory responses and tissue damage that occur with age. (**A**) Plasma IL-6 in young and old WT mice (*n* = 6). Significant changes shown are relative to young WT mice. (**B**) H&E stain of formalin-fixed histological sections from the lungs of young and old WT mice at 20X magnification. Each representative image is of a biological replicate. (**C**) IL-6 levels as detected by ELISA in whole lung tissue homogenates from young and old WT and TNF KO mice (*n* = 6). Significant changes shown are relative to young WT mice. (**D**) H&E stain of formalin-fixed histological sections of lungs of young and old TNF KO mice (20X magnification; each image represents a biological replicate). (**E**) Circulating muramyl dipeptide (MDP) in the plasma of young and old WT and TNF KO mice as measured by NOD-NF-κB promotor bioassay. Significant changes shown are relative to young WT mice. (**F**) Mucosal-to-serosal flux of ^{51}Cr-EDTA as measured in Ussing chambers was used to measure the paracelluar permeability of ileums and colons from young and old WT mice (*n* = 6 to 12). (**G**) Log_2 normalized relative expression of key genes related to cellular junctions showing age-dependent alterations in expression. Genes listed were found to be altered with a significance of $p < 0.01$ by Student's *t*-test. Genes expressed at higher levels in old mice as compared to young controls are depicted above the *x*-axis, while those with decreased relative expression are below the *x*-axis. (**H**) Intestinal permeability as measured by plasma FITC-dextran following oral gavage in young and old WT, TNF KO and germ-free (GF) mice (*n* = 5 to 10). Results represent pooled data and are shown as mean ± SEM. Significant changes shown are relative to young WT mice. Statistical significance was determined using the Mann-Whitney test or two-way ANOVA with Fisher's post-test where appropriate. $p < 0.05$ is indicated by *; $p < 0.005$ by ** and $p < 0.0005$ by ***.

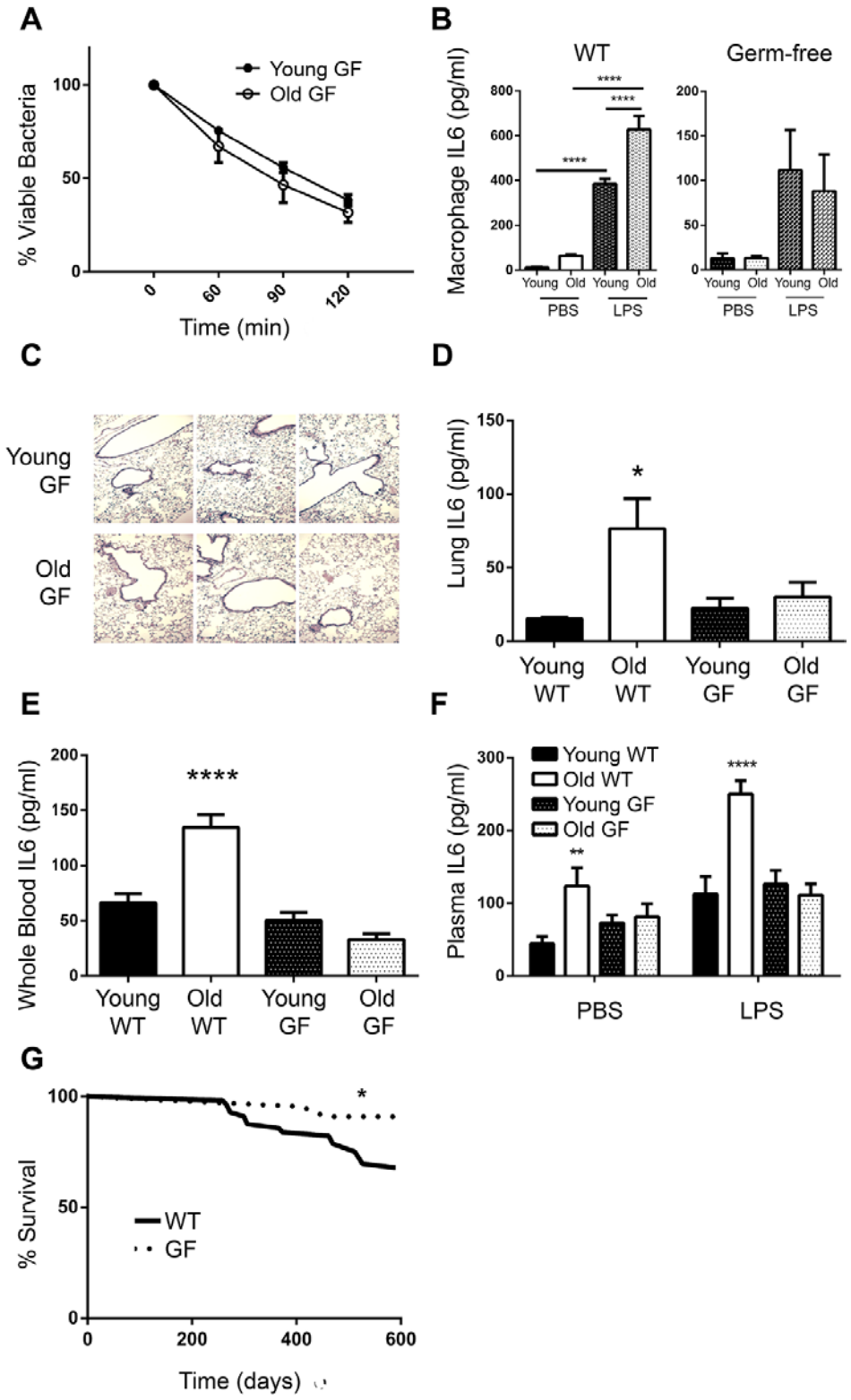

A
% Viable Bacteria
Young GF
Old GF
Time (min)
B
WT
Germ-free
Macrophage IL6 (pg/ml)
Young Old Young Old
PBS LPS
C
Young GF
Old GF
D
Lung IL6 (pg/ml)
Young WT
Old WT
Young GF
Old GF
E
Whole Blood IL6 (pg/ml)
F
Plasma IL6 (pg/ml)
Young WT
Old WT
Young GF
Old GF
PBS
LPS
G
% Survival
WT
GF
Time (days)

Fig. 3. Germ-free mice do not develop age-associated inflammation or macrophage defects, and have improved longevity. (**A**-**B**) *S. pneumoniae* killing (**A**) and IL-6 production following stimulation with LPS, *S. pneumoniae* or a vehicle control (PBS) (**B**) in macrophages from young and old GF mice (n = 5). Significant changes shown are relative to young WT mice. (**C**) Histological analysis of lung sections stained with H&E from young and old germ-free mice (20X magnification; each image represents a biological replicate). (**D**) IL-6 production in the lungs of young and old GF mice (n = 3 to 5). Significant changes shown are relative to young WT mice. (**E**) Plasma IL-6 in young and old GF mice (n = 5). Significant changes shown are relative to young WT mice. (**F**) IL-6 production in the whole blood of young and old WT and GF mice following stimulation with LPS or a vehicle control (PBS)(n = 5 to 9). Significant changes shown are relative to young WT mice. (**G**) Survival analysis showing all-cause mortality of WT and GF mice up to 600 days of life. Results represent pooled data and are shown as mean ± SEM. Statistical significance was determined using the Mann-Whitney test or two-way ANOVA with Fisher's post-test where appropriate. $p < 0.05$ is indicated by *; $p < 0.005$ by ** and $p < 0.0005$ by ***.

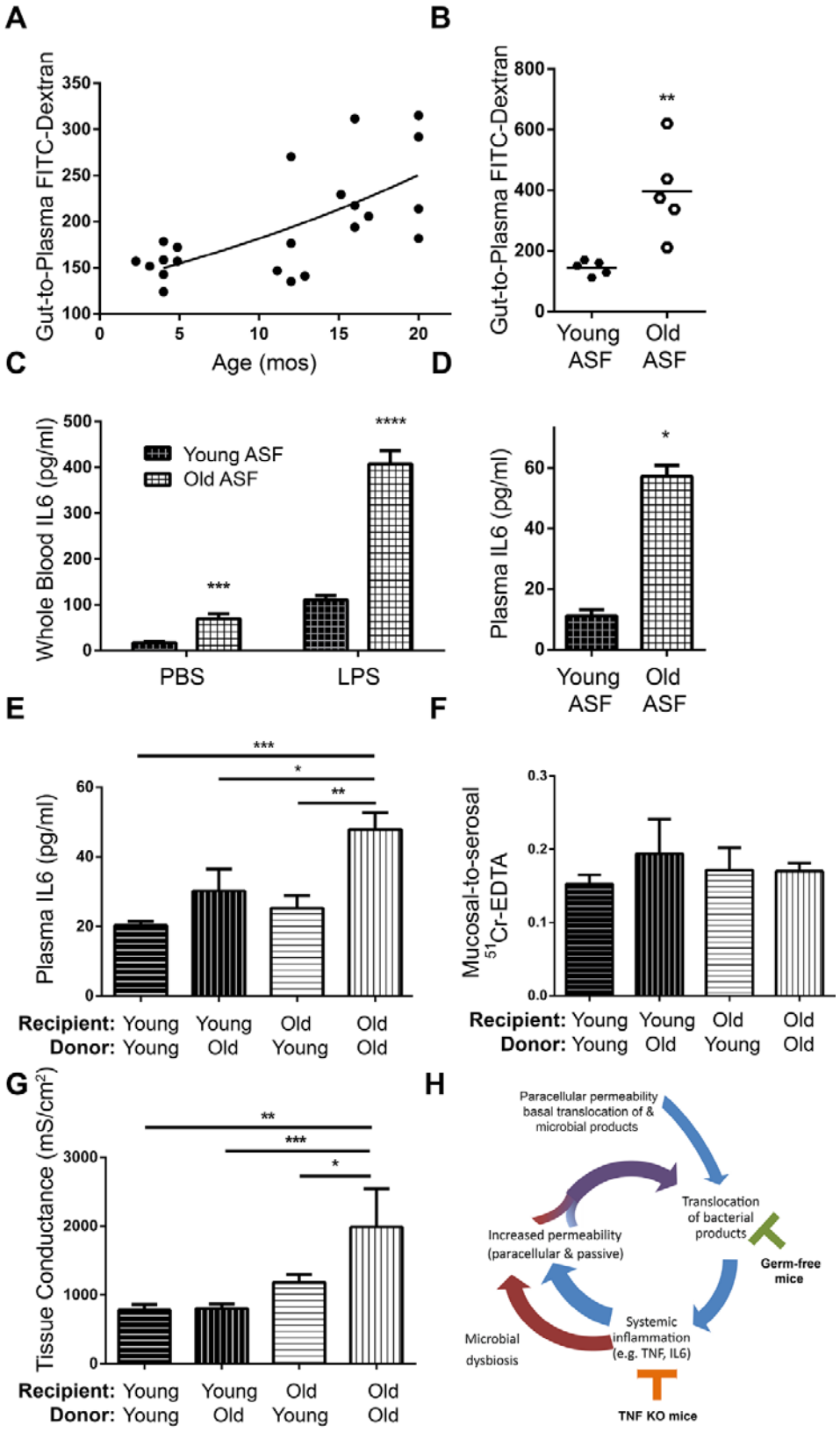

A
Gut-to-Plasma FITC-Dextran
Age (mos)
B
Gut-to-Plasma FITC-Dextran
Young ASF
Old ASF
C
Whole Blood IL6 (pg/ml)
Young ASF
Old ASF
PBS
LPS
D
Plasma IL6 (pg/ml)
Young ASF
Old ASF
E
Plasma IL6 (pg/ml)
Recipient: Young Young Old Old
Donor: Young Old Young Old
F
Mucosal-to-serosal 51Cr-EDTA
Recipient: Young Young Old Old
Donor: Young Old Young Old
G
Tissue Conductance (mS/cm2)
Recipient: Young Young Old Old
Donor: Young Old Young Old
H
Paracellular permeability basal translocation of & microbial products
Translocation of bacterial products
Germ-free mice
Systemic inflammation (e.g. TNF, IL6)
Microbial dysbiosis
Increased permeability (paracellular & passive)
TNF KO mice

Fig. 4. The extent of age-associated inflammation is dependent on the composition of the intestinal microbial community. (**A**) Intestinal permeability of aging mice (3, 12, 15 and 18 months old) as measured by FITC-dextran translocation to the circulation following oral gavage (*n* = 4-8; bars represent mean+/-STDEV). **(B**) Mice colonized with an ultra-clean ASF-derived microbiota were aged and their intestinal permeability was measured via FITC-dextran oral gavage assay (n = *5*). (**C-D**) measures of systemic inflammation in young and old ASF mice as determined by (**C**) IL-6 production in whole blood after 24 hours of stimulation with PBS or LPS and (**D**) plasma IL-6 (*n* = 3 to 5). (**E**) IL-6 production in the plasma of young and old GF mice recolonized with young or old donor microbiota (*n* = 3 to 6). (**F-G**) Sections of colon were mounted in Ussing chambers and (**F**) paracellular permeability (via ^{51}Cr-EDTA mucosal-to-serosal flux) and (**G)** tissue conductance were measured in young and old GF mice recolonized with a microbiota derived from young and old WT donor mice for a minimum of 3 months (*n* = 4 to 8). Significant changes shown are relative to all other treatments. Results represent pooled data and are shown as mean ± SEM. Statistical significance was determined using the Mann-Whitney test or two-way ANOVA with Fisher's post-test where appropriate. $p < 0.05$ is indicated by *; $p < 0.005$ by ** and $p < 0.0005$ by ***. (**H**) Proposed model of gut-microbe dependent induction of age-associated inflammation. Basal translocation of microbial products and increased intestinal permeability precede larger scale microbial product translocation. Systemic localization of these products elicits systemic inflammation, resulting in a microbial dysbiosis and increased permeability, which drive additional translocation of bacterial products, thereby promoting a self-sustaining cycle.

Supplementary Materials

Materials and Methods

Figs. S1 to S6

Table S1

References (*59-78*)

Supplementary Materials and Methods

Materials and Methods

Animals

Wild-type C57BL/6 mice were purchased from Charles River or Jackson Laboratories, while TNF knockout mice were obtained from the breeding colony of Dr. Zhou Xing. Pathogen-free status of mice within the aging colony was confirmed in mice through constitutive monitoring of sentinel mice and specific testing of fecal samples for common mouse pathogens. All of the animals that were used were sex-matched to their controls and maintained in the same animal room, with the exception of germ-free and ASF mice, which were bred in the Gnotobiotic Facility of McMaster. 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.

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.

Histology

Histopathological analysis was carried out on samples from the lungs of old WT, TNF KO and germ-free mice, and their young controls. Upon collection, lungs were formalin-inflated and these, alongside formalin fixed spleens, were paraffin-embedded. Tissue blocks were cut into 4-µm sections that were stained with hematoxylin-eosin (HE). For immunohistochemistry, antibodies against mouse CD3 were used. Images were acquired with a Leica DM LB2 microscope at a magnification of 20X and captured using a Leica DFC 280 camera.

Measurement of cytokine production

For circulating levels of cytokines, blood samples from naive animals were collected into heparin, and spun at 15000 x *g* for 5 minutes. 100 µl of plasma was then collected, and IL-6 levels assayed using ELISA as per the manufacturer's direction (eBioscience). For whole blood stimulation studies, 100 µl of whole blood samples collected in heparin from young and old WT, TNF KO and germ-free mice were stimulated with 100 ng/ml of LPS, or left unstimulated. Samples were incubated for 24 hours at 5% CO_2 and 37°C, then centrifuged at 15000*g* for 5 minutes. 50 µl of plasma samples were assayed for the presence of IL-6 using ELISA. To measure constitutive levels IL-6 in the lung, right lobe samples of lung were mechanically homogenized in 500 µl of PBS and assayed by ELISA. To measure inducible cytokine production in lung tissue, , lungs were perfused with low melt agarose and sliced into 10 micron sections as previously described(*59*)). 3 slices were cultured in 1 ml of media for 24 hours; supernatants were then removed and assayed for IL-6 production using 100 µl of sample ELISA. To measure cytokine production by bone marrow macrophages, 3.5 x 10^5 mature bone marrow-derived macrophages were seeded in a 24-well tissue culture-grade plate (Fisher) in 1.5 ml of media and allowed 24 hours to recover. Cells were then stimulated with either 100 ng/ml of LPS, whole heat-killed P1547 at an MOI of 50 or 50 µl of media control. Supernatants were collected at 24 hours post-stimulation. Levels of TNF or IL-6 were measured by ELISA.

Macrophage culture

Bone marrow derived-macrophages were isolated according to previously published methods(*60*) and differentiated in the presence of L929 conditioned media for 8 days as per standard protocols. After 8 days the cells were incubated with 4 mg/ml lidocaine (Sigma) for 15 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 maturation was assessed by flow cytometry using APC-conjugated anti-F4/80, PE-conjugated anti-Ly6G or -CCR2, FITC-conjugated Ly6C, eFluor 450-conjugated CD45 and PE-Cy7-conjugated CD11b, or corresponding isotype controls. PRR expression was measured using anti-TLR4-FITC, anti-TLR2-PE-Cy7 and anti-CD14-PerCpCy5.5 (eBioscience), as well as anti-MARCO-PE (RND systems)

Bacterial killing assays

To measure macrophage killing of *S. pneumoniae,* 5 x 10^5 bone marrow derived macrophages from were pre-incubated with an MOI of 10 bacteria per macrophage for 60 minutes at 37°C with gentle inversion as outlined above to allow for internalization of bacteria. Samples were then washed with PBS three times to remove unbound bacteria and bacterial killing was measured over the course of 120 min. The number of bacteria remaining after washing (t = 0 min) were normalized to 100%. At 30, 60, 90 and 120 min

the macrophages were lysed by osmotic rupture in distilled H_2O for 15 min and viable CFUs were determined by culturing of supernatants on TS agar plates as described above. To visualize *S. pneumoniae* uptake by macrophages, TRITC labelled bacteria were incubated with bone marrow derived macrophages for 2h at an MOI of 200. Cells were fixed and stained using an anti-beta actin antibody (Cell Signaling). Images were acquired at 40X magnification using an inverted Zeiss LSM510 laser confocal microscope.

In vivo permeability

Sections of colon and ileum were excised, opened along the mesenteric border, and mounted in Ussing chambers (World Precision Instruments, Sarasota, Florida). Recordings were performed as described in detail before(*61–63*). Briefly, tissues were allowed to equilibrate for 15-25 min before baseline values for potential difference (PD) and short circuit current (Isc) were recorded. Tissue conductance (G) was calculated by Ohm's law using the PD and Isc values. Mucosal to serosal flux of the small inert probe (360 Da) 51-*chromium*-ethylenediaminetetraacetic acid (^{51}Cr-EDTA) was used to assess paracellular permeability. After equilibration, time zero samples were taken from the serosal buffer and 6μCi/ml ^{51}CR-EDTA was added to the mucosal compartment. A "hot sample" was taken from the mucosal buffer then samples were then taken every 30 minutes from the serosal buffer for 2 hours and counted in a liquid scintillation counter (Beckman). Counts from each 30 min were averaged and compared to the "hot sample"(100%). Data expressed as mucosal-to-serosal flux (%flux/cm^2/hr).

For non-terminal studies, tracer FITC-labeled dextran (4kDa; Sigma-Aldrich) was used to assess intestinal permeability. Mice were deprived of food and water for 4 h following an oral gavage using 200 μl of 0.8 mg/ml FITC-dextran. Blood was retro-orbitally collected after 4 h, and fluorescence intensity was measured on fluorescence plates using an excitation wavelength of 492nm and an emission wavelength of 525 nm.

Gene expression analysis in the murine intestinal tract

Colons from three young (10-14 wk) and 3 old (16-18 mo) mice were collected and total RNA was extracted using the RNeasy Mini Kit (Qiagen). 4 μg of total RNA was used to prepare cDNA libraries which were sequenced (250 bp, paired-end or single-end) by Illumina MiSeq. Read alignment was performed using Bowtie followed by mapping to the murine reference genome (mm10) by TopHat. Transcript assembly, abundance estimation and comparison was performed using Cufflinks. Transcript abundance is presented as fragments per kilobase of exon per million fragments mapped (FPKM), and the significance of $\log_2$ fold-differences were determined by Student's *t*-test, with experimental-wise thresholds determined by Benjamini-Hochberg's procedure for controlling false discovery rate (FDR).Genes from old mice with a significant change compared to young controls (p at least 0.01) were selected and annotated using the DAVID Bioinformatics Resource (http://david.abcc.ncifcrf.gov/). Unique cellular junction related genes were compared in

whole colonic tissue from young and old WT mice using the KEGG pathway and Gene Ontology (GO) Cellular Compartment (GO_TERM_CC_FAT) ontology groups.

MDP Detection Bioassay

HEK293T cells stably were transfected with mNod2 (a kind gift from Dr. Jonathan Schertzer) and pNifty2-SEAP plasmids (Invivogen) to create a reporter system. Binding of the intracellular mNod2 receptor with its ligand, MDP, results in downstream activation and translocation of NF**κ**B. Activation of this transcription factor leads to SEAP expression via the ELAM proximal promoter, which is detected via absorbance spectroscopy. Plates were seeded with cells 24 hours prior to addition of heat-inactivated mouse plasma, diluted 1 in 200 in HEK Blue Detection Media (Invivogen) to a final volume of 200 µl, in a 96-well plate format. Readings were performed at 630nm, 24 hours subsequent to stimulation.

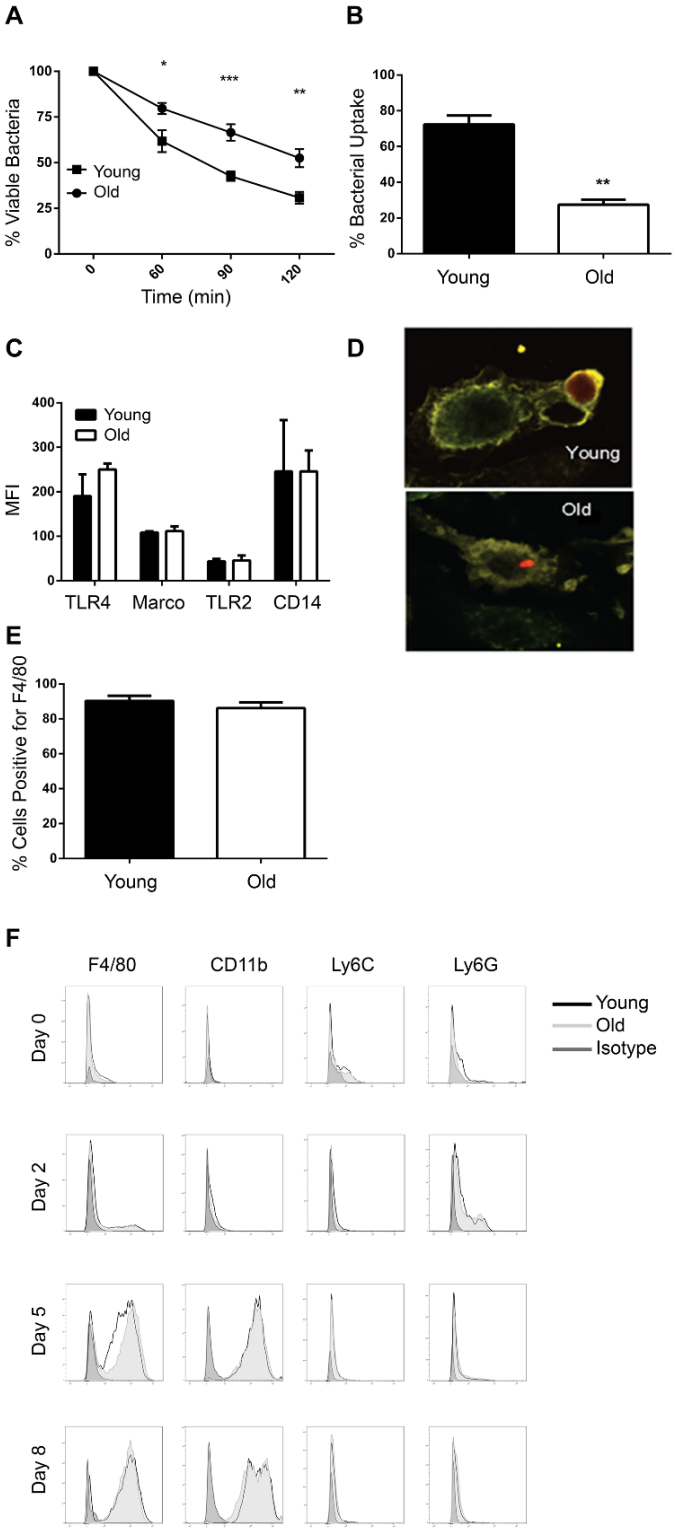
Germ-free Mouse Recolonization

For recolonization studies, young and old germ-free mice were transferred to specific-pathogen free housing and immediately given oral gavage of mouse fecal pellet slurry. Slurry was comprised of fecal pellets from 5 young or 5 old WT mice suspended in sterile PBS. Mice were monitored for signs of recolonization (reduction in cecal enlargement) and were maintained for a minimum of 3 months after initial recolonization at which point fecal pellets were collected for microbiome analysis (as described below), plasma IL-6 was assayed and intestinal permeability was measured as described above.

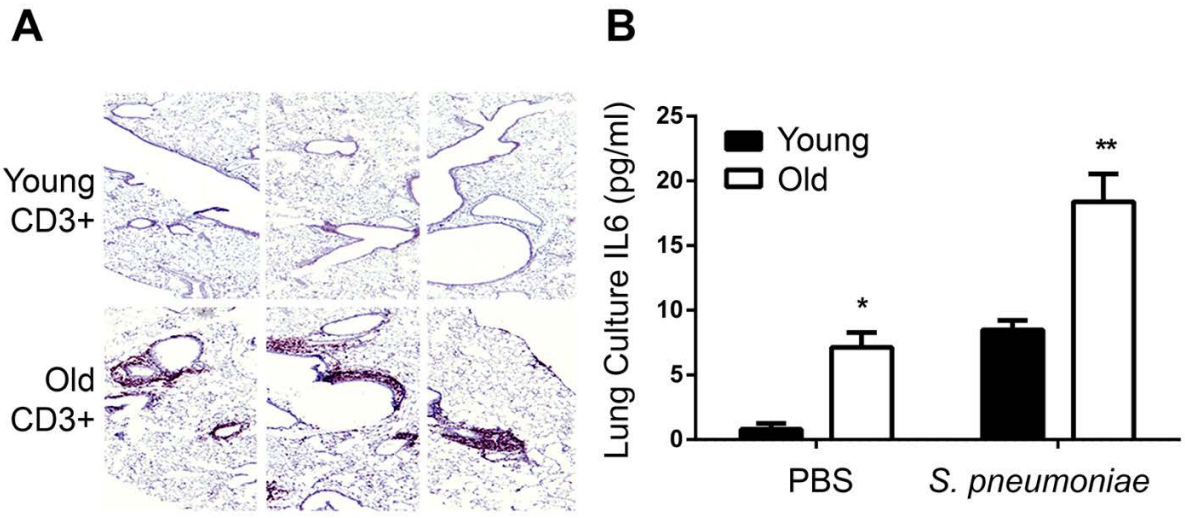
Statistics

Unless otherwise mentioned in the figure legend, statistical significance was determined by two-way analysis of variance with Fischer's post-test and unpaired *t* tests (two tailed). Statistical significance was defined as a p value of 0.05. All data were analyzed with Prism (Version 6; GraphPad). Statistical analysis of microbiome changes was conducted using principal component analysis as measured by weighted UniFrac distance and Bray-Curtis.

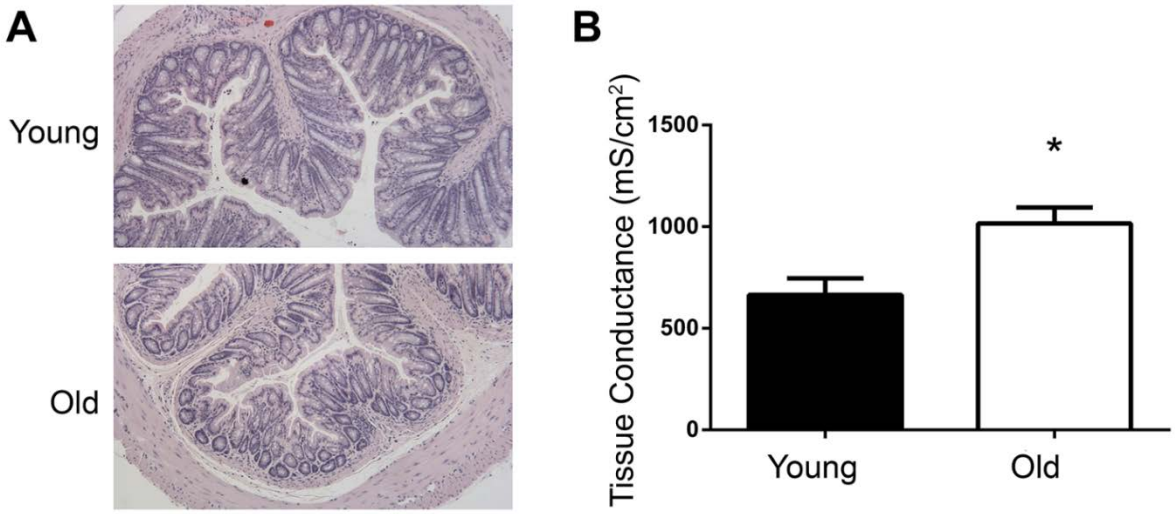
Supplementary Figures



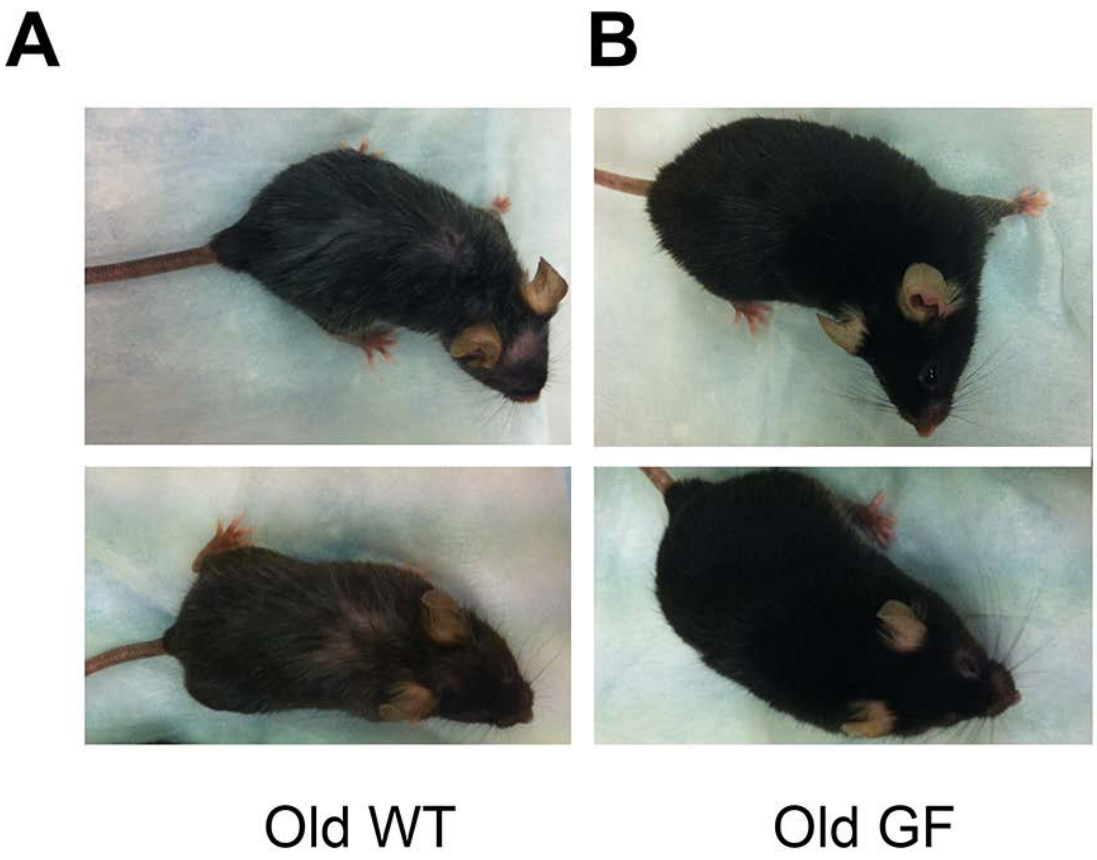
Supplementary Figure 1. Maturation status and pattern recognition receptor expression of macrophages does not differ with age, but bacterial uptake and killing does. (**A**) Killing of *S. pneumoniae* by resident peritoneal macrophages isolated from young and old mice (*n* = 5). (**B**) Uptake of heat-killed, TRITC-labelled *S. pneumoniae* by bone marrow derived macrophages from young and old mice as measured by flow cytometry (*n* = 6). (**C**) Surface expression of pattern recognition receptors on bone marrow derived macrophages from young and old mice, as measured by flow cytometry (*n* = 5). (**D**) Immunoflourescent microscopy (20x) of bone marrow macrophages stained for beta-actin (green) from young and old mice following 2 h incubation with live *S. pneumoniae* (red). (**E**) Percentage of cells positive for F4/80 (as determined by flow cytometry) following young and old WT mouse bone marrow macrophage differentiation for 8 days using M-CSF (*n* = 5 to 6). (**F**) Longitudinal assessment of bone marrow macrophage differentiation. Histograms showing expression of key maturity markers (CD11b, F4/80, Ly6C and Ly6G) on differentiating bone marrow at days 0, 2, 5 and 8. Bone marrow was derived from young (3 mo) and old (18-20 mo) mice and differentiated in M-CSF-enriched media. Bone marrow from one representative mouse of at least three is shown; cells were analyzed using flow cytometry. Statistical significance was determined using the Mann-Whitney test or two-way ANOVA with Fisher's post-test where appropriate. $p < 0.05$ is indicated by *; $p < 0.005$ by ** and $p < 0.0005$ by ***.



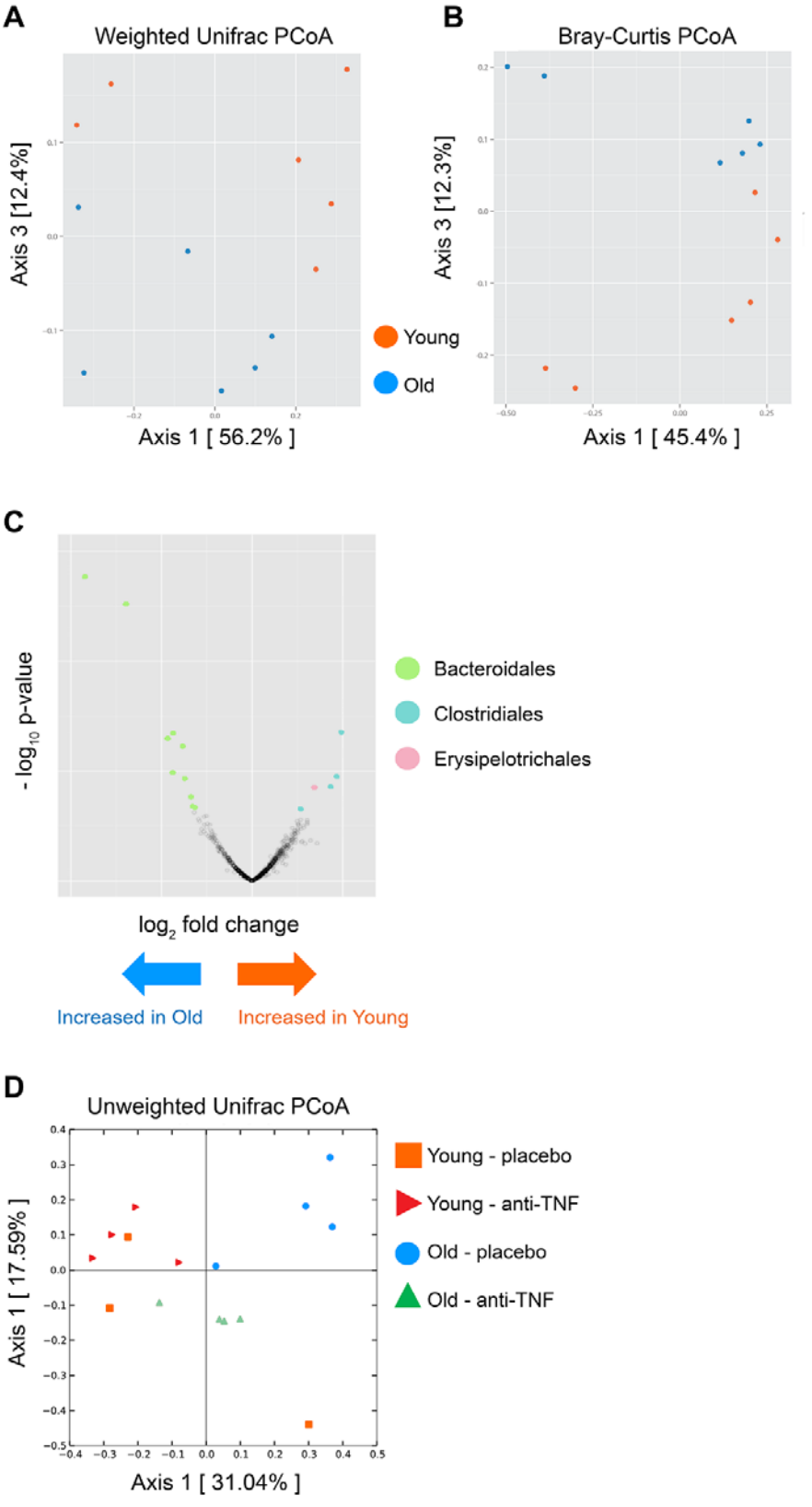
Supplementary Figure 2. Lungs from old mice have more CD3+ leukocytic infiltration and produce more IL-6 basally and in response to *S. pneumoniae* as compared to those from young mice. (**A**) Immunohistochemistry of lung slices from young and old mice stained with CD3 antibody. Lung slices were derived from the lungs of young and old mice and cultured in media. IL-6 production was subsequently measured in the supernatant at 4 hours following stimulation with heat-killed *S. pneumoniae* or PBS control (n = 3).



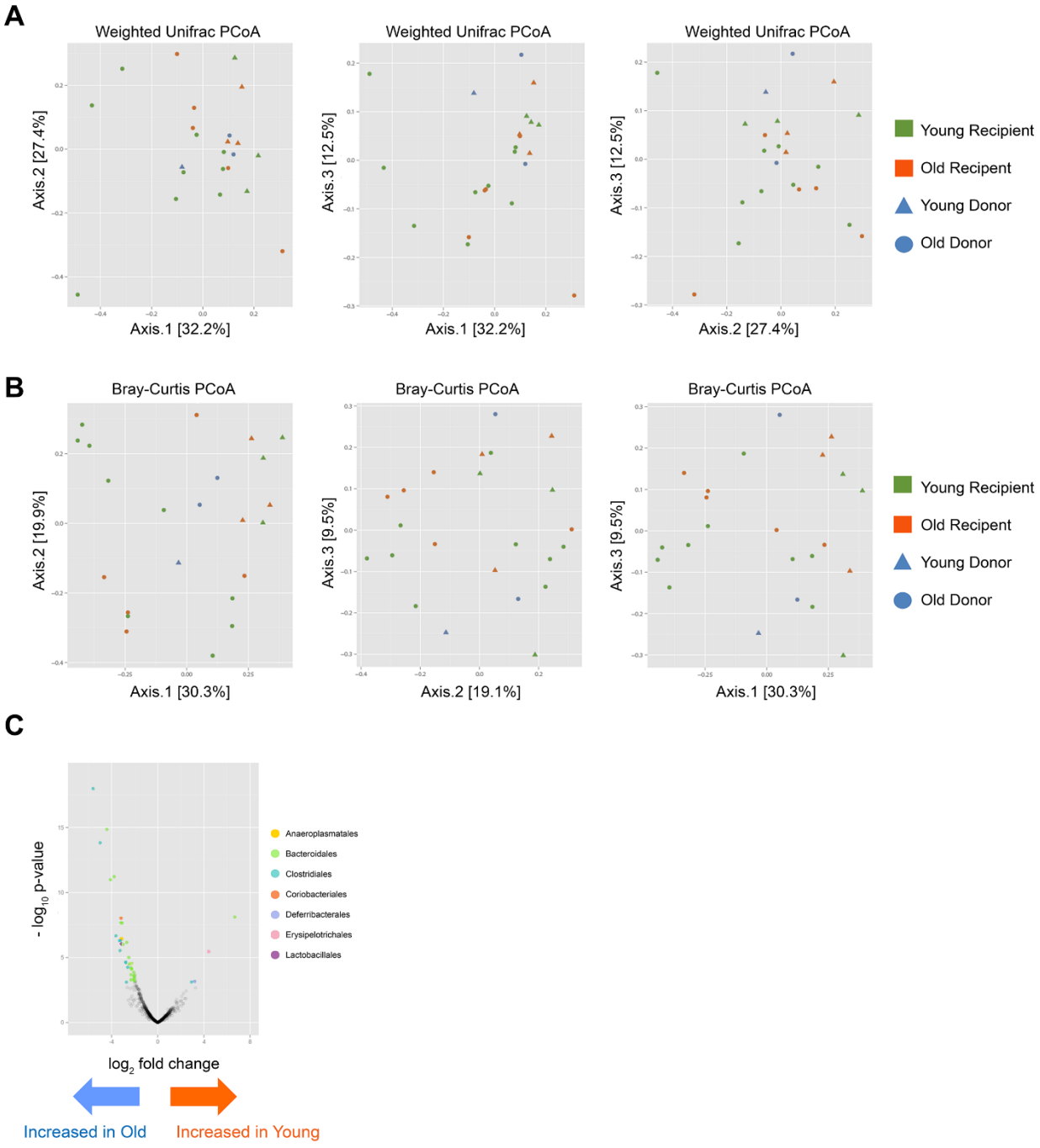
Supplementary Figure 3. Intestines from old mice have increased tissue conductance and do not show gross histological changes as compared to young mice. (A) Colons of young and old mice do not have gross changes in architecture (**B)** Tissue conductance as measured in Ussing chambers in colons from young and old WT mice indicate that tissue conductance is increased in old mice(*n* = 6 to 12).



Supplementary Figure 4. Visual comparison of old germ-free mice and old WT mice. (**A**) Photographs of old WT (left) and GF (right) mice. Germ-free mice exhibit fewer outward manifestations of aging such as grey fur, balding and dandruff.


A
Weighted Unifrac PCoA
Axis 3 [12.4%]
Axis 1 [56.2%]
Young
Old
B
Bray-Curtis PCoA
Axis 3 [12.3%]
Axis 1 [45.4%]
C
- log10 p-value
log2 fold change
Increased in Old
Increased in Young
Bacteroidales
Clostridiales
Erysipelotrichales
D
Unweighted Unifrac PCoA
Axis 1 [17.59%]
Axis 1 [31.04%]
Young - placebo
Young - anti-TNF
Old - placebo
Old - anti-TNF

Supplementary Figure 5. Microbial communities of the gut are distinct in young and old mice. Principal coordinate analysis based on **(A)** weighted UniFrac distance and **(B)** Bray-Curtis. Chi-squared of the likelihood ratio test in DESeq2 shows old and young microbiomes are significantly different ($p < 0.001$, not shown). (**C**) The OTUs that were significantly different between young and old mice and had at least a four-fold change between the ages are plotted. OTUs representing Bacteroidales (green) were higher in young mice and OTUs representing Clostridales (blue) and Erysipelotrichales (pink) were higher in old mice. (D) Although the microbial communities of old and young WT mice were distinct when visualized using Bray-Curtis PCoA plots, the microbial communities of young and old TNF KO mice did not diverge with age.


A
Weighted Unifrac PCoA
Axis.2 [27.4%]
Axis.1 [32.2%]
Weighted Unifrac PCoA
Axis.3 [12.5%]
Axis.1 [32.2%]
Weighted Unifrac PCoA
Axis.3 [12.5%]
Axis.2 [27.4%]
Young Recipient
Old Recipent
Young Donor
Old Donor
B
Bray-Curtis PCoA
Axis.2 [19.9%]
Axis.1 [30.3%]
Bray-Curtis PCoA
Axis.3 [9.5%]
Axis.2 [19.1%]
Bray-Curtis PCoA
Axis.3 [9.5%]
Axis.1 [30.3%]
Young Recipient
Old Recipent
Young Donor
Old Donor
C
- log10 p-value
log2 fold change
Anaeroplasmatales
Bacteroidales
Clostridiales
Coriobacteriales
Deferribacterales
Erysipelotrichales
Lactobacillales
Increased in Old
Increased in Young

Supplementary Figure 6. The age of mice influences microbiota reconstitution. Young (6-10 wk) and old (18-22 mo) germ free mice were recolonized with fecal slurries derived from young mice (purple) or old mice (green and red). (**A**) Weighted UniFrac distance and (**B**) Bray-Curtis PCoA plots demonstrate that although there is no clear partitioning between mice colonized with young or old microbiomes, or between young and old recipient mice, the microbiomes of the recipient mice cluster more closely with the donor microbiomes with which they were colonized. (**C**) As in young and old SPF mice, mice recolonized with microbiomes from young and old donor had differences in abundances of specific OTUs. The OTUs that were significantly different between young and old microbiota after 12 weeks of re-colonization are plotted by log2 fold change vs. -log10 p-value.

Supplementary Tables

Supplementary Table 1. Significantly changed OTUs between fecal microbiomes of young and old mice.

OTU	Phylum	Class	Order	Family	baseMean	log_2FoldChange	pvalue	padj
20	Bacteroidetes	Bacteroidia	Bacteroidales	NA	340.78868	-9.235547	1.44E-14	1.96E-11
44	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	128.87719	-6.951082	2.48E-13	1.69E-10
140	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	17.804461	4.9164107	1.66E-07	6.36E-05
183	Bacteroidetes	Bacteroidia	Bacteroidales	NA	12.140085	-4.358555	1.87E-07	6.36E-05
145	Bacteroidetes	Bacteroidia	Bacteroidales	NA	17.287917	-4.659503	3.22E-07	8.77E-05
214	Bacteroidetes	Bacteroidia	Bacteroidales	NA	8.5888366	-3.828773	7.42E-07	0.000169
179	Bacteroidetes	Bacteroidia	Bacteroidales	NA	12.333908	-4.382969	1.17E-05	0.002288
157	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	15.024023	4.6623803	1.76E-05	0.002993
223	Bacteroidetes	Bacteroidia	Bacteroidales	NA	7.9922455	-3.717984	2.16E-05	0.003278
162	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	14.091852	4.3356768	5.30E-05	0.00694
166	Tenericutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	13.392079	3.4324206	5.60E-05	0.00694
254	Bacteroidetes	Bacteroidia	Bacteroidales	NA	6.4152657	-3.375369	0.000148	0.01678
263	Bacteroidetes	Bacteroidia	Bacteroidales	NA	6.0475407	-3.281328	0.000399	0.041798
279	Bacteroidetes	Bacteroidia	Bacteroidales	NA	5.5573464	-3.147163	0.000457	0.044512
226	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	7.7119465	2.6701394	0.000538	0.048913

Supplementary Materials & Methods

Bacterial DNA isolation and Illumina sequencing of bacterial tags

DNA was extracted from fecal samples with a custom DNA extraction protocol involving mechanical and enzymatic lysis followed by a phenol:chloroform extraction and a clean-up step. First, approximately four fecal pellets were added to a tube containing 0.1 mm glass beads within a 2 mL plastic screw top tube (MoBio Laboratories Inc. Carlsbad, CA, USA) along with 800 µl of 200 mM sodium phosphate monobasic (pH 8) and 100 µl GES buffer (50.8 mM guanidine thiocyanate, 100 mM EDTA and 34 mM N-lauroylsarcosine). These were then homogenized in the PowerLyzer 24 Bench Top Homogenizer (MoBio Laboratories Inc. Carlsbad, CA, USA) for 3 minutes at 2500 RPM.

Next, two enzymatic lysis steps were performed. In the first, the sample was incubated with 50 µl of 100 mg/ml lysozyme (100 mg/ mL), 50 µl mutanolysin (10U/ µl) and 10 µl of 10 mg/ml RNase (10 mg/ mL) for 1 hour in a 37 °C waterbath. In the second, the sample was incubated with 25 µl 25% SDS, 25 µl of 20 mg/ml Proteinase K and 100 µl of 5 M NaCl at 65 °C for 1 hour. Next, debris was pelleted in a table-top centrifuge at maximum speed for five minutes and 900 µl of the supernatant was added to 900 µl of phenol:chloroform:isoamyl alcohol (25:24:1). The sample was then vortexed and centrifuged at maximum speed in a tabletop centrifuge for 10 minutes. The aqueous phase was removed and the sample run through the Zymo Research Clean and Concentrator-25 column (Irvine, CA, USA) according kit directions except for elution which is done with 50 µl of ultrapure water and allowed to sit for 5 minutes before elution. The DNA was quantified using a Nanodrop 2000c Spectrophotometer.

Amplification of bacterial 16S rRNA gene V3 region tags was done as in (Bartram et al, 2011) with the following changes: 5 pmol of primer, 1 µl of 10 mM dNTPs, 1.5 µl of 50 mM $MgCl_2$, 2 µl of 10 mg/ml BSA and 0.5 µl of Taq polymerase were used in a 50 µl reaction volume. The reactions were then split into three equal volumes. The PCR program used was as follows: 94 °C for 2 minutes followed by 30 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 30 seconds. Finally, there was a final extension step at 72 °C for 10 minutes.

Illumina libraries were sequenced in theMcMaster DNA Sequencing Facility with the following steps. Pooled libraries were first tested on an Agilent BioAnalyzer High Sensitivity DNA chip then quantified with qPCR using Illumina's PhiX control library as a standard, SYBR fast 2x qPCR mastermix (KAPABiosystems) and primers that bind to the distal ends of the adaptors (flowcell 155 binding regions): P5 5'-AATGATACGGCGACCACCGA-3', P7 5'-CAAGCAGAAGACGGCATACGA-3'. 16S rRNA gene v3 region pools were then combined with PhiX control DNA in a 9:1 ratio and 250 bp were sequenced in the forward and reverse direction on the Illumina MiSeq instrument. The completed run was demultiplexed with Illumina's Casava software (version 1.8.2).

Sequence Processing & Data Analysis

Custom, in-house Perl scripts were developed to process Illumina sequences and are available from the authors(*64*). First, Cutadapt(*65*) was used to trim any reads that exceeded the v3 region of the bacterial 16S rRNA gene. The resulting paired-end sequences were aligned with PANDAseq(*66*) and sequences with any mismatches or ambiguous bases were culled. Input sequences from all samples were clustered into operational taxonomic units (OTUs) using AbundantOTU+(*67*) with a clustering threshold of 97%. Output from this tool was then formatted for input into QIIME, where taxonomy was assigned using the Ribosomal Database Project (RDP) classifier(*68*) with a minimum confidence cutoff of 0.8 (QIIME default) against the Greengenes (Feb 4th 2011 release) reference database to the genus level(*69*). All OTUs classified as "Root:Other" were excluded. All OTUs represented by only a single sequence were excluded for all analysis except α-diversity.

Statistical analysis was performed in R(*70*) (scripts available from the authors). The α-diversity was measured by Shannon diversity(*71*), because its insensitivity to low-abundance species makes it the diversity measure which can be most accurately estimated from a sample with microbiome data(*72*). β-diversity was calculated using both weighted UniFrac distance(*73*) and Bray-Curtis distance(*74*) on relative abundance profiles, since these have been found to give the most accurate clustering of microbiome data(*75*). β-diversity was plotted by PCoA.

The alpha and beta diversity measures were calculated using the phyloseq package in R(*76*) and plotted with ggplot2(*77*). Analysis of the differential abundance of OTUs by age was performed using the negative binomial-based generalized linear model implemented in the DESeq2 package in R(*78*). Data were entered into DESeq2 using the wrapper provided by phyloseq(*75*). We used a likelihood ratio test (LRT) to determine whether mouse age generated a significantly better model fit than a simple intercept model for each OTU. P-values were corrected using a Benjamini-Hochberg false discovery rate (FDR) of 10% without independent filtering based on mean abundance. To determine whether the microbiomes were different overall, we summed the Chi-squared test statistics provided by each individual OTU, and determined the p-value by summing the degrees of freedom of all the individual OTU tests.

Chapter 5. Discussion

Inflammaging and morbidity and mortality in the elderly are tightly linked, with inflammaging underlying almost every chronic disorder for which aging is a risk factor(*13*). Inflammaging is not limited to chronic inflammatory disorders, but also increases the risk of infectious disease acquisition and pathogenesis. Since inflammaging has emerged as a unifying process governing disease and illness in the elderly, it serves a prime target for intervention strategies to promote healthy aging.

The work presented in this thesis is focused on studying inflammaging, both its origins and consequences. Chapter 2 provides a comprehensive technical description of *Streptococcus pneumoniae* intranasal colonization in mice, as well as an overview of its impacts on immune responses in the nasopharynx. Chapter 3 is centered on understanding the role of TNF as a fundamental mediator of inflammaging, particularly as it shapes Ly6C^{high} monocyte phenotype, function and recruitment during the steady state and following *S. pneumoniae* colonization. Chapter 4 focuses on investigating the intestinal microbiota as the initiating factor leading to inflammaging, and its downstream effects on macrophage function. Together, these studies advance our understanding of the interactions between the host and the aging microenvironment, and uncover the driving factors behind the etiology of inflammaging.

Each individual manuscript presented within this thesis is complete with its own discussion. Therefore, the intent of the following discussion section is to extend and integrate interpretation of these collective findings, provide justifications for experimental design, highlight limitations and outline future avenues of work.

Modelling Aging

In humans, aging is a process that includes: 1) altered cellular function, 2) decreased physiological capacity and 3) reduced ability to respond to environmental stressors such as pathogens(*195*). With the passage of time, these changes accumulate and lead to increased susceptibility and vulnerability to infection and disease, ultimately resulting in increased mortality. Although inevitable, aging is also highly mutable, arising from a mix of diverse interactions between biological changes in the host (based on genetic background and epigenetic regulation) and environmental factors (including diet, history of antigenic exposure and levels of physical activity). This multitude of individual variables results in a complexity which limits attempts to understand the aging process itself(*195*).

In a perfect world, aging research would employ humans as subjects to model aging. Unfortunately, use of humans in this manner becomes far from ideal when one considers the long natural life span that humans enjoy, the large amount of genetic variation between individuals and the numerous environmental influences that humans are exposed to (which have effects on the aging process that are difficult to properly quantify and understand). Furthermore, while humans may suffice for observational studies, ethical considerations make it unrealistic to gain more mechanistic understanding from human aging models. Therefore, many in the aging research field, our laboratory among them, have turned to the use of animal models to study the biology of aging.

Animal models used to understand the basis of aging should try to faithfully reproduce the aging process while controlling for confounding intrinsic and extrinsic factors. Employing mice as model organisms for the study of mammalian aging allows one to limit the degree of genetic variability, while strictly controlling for environmental factors like diet, housing and microbial exposure. It also allows one to study mammalian aging in a relatively short time span; unlike primates, which may be closer to humans genetically but still possess relatively long lifespans, most standard strains of laboratory mice live an average of 2 years. As such, mouse models have proven to be indispensable for the study of aging, providing researchers with subjects that have short lifespans, are low-cost, and that lend themselves well to genetic manipulation and therapeutic intervention(*196*). And although mice do differ from humans biologically, they are still remarkably similar with regards to gross anatomy, physiology and cellular functions(*197*, *198*). Unlike some other mouse models of disease and pathology, the induction of aging in mice requires no pre-existing genetic bias or additional interference, merely the passage of time. And, importantly for this thesis, the immune systems of mice and men are for the most part analogous in development, architecture and function(*199*), with many age-associated changes that occur in humans also characterized as occurring in mice. For example, both murine and human aging is accompanied by myeloid skewing in circulating leukocyte populations(*200*). From the standpoint of the work outlined in this thesis, one of the most crucial similarities that exists between mice

and humans with regards to aging is the advent of an inflammaged state with advancing age(*13*).

In order to perform the experiments described in this thesis, we established an aging mouse colony that could serve as a source of aging subjects. Upon its inception, many considerations were made as to how to best extract information that showed true changes in the normal aging process. The consideration of age was of prime importance. Consistent with what has been reported by others, we used mice that were a minimum 10-12 weeks old as young adult controls, to ensure that we were studying developmentally mature mice, and not juveniles(*201*). Our old mice were limited to an age of 22 months, so that we were not artificially selecting for survivor mice who reached extreme old age. Mice with confounding conditions, such as the development of extensive skin lesions, cancer or kidney disease were also excluded from study(*201*). Aging mice were also raised in vent racks, under specific pathogen-free conditions, and tested for any evidence of common murine pathogens via fecal and tape stripping analyses.

Caloric restriction has long been known to extend murine and human lifespans(*202*); conversely, in elderly populations, obesity correlates strongly with earlier death(*203*). To minimize the effects of an *ad libitum* diet, as well as cage dynamics (wherein one or two mice tend to have much higher weight than subordinate cage mates), we developed a specialized low-fat, twice autoclaved diet.

This diet is more difficult to ingest and therefore limits overfeeding without compromising nutrition.

Because stress can negatively impact immunity and disease susceptibility in mice(*204–206*), we also provided members of our aging mouse colony with environmental enrichment. In laboratory mice, the most common cause of stress is the inability to control one's environment, arising from boredom in a barren setting over which the animal can exercise little control(*207*). An abundance of literature now exists demonstrating that housing enrichment reduces stress in mice, providing them with stimulation and some degree of control over their environment(*207*), with preference given to setups with the most complexity. In addition to reducing the confounding factor of stress, enrichment also reduces stereotypic behavior in mice, such as in-fighting and barbering(*208*, *209*), thereby decreasing the incidence of lesions. With these studies in mind, we utilized housing domes, exercise wheels and nesting substrate in our aging colony.

Experimental parameters are often set with the goal of segregating the mechanistic properties of the phenomena under study, which is particularly vital when the phenomenon of study is one as complex as aging. Because of this, despite a researcher's best intentions, experimental models are ultimately limited. On one hand, exclusion of variables is necessary to confidently isolate biological mechanisms. On the other, this imposes an artificial layer to the process being studied. For example, beyond the obvious limitations in using mice to model human aging, our mouse model of aging is solely based on data using one mouse strain (C57Blk/6J), represented

mostly by one sex (females), with a limited representation of age ranges (10-14 wo adult mice; 18-22 mo old mice), housed under pathogen-free conditions (and therefore without the natural extent of pathogenic exposure mammals experience in the real world), with infectious disease responses studied in the context of one pathogen (*S. pneumoniae*). Even with this limited number of variables, due to the time that aging colonies demand (a minimum of 18 months of life from birth onwards before classification of "old"), as well as the inherent expense of housing mice for a minimum of 18 months, the studies outlined in this thesis were conducted under constant constraints on experimental replication.

TNF as a Central Mediator of Inflammaging

Despite aging being a subject of interest to humans since the beginning of recorded history, we are still far from a full understanding of which factors contribute to aging and how they may interact(*210*). In Chapters 3 and 4, we provide data that lend insight to this fundamental mystery, by unravelling the role of age-associated inflammation as a driver of the aging process. To study inflammaging in isolation of other variables that may influence the aging process, we employed aged TNF KO mice. We hypothesized that TNF is a central mediator of inflammaging (for reasons outlined below), and that therefore TNF KO mice are protected from the effects of inflammaging. By comparing immunological outcomes from old TNF KO mice to old WT mice, we reasoned we could gain a better understanding of which aspects of aging are governed by inflammaging and which occur due to other, outside processes.

We chose to focus on TNF as a major mediator of inflammaging for a number of reasons. As outlined in the Introduction section of this thesis, increased circulating levels of proinflammatory cytokines are most often used as markers of inflammaging in humans(*211–215*). Of these, it is TNF and IL-6 that are considered to be the most robust predictors of risk of chronic disorders and infection in elderly populations, and therefore they are also the most frequently measured. Additionally, TNF is known to have pleiotropic effects on a number of cytokines, including, critically, IL-6(*216–218*). TNF has been shown to amplify IL-6 production in the blood in other models of chronic inflammation(*219–221*) (while administration of IL-6 has no effect on circulating levels of TNF(*222*)). As such, we reasoned that age-associated initiation of basal TNF production might, in turn, initiate amplification of other proinflammatory cytokines downstream. Once established, basal proinflammatory cytokine production could be self-sustaining, by constantly promoting its own maintenance via positive feedback mechanisms.

The work entailed in both Chapters 3 and 4 substantiates a critical role for TNF as a mediator of inflammaging. The majority of the immunological changes we detected with age were absent in old TNF KO mice and could be reversed through use of adalimumab, a TNF blocking antibody used frequently in clinical settings. Consistent with an inflammaged phenotype in humans, we found that old WT mice produced heightened levels of plasma IL-6 and had hyper-inflammatory responses in whole blood following stimulation with LPS. Furthermore, in agreement with

previous reports, the aged lung microenvironment had higher levels of IL-6 and histological evidence of leukocytic infiltration(*223*). Importantly, these data demonstrate that old TNF KO mice are protected from major markers of inflammaging, indicating that TNF either directly or indirectly drives inflammaging.

In agreement with our data, the importance of TNF in mediating systemic inflammation has been demonstrated in models of sepsis. Mice deficient in either TNF or TNF receptors are less susceptible to endotoxin shock from high-dose LPS challenge and have increased survival compared to WT mice(*224*–*227*). Although sepsis is typically thought of as an acute condition, studies of chronic inflammatory disorders (including obesity(*228*–*230*), rheumatoid arthritis(*231*–*233*), psoriasis(*234*, *235*) and inflammatory bowel disorders(*236*, *237*)) have corroborated the central role of TNF in the induction of systemic inflammation. Of particular interest is a study published at the time our manuscripts were being prepared for publication. This study, conducted by Bouchlaka *et al*., characterized TNF as promoting a basal state of inflammation exclusively in old mice, which could exacerbate tissue damage and cytotoxicity following cancer immunotherapy regimes(*127*). Much like our studies, the authors also demonstrated that TNF regulates IL-6 in the plasma and the tissues affected by cytotoxicity (the intestines and liver). Furthermore, consistent with our findings, they found that this age-associated inflammation could be reversed by TNF blockade.

We demonstrated that one of the pathways through which TNF mediates inflammaging is through its actions on the myeloid compartment. Although increased monocyte frequency in the blood has been understood to be a consequence of the aging process for a number of years, for the first time, we demonstrated that it occurs in part due to systemic exposure to TNF. Numbers of total monocytes, and in particular, the Ly6C^{high} monocyte subset were significantly increased in the blood of old WT mice, but not in old TNF KO mice. Intraperitoneal delivery of TNF could mimic this age-associated phenotype, resulting in an increase of both circulating Ly6C^{high} monocytes and IL-6. More importantly, treatment of old WT mice with adalimumab, a TNF blocker, was sufficient to completely reverse the increase in circulating Ly6C^{high} monocyte numbers we observed with age.

Interestingly, both old WT and old TNF KO mice had an increase in Ly6C^{high} monocytes in the bone marrow, indicating that expansion of this population occurs independent of inflammaging. Numerous studies have demonstrated that with age, the hematopoietic stem cell (HSC) pool becomes more myeloid biased, due to the high intrinsic self-renewal capacity of myeloid-biased HSCs as compared to lymphoid-biased HSCs(*238*, *239*). It has been theorized that, because this shift starts during adolescence, it is a result of the developmental programs associated with maturity, and not degenerative mechanisms associated with aging *per se*(*240*)*;* therefore, these changes may be a cause, not an effect, of aging. This may explain why expansion of myeloid cells in the bone marrow occurs independently of TNF.

Furthermore, we did find that although both old WT and TNK KO mice had increased Ly6C^{high} monocytes in the bone marrow, only the former also had increased Ly6C^{high} monocytes in the circulation. This difference was attributable to heightened expression of CCR2, which occurred on bone marrow-localized monocytes in old WT, but not old TNF KO mice. As CCR2 is a receptor that is essential for monocyte egress from the bone marrow(*64*, *65*), we hypothesized that higher CCR2 expression could result in a higher number of monocytes egressing from the bone marrow (even in the absence of a systemic increase of MCP1, which we found did not occur with age during the steady state). In support of this hypothesis, we found that monocyte trafficking to the peritoneum following intraperitoneal injection of an equivalent dose of MCP1 was greatly increased in old WT mice as compared to young controls.

Old mice treated with anti-TNF demonstrated a decrease in both plasma IL-6 and hyper-inflammatory production of IL-6 in whole blood in response to LPS, suggesting that monocytes may be a major source of plasma IL-6 in old hosts. Immune cell depletion experiments targeting Ly6C^{high} monocytes solidified the link between these cells and heightened plasma IL-6. This confirms that one of the mechanisms behind the TNF-mediated amplification of downstream IL-6 is through an up-regulation of circulating Ly6C^{high} monocytes. Additionally, intracellular staining demonstrated that with age, more monocytes (regardless of subset) were positive for IL-6 and TNF. This finding was mirrored in *ex vivo* macrophages from

old WT mice, which produced higher levels of TNF and IL-6 as compared to macrophages from young WT mice (independent of expression of key PRRs, such as TLR2 or 4).

These data provide experimental evidence for the involvement of monocytes and macrophages as a central cell type in the pathogenesis of inflammaging. Franceschi *et al.*, who first advanced the theory of inflammaging in 2000(*241*), have maintained that macrophages are the central cell type in the generation of the inflammaging(*242*, *243*). Despite this, the involvement of myeloid cells in promoting inflammaging remains ambiguous. Monocytes are generally accepted to be key responders to microbial product stimulation – for example, they are the chief source of TNF in the blood in response to LPS(*244*, *245*). Our data confirm that they are also important cellular mediators of inflammaging, furthering its progression via their contribution to the systemic proinflammatory milieu. Although these findings must be further explored, they suggest that myeloid cells serve as prime targets for interventions intended to prolong healthy aging.

Our data imply that monocytes/macrophages promote inflammaging through a variety of mechanisms. For one, it may simply be a matter of sheer numbers: because the total amount of circulating monocytes is increased in the aged host, the total output of monocyte produced cytokines is as well. Additionally, the enrichment of $Ly6C^{high}$ monocytes, which are characterized as more proinflammatory than their $Ly6C^{low}$ counterparts, likely compounds this. Finally, on a per cell basis, aged

monocytes and macrophages seem to have a functional bias towards proinflammatory cytokine production and are more responsive to stimulation (possibly due to a lowered activation threshold).

Beyond providing important novel data to the study of inflammaging and immunity, these studies also impart important justifications for the consideration of age parameters in models that investigate chronic inflammatory diseases. For example for individuals over the age of 65, cancer incidence (based on time of initial diagnosis) is increased over 9-fold(*246*), stroke incidence is increased over 10-fold(*247*) and myocardial infarction incidence is increase over 8-fold(*248*) as compared to young adults. Despite this, the vast majority of mouse studies for these diseases are conducted with little-to-no consideration of this age-dependent susceptibility, using mice of 2–3 months of age, the equivalents of humans who are adolescents or young adults. As our work demonstrates, the immune system is markedly different in old mice, with inflammaging influencing the degree of inflammatory responses at baseline. Therefore, to more accurately reflect typical patients for the majority of chronic inflammatory disorders, our work indicates that it may be more appropriate to perform, or at least validate, models of chronic inflammatory disorders in aged mice.

Our studies have been centred on understanding the interplay between TNF-driven inflammaging and myeloid cells, namely monocytes and macrophages. Consequently, our data does not address how inflammaging impacts – and is impacted

by – other immune cell populations. Although, consistent with previous reports, we did find that in the steady state circulating numbers of lymphoid cells are decreased with age, and that neutrophils are increased, a better understanding of how these, and other leukocytes, play into the TNF-driven inflammaging framework is needed. Furthermore, although our work has focused mainly on the role TNF plays in inflammaging from an immunological standpoint, it is important to eventually translate this work beyond the constraints of this perspective. After all, TNF is a pleiotropic cytokine, whose effects on physiological balance extend beyond its role in mediating inflammation and immunity(*249*). For example, TNF has also been implicated in the interference of metabolic processing of lipids and glucose, which is believed, in part, to mediate its involvement in the promotion of atherosclerosis and diabetes(*228–230*, *250–252*). As both of these conditions fall under the constellation of disorders for which aging is a major risk factor, understanding additional pathways through which TNF may promote biological aging, and how they intersect with its role in promoting inflammatory cascades and shifts in myeloid function is an important avenue for future work.

The Consequences of Inflammaging for Anti-Bacterial Immunity

Respiratory infections are among the most important causes of morbidity and mortality from infectious diseases worldwide, with *Streptococcus pneumoniae*

identified as one of the most common causative pathogens(*253*). Although both the very young (4 years and under) and old (65 years and older) are at increased risk for infection by *S. pneumoniae* and represent the majority of individuals hospitalized due to pneumococcal infection(*254*), the overwhelming majority of deaths occur in elderly individuals. The Centres for Disease and Control report that while case fatality rates for infants and young children in the United States are 0.22 and 0.15 per 100,000 individuals, respectively, in the elderly, this rate is 5.61(*255*). Because *S. pneumoniae* represents a major source of morbidity and mortality in the elderly, it serves as a highly relevant model pathogen in the study of infection in aged subjects.

Colonization of the nasopharynx by *S. pneumoniae* is a mandatory prerequisite to systemic infection in humans(*256*). The local immune response of the nasopharynx is the "front-line" of control of bacterial carriage; failure to contain colonization results in bacterial invasion to regions like the lungs (resulting in pneumonia) and the blood (resulting in bacteremia and sepsis). Thus, studying colonization is highly relevant to understanding the immunological processes that lead to pneumococcal infection.

In children, colonization of the nasopharynx occurs often but is generally asymptomatic, with carriage rates shown to be as high as 50%. Infrequently, nasal immunity fails to control carriage events in this population, resulting in bacterial spread (pneumonia, meningitis or septicaemia)(*257*). In adults, carriage has been demonstrated to be less frequent (1-10% of the population is colonized at any given time) and of a shorter duration. Consequently, in this age group, *S. pneumoniae* disease

is limited almost exclusively to individuals with immunosuppressive co-morbidities(*258*). In the elderly, the rate of carriage has been demonstrated to be similar in adults, occurring with a relatively low frequency(*259*). The combination of low carriage rates and high incidence of invasive disease (manifesting most commonly as pneumonia) implies that breakthrough events are more frequent in aged hosts(*257*).

In order to test the hypothesis that the elderly are at increased risk for developing invasive disease following *S. pneumoniae* colonization due to defects in nasal control of the bacteria, we developed a mouse model of intranasal colonization, as described in Chapter 2. This model is based on human studies that have demonstrated that young adult mice faithfully mimic *S. pneumoniae* carriage in healthy adults with regards to parameters that include the duration of carriage and the kinetics of host responses(*144*, *145*). Before adapting our model to old mice, we needed to acquire a comprehensive understanding of the relationships between pneumococcal strain of choice, kinetics of carriage (including bacterial load, colonization duration and incidence of invasion) and host responses (including local and systemic cytokine responses and cellular recruitment). Understanding the details of these processes in young mice served as an important antecedent to understanding how immune responses change with age and allowed us to clarify the conditions under which defects in control of pneumococcal colonization occur in old mice. This was the central goal of the work presented in Chapter 2.

Initially, we focused on characterization of immune responses in immunological component young mice using our model of S*. pneumoniae* colonization. Mice were exposed intranasally to a static dose of live *S. pneumoniae* (10^7 colony forming units (CFU)) demonstrated by others to result in intranasal carriage(*145*). We found that this dose consistently yielded early stage (day 3) bacterial load in the range of 10^4-10^5 CFU in adult mice, depending on the strain employed. We also found that the length of colonization (time for the host to clear the bacteria to below the limit of detection, 100 CFU) was dependent on the pneumococcal strain employed. Consistent with what has been previously published, we also demonstrated that clearance could be influenced by the strain of mouse used in the studies, with C57Blk/6 mice clearing colonization more readily than their BALB/C counterparts. Furthermore, although the main three bacterial strains we tested in our model (classified as P1121, P1542 and P1547) were clinical isolates, we found that each was characterized by a different frequency of breakthrough events and resultant invasive disease (as determined by detection of bacteria in the spleen and/or lungs of colonized mice). For example, we found that P1121 was never accompanied by breakthrough events, and therefore represents a stable strain that models colonization well but is impractical for use in the study of invasive disease. In contrast, P1547 was associated with invasive disease in 20-50% of young mice studied, but was not as aggressive in mice as other strains used commonly in the literature (such as D39 which leads to the induction of a rapid, and for the most part lethal, cytokine storm). After contrasting host survival, length of carriage and incidence of invasion, we chose to focus our subsequent studies in old

mice using P1547, because of its heterogeneous capacity to act as a reliable colonizing strain (that nonetheless reaches nearly full clearance within a relatively measurable time span, ~28 days) and that leads to invasive lung infection in some, but not all young mice, making it possible to determine if this property is increased in old mice.

Employment of P1547 in our model allowed us to understand the nature and degree of immunological responses to *S. pneumoniae* colonization in young mice, as described in Chapter 2 and further extended in Chapter 3. For example, our data demonstrated that in young mice, the cellular response was mostly dependent on innate immune cells (monocytes, macrophages, neutrophils), peaked at day 7, and showed resolution by day 14, concurrent with bacterial clearance from the nasopharynx. Recruitment of cells to the nasopharynx was preceded by up-regulation of these cell populations in the blood, as well as systemic cytokine responses. Similarly, early local inflammatory production was a hallmark of intranasal *S. pneumoniae* colonization in young mice, but was down-regulated towards the later stages of colonization (days 14-21).

Application of our *S. pneumoniae* intranasal colonization model to old mice demonstrated key differences in the carriage kinetics with age, including the findings that: 1) clearance of the bacteria from the nasopharynx was significantly delayed with age and 2) bacterial invasion to systemic tissues (particularly the lungs) occurred in a higher proportion of aged mice (nearly 100% as opposed to in young mice), resulting in higher mortality. Furthermore, the immunological profile (the degree of cytokine production and cellular recruitment) was also quite dramatically altered

with age. At the time these studies were undertaken, the prevailing dogma was that systemic inflammation with age translated to globally hypo-inflammatory responses following pathogenic exposure(*117*). Surprisingly, we found the opposite to be true: defects in bacterial clearance in old mice were accompanied by an enhancement of local and systemic cytokine production (particularly with regards to TNF) relative to young mice.

These novel data, detailed in Chapter 3, present an interesting paradox: despite intact, and in fact, hyper-inflammatory immune responses, bacterial control was compromised with old age. Since serum cytokines levels correlate with susceptibility to, and poor prognosis during pneumonia in the elderly(*156*, *157*), this raised the possibility that enhanced inflammatory responses were responsible for poor bacterial control in old mice. This hypothesis was validated by use of old TNF KO mice in our *S. pneumoniae* colonization model, which demonstrated that bacterial load in the nasopharynx was in fact lower in the absence of TNF. Interestingly, in young mice, TNF had no overall impact on bacterial load in the *S. pneumoniae* colonization model, suggesting that it is only age-associated (ergo exacerbated) production of TNF that is detrimental to bacterial clearance. The notion that TNF is not always protective during pathogenic challenge, and may, in fact, hinder clearance or control of an invader is supported by other models of infection, such as those following exposure to certain strains of viral influenza(*260*), *C. albicans* (*261*) and bacteremia(*262*, *263*) Although our data are the first to demonstrate that age-associated susceptibility to *S. pneumoniae* infection is

mediated by TNF, others have previously shown that systemic infusion of young mice with TNF has a direct effect on increasing bacterial load following *S. pneumoniae* intranasal colonization(*254*). These observations are also consistent with reports that aged individuals (60+ years of age) who are on anti-TNF therapy for the treatment of intestinal bowel disorders (IBD) are less vulnerable to pneumococcal infection as compared to an age-matched cohort on glucocorticoid therapy (who demonstrated a ~2 fold increase in the incidence of pneumococcal infection)(*265*).

Concurrent with the increase in local and systemic cytokines, we detected increased recruitment of leukocytes. Although both local and systemic recruitment of most immune cells we studied was increased (including that of T cells and neutrophils), we chose to focus subsequent investigations primarily on monocytes and macrophages for several reasons. For one, these cells have been demonstrated in numerous studies to be the central mediators of immune control of intranasal *S. pneumoniae* colonization. Secondarily, our earlier data demonstrated these cells were involved in promoting inflammaging during the steady state, hinting at their involvement in hyper-inflammation following infection.

Indeed, the heightened numbers of Ly6C^{high} monocytes we detected with age during the steady state were maintained during the course of colonization, with old mice having consistently higher circulating numbers of these cells at all time points (days 3, 7, 14 and 21) studied. This correlated with populations of monocytes and macrophages that were enriched in the nasopharynx with age during *S. pneumoniae*

colonization. And, as was the case during the steady state, increased monocyte recruitment during *S. pneumoniae* colonization was dependent on TNF, as demonstrated by the decrease in circulating monocytes in old TNF KO mice as compared to old WT mice.

During acute infection, the recruitment of innate immune cells serves as an initial defense against a pathogen, and this response is crucial for control of the invader. Nonetheless, in Chapter 3, we have uncovered a scenario where robust recruitment of monocytes does not promote bacterial clearance. Instead, continued recruitment of these cells was associated with the sustained presence of bacteria in the nasopharynx of the host. In fact, as evidenced by our experiments selectively diminishing the recruitment of Ly6C^{high} monocytes, these cells are detrimental to host removal of the bacteria. Although enhanced recruitment of Ly6C^{high} monocytes has been demonstrated to play a role in the pathogenesis of numerous chronic inflammatory disorders(*59*), this is for the first time, to our knowledge, that they have been demonstrated to impair anti-bacterial immunity in aged hosts.

This finding raises an obvious question: *what is the mechanism behind the impairment of anti-bacterial responses enacted by Ly6C^{high} monocytes with age during S. pneumoniae colonization?* Based on our work in Chapter 3, it was evident that the Ly6C^{high} monocytes that were present in the steady state of old animals were more frequently positive for TNF and IL-6 and contributed to systemic levels of proinflammatory cytokine. Thus, if this predisposition towards increased cytokine production is maintained (or increased) in response to infection, it may play a role in

the mechanisms underpinning why Ly6C^{high} monocytes from aged hosts contribute to defects in anti-bacterial clearance. In other models of disease, this is certainly the case, with Ly6C^{high} monocytes promoting pathogenesis via cytokine production that results in tissue damage and dysregulation of immune responses(*59*). Even if it does prove to be the case that Ly6C^{high} monocytes impair bacterial responses by contributing to inflammation, understanding exactly how excessive inflammatory cytokine production impairs host responses remains to be elucidated.

The general consensus is that age-associated changes in myeloid function are due to changes in the aging microenvironment(*97*). Thus, another potential mechanism through which TNF might negatively affect anti-bacterial immunity in the context of age is through modulation of the anti-bacterial function of myeloid cells. Although we did not examine the effects of TNF on Ly6C^{high} monocyte anti-bacterial capacity directly, we did observe, in data presented in Chapter 4, that unlike cultured macrophages from old WT mice, those from old TNF KO mice were protected from defects in killing of *S. pneumoniae*. These changes, at least in part, seemed to be mediated by acute exposure to a relatively high (10 ng/ml) dose of TNF, as spiking the macrophage culture could directly impair killing of *S. pneumoniae* in both young and old macrophages (after 2 hours). Thus, it may be possible that, in addition to promoting a proinflammatory phenotype in monocytes and macrophages, elevated TNF exposure, be it chronic or acute, can adversely affect that capacity of these cells to kill bacterial pathogens.

Although TNF is classically thought of as a cytokine that globally promotes bacterial killing in phagocytes like macrophages and neutrophils, there is evidence that in fact, anti-bacterial responses in cells to TNF are not linear. For example, others have demonstrated that bacterial killing efficacy in neutrophils as a function of TNF dose followed a sigmoidal-pattern, with higher concentrations of TNF resulting in a decline in bacterial killing(*266*). In a study examining the effects of *Mycobacterium avium* on decreased anti-microbial functions in macrophages, researchers demonstrated that reduction of anti-bacterial killing occurred following induction of heightened TNF production, and was dependent on activation of MAPK-p38 signaling (a pathway downstream of TNF)(*267*). Similarly, predictive modelling suggests that high levels of TNF preceding *Mycobacterium tuberculosis* infection will down-regulate macrophage killing of the bacteria(*268*). In a study of macrophage responses to *Legionella pneumophilia*, macrophages which could not control bacterial replication in fact produced 4-fold more TNF than non-permissive macrophages(*269*). Interestingly, the elderly are at a higher risk of both *Legionella* infection and *Tuberculosis* reactivation(*270*, *271*). Taken as a whole, these studies support the role of TNF in anti-microbial response as a complex one, demonstrating that under certain conditions (such as during sustained chronic inflammation as per the inflammaged state), TNF may inhibit bacterial killing.

Consistent with our own findings in old mice, a study was recently published examining aged mouse responses to *M. tuberculosis* that revealed many parallels to

our own work(*272*). The researchers found that macrophages isolated from the lungs of old mice displayed increased proinflammatory cytokine production basally or following stimulation with *M. tuberculosis.* Furthermore, these cells had decreased killing of *M. tuberculosis* as compared to macrophages from young mice. This defect was reversible through short-term use of a broad spectrum anti-inflammatory compound (ibuprofen), providing support for our own findings that inflammation can directly impact anti-bacterial responses in macrophages. Taken as a whole, this demonstrates that the functional shift of aged macrophages towards increased cytokine production concurrent with decreased bacterial killing may be a global age-associated phenomenon, occurring across different macrophage populations and in response to multiple bacterial pathogens. Further study of monocytes/macrophages and inflammaging against the background of other microbes common with advanced age would help validate this observation.

The mechanisms behind the phenomena of TNF negatively regulating bacterial killing are unclear, but may be related to effect high levels of TNF have on inducing macrophage apoptosis. This has been implicated to occur in sepsis(*273*). Another putative mechanism may lie in indirect processes, as mediated by TNF's pleiotropic effects on other cytokines. For example, TNF is capable of inducing production of IL-10 in human PBMCs(*274*, *275*), and in fact, heightened TNF production in PBMCs from elderly individuals has been demonstrated to occur concomitant to increases in IL-10 production(*276*). Interestingly, IL-10 is known

abrogate macrophage killing of a number of pathogens(*277–279*). In this proposed scenario, potent induction of TNF production in aged macrophages may result in complete dysregulation of both proinflammatory and anti-inflammatory cytokine cascades at the cellular level. This would result in a "mixed message" wherein autocrine up-regulation of self-sustaining cytokine production occurs (through the actions of TNF/ IL-6) simultaneous to the down-regulation of bacterial killing (through IL-10). Studies into the synergistic relationships between TNF overproduction and IL-10 in the context of the aged macrophage would shed light into this hypothesis.

The Interplay between the Microbiota and Inflammaging

Although our identification of TNF as a primary driver of inflammaging is an important advancement in the study of aging, it raises one main question: *what initiates the systemic increase in TNF production that predicates chronic, age-associated inflammation*? Indeed, the source of inflammaging has been a much speculated and debated topic in the aging literature. Chapter 4 of this thesis resolves this major disparity by demonstrating that inflammaging is initiated by increased microbial translocation of bacterial by-products with age.

When designing the experiments outlined in Chapter 4, we turned to century-old insights from Elie Metchnikoff's penultimate publication, "The Prolongation of

Life: Optimistic Studies". In it, Metchnikoff proposed the theory that, because the digestive tract of humans and other mammals is particularly long and harbours extensive microbial load, lifelong exposure to the onslaught of these microbes and their by-products (which he termed toxins) furthers the aging process. In laying out his hypothesis, he wrote,

"The intestinal flora is an extremely important factor in the causation of senility. Amongst the microbes of the gut, there are some that are inoffensive, but others are known to have pernicious properties. It is generally believed that they form poisonous substances which are absorbed by the walls of the intestine and so pass into the system. Although the intestinal wall in an intact state offers a substantial obstacle to the passage of bacteria, it is incontestable that some of these pass through it into the organs and the blood. It is known that the wall of the gut is damaged extremely easily. In the ordinary course of life, the delicate wall of the gut must often undergo slight wounding, and the frequent presence of microbes in the mesenteric ganglia of healthy animals shows clearly what takes place. It is indubitable, therefore, that the intestinal microbes or their poisons may reach the system generally and bring harm to it. I infer from the facts that the more a digestive tract is charged with microbes, the more it is a source of harm capable of shortening life. As the large intestine not only is the part of the digestive tube most richly charged with microbes, but is relatively more capacious in mammals than in any other vertebrates, it is a just inference that the duration of life

of mammals has been notably shortened as the result of chronic poisoning from an abundant intestinal flora."(*168*)

In recent years, a link has been unveiled between the phenomenon of microbial translocation (resulting in the systemic "poisoning" that Metchnikoff envisioned) and the pathogenesis of two chronic conditions, human immunodeficiency virus (HIV) and chronic graft-versus-host disease (cGVHD). While HIV and cGVHD might seem, from the outset, as very distinct disorders, they share two important commonalities: 1) they result in the manifestation of immunological signs of premature aging(*280–282*) and 2) their progression is promoted by systemic inflammation that arises from microbial translocation due to intestinal immunity failure(*192*, *283–286*).

In an age of retroviral therapy, significantly extended survival for HIV patients has led to the emergence of a state of accelerated aging, to the extent that the use of HIV as a potential model for the study of biological aging has been proposed by others(*282*, *287*). For example HIV carriers possess immunophenotypic changes in their peripheral blood lymphocytes that show a tendency towards T cell senescence with an inverted ratio of naïve/memory T cells(*288*, *289*). These changes appear in HIV-carriers 20-30 years earlier than in non-carriers. Peripheral blood monocytes from young HIV-positive individuals also exhibit a more activated phenotype with reduced antimicrobial activity, similar to that observed in elderly

controls aged 30 years older(*243*, *290*). Furthermore, it has been demonstrated that the consequences of cGVHD (increased leukocytic infiltration, increased oxidative stress in macrophages, increased positivity of endothelial markers of aging) for the host appear identical to those exhibited by old mice(*291*). Both cGVHD and HIV are also associated with higher incidence of disorders commonly associated with age, including frailty, osteoporosis and neurocognitive changes(*292–294*), as well as other disorders for which aging is a major risk factor including cancer, diabetes and cardiovascular disease(*287*, *295*).

What is more telling is evidence that the consequences of HIV and cGVHD outlined above are the result of a low-grade, chronic proinflammatory state (described commonly "chronic immune activation" in the HIV literature) that is characterized by increased IL-6 and TNF in the circulation(*296*, *297*). The roots of this chronic proinflammatory state in HIV and cGVHD have been demonstrated to occur following damage to the intestinal barrier. In the case of HIV this occurs due to a loss of CD4+ T cell immunity(*298*, *299*); in the case of cGVHD excessive intestinal inflammation secondary to transplantation results in a dysbiosis that promotes intestinal destruction(*192*, *283*)). In both cases, poor intestinal integrity facilitates the translocation of microbial products (such as LPS) across the mucosa and into the systemic circulation, ultimately providing a continuous source of activation for innate immune cells (including, in particular, monocytes and macrophages). These microbial products induce basal production of proinflammatory cytokines, resulting

in further activation of the immune system and thereby establishing a self-perpetuating cycle(*300*).

The parallel between chronic immune activation in HIV and cGVHD and inflammaging, particularly when considered against the backdrop of Metchnikoff's early predictions, makes a strong case for the argument that the microbiome is the ultimate root of inflammaging. Consistent with this theory, we found that markers of inflammaging in our old mice correlated with increased permeability in the gastrointestinal tract and increased bacterial productions in the circulation. While old WT mice demonstrated evidence of increased permeability relative to young controls, and had higher systemic levels of muramyl dipeptide (MDP, a major component in the cell walls of both Gram-negative and Gram–positive bacteria), old TNF KO mice exhibited neither of these changes. In agreement with this, old mice raised under entirely germ-free conditions (i.e. mice without a microbiota) had less overall mortality (compared to WT mice up to 600 days), demonstrated preservation of intestinal integrity, and, importantly, had no evidence of inflammaging, as assessed by plasma IL-6, whole blood LPS stimulation assay, lung IL-6 levels and lung histology. Furthermore, these mice were also protected from age-associated defects in macrophage function (increased cytokine production, decreased bacterial killing) that we showed arise from chronic TNF exposure in our studies utilizing old TNF KO mice.

The mechanism behind the loss of epithelial integrity with age we evidenced may be host-dependent or microbiome-dependent. Our data indicated that

permeability changes mainly occurred in the colon (which represents the lower part of the intestinal tract and has the highest density of colonizing microbes) through paracellular (between-cell) pathways. Consistent with this finding, using gene ontology analysis, we demonstrated that major pathways involved in cell-to-cell interactions (including those involved in the regulation of adherens and tight junctions) were dysregulated within the colons of old mice. Age-associated reduction in intestinal permeability was dependent on the presence of a microbiome, as old germ-free mice did not develop increased permeability. Furthermore, TNF KO mice were also protected from the development of increased intestinal permeability. In conjunction, these findings indicate that a disruption of barrier function with age requires a host-microbe interaction (potentially through microbe-induced inflammatory responses in host immune cells).

Consistent with this, others have characterized changes that occur in gut immunity with age. With age, the intestinal mucosa demonstrates a decreased capacity to synthesize strain-specific secretory antibody responses and to generate tolerance against harmless antigens(*301*–*304*). CD4+ T cell numbers are also reduced in the aged gut, compounded by a diminished cytokine response in these cells(*304*, *305*).

In models of HIV, targeting of CD4+ T cells by the virus leads to a disruption of immunity leading to barrier integrity breakdown. This results in failure to control the resident microbiota, allowing for uncontrolled microbial growth, and a

subsequent shift from and tolerant immunophenotype, to a strongly proinflammatory one; a similar progression of pathogenesis may occur in elderly individuals. Alternatively, age-associated changes in gut mucosal immunity could occur downstream of an initial shift in microbial composition. For example, in inflammatory bowel disorders, microbial dysbiosis has been demonstrated to trigger the creation of an inflammatory milieu in the gut, which then shifts immune cell behaviour away from tolerance(*306*, *307*). In support of the possibility of a similar mechanism occurring with age, we found that the gut microbiome of old mice had a marked shift in composition. This is concurrent with findings that age is associated with gut dysbiosis, which in turn correlates with increased gut inflammation, the degree of systemic inflammation and the health status of the host(*189*, *308*, *309*). Further studies will have to be done to better understand the interplay between host immune defenses in the gut and alterations in the microbial composition with age.

Aging is inherently a process dependent on the passage of time; as such, we wondered whether the basis of increased permeability might simply be due to immune changes elicited by prolonged exposure to the microbiome. Our cross-sectional analysis of longitudinal changes in intestinal permeability demonstrated that intestinal permeability increased in an exponential manner as a function of time, with very little increase in permeability evidenced as late as 12 months of age. This was supported by our recolonization studies in young and old-germ free mice, wherein we recolonized GF mice via oral gavage with a fecal suspension sourced

from either young or old conventionally colonized, specific pathogen-free mice. Recolonized mice showed no increase in intestinal permeability after a minimum recolonization period of 3 months, regardless of age of recipient or donor, indicating that in order to elicit increased gut permeability, host exposure to the microbiome must be long-term. Although beyond the scope of this thesis, it would be beneficial to determine the length of time of microbial exposure required to disrupt intestinal barrier integrity.

Interestingly, when we examined IL-6 production in the plasma of our recolonized GF mice, we found that old GF mice recolonized with an old microbiota, but not ones recolonized with a young microbiota, had IL-6 levels above baseline. Therefore, microbiome composition can influence the degree of downstream systemic IL-6 production (and presumably systemic inflammation), independent of changes in intestinal permeability. In support of this, we found that recolonized mice maintained microbial divergence from one another. This suggests that even in the absence of increased bacterial translocation, age-associated microbial dysbiosis may result in enrichment of bacterial species that are capable of stimulating systemic inflammatory responses more strongly than members of a normal flora. Therefore, in hosts with a lifelong exposure to the microbiome, microbial dysbiosis likely acts to compound age-associated disruption in intestinal permeability, resulting in a host with both 1) higher total systemic load of bacterial products and 2) increased representation of immunogenic bacterial products.

Concluding Remarks

The work presented in this thesis contains important breakthroughs on the understanding of inflammaging, its corollaries and its causes. Herein we demonstrated that TNF is a major driver of inflammaging, via its modulation of monocyte and macrophage recruitment, phenotype and function. Through these effects, chronic exposure to TNF has negative consequences not only for the steady state, but also bacterial infection with *S. pneumoniae*. Furthermore, we showed that the microbiota is a chief contributor to inflammaging and age-associated decline in macrophage function. Changes in permeability occur with age, promoting the translocation of microbial products which can then stimulate systemically-localized immune cell. In turn, this can initiate inflammatory cascades that provide the basis of age-associated inflammation.

For many researchers in the aging field, ourselves included, the end-goal of our work is to apply bench-side findings to bedside strategies that may promote either a prolongation of life or healthy aging (or, ideally, both). Based on the data we present here, one of the most obvious avenues for this is through manipulation of the microbiome, through the use of pre- and probiotics. These types of intervention are especially attractive because of their low-cost, high patient compliance and ease of delivery. Our studies suggest that age-associated increases in permeability occur due to lifelong exposure of the host to the gut microbiota. Furthermore, we found that ASF-colonized mice, with a very limited 8-species microbiota, demonstrated an

increase of intestinal permeability on par with that found in old conventionally colonized mice. This suggests that microbial dysbiosis may not necessarily modulate increased permeability. Therefore, maintenance of a normal microbiota may not be an effective strategy in the abatement of intestinal permeability changes. Nonetheless, our data did indicate that age-associated changes in the microbiome can mediate the extent of downstream systemic inflammation (independent of changes in permeability). As such, the use of probiotics may be beneficial in preventing the enrichment of more immunogenic species, and therefore help minimize the extent of systemic immune stimulation and subsequent inflammaging. Current research into how individual microbial genera/species are altered in with age is an important first step in tailoring probiotics for the prevention of inflammaging.

It should be noted that gut species do not exist in isolation; rather, the microbiome is, by definition a microbial community, under the influence of interspecies interactions(*310*). As such, a comprehensive understanding of the microbiome as a cohesive unit, particularly in the context of the aged microenvironment, is necessary in the long run. Even without this full understanding of the nuances of microbe-to-microbe, and microbe-to-host dynamics, early studies in the use of probiotics to promote healthy aging have shown promise. Work in invertebrates demonstrates that there is a direct link between microbial composition, the remodelling of physiological processes, and ultimately, the rate of aging(*311*). Intervention studies of probiotics in the elderly

have demonstrated that limited probiotic mixes, using members of genera like *Bifidobacterium* seem to have a negative effect on overrepresented genera, like the *Enterobacteria*, thereby restoring bacterial representation to a more "young-like" microbiome.

More dramatically, there are reports that direct manipulation of the gut microbiome in the elderly through probiotic use has far-reaching consequences for systemic immunity, including a decrease in circulating IL-6 and TNF levels, an improvement in antibacterial activity in macrophages, neutrophils and NK cells, a restoration of circulating lymphocyte numbers, and even an enhancement of vaccine responsiveness [reviewed in (*312*)]. Although these studies represent only the beginning, they nonetheless serve as proof of principle for the idea that microbial manipulation can promote host longevity and health in old age.

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Appendix I.

The Macrophage.

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The Macrophage

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Abstract

Macrophages are a diverse phenotype of professional phagocytic cells derived from bone marrow precursors and parent monocytes in the peripheral blood. They are essential for the maintenance and defence of host tissues, doing so by sensing and engulfing particulate matter and, when necessary, initiating a proinflammatory response. Playing such a vast number of roles in both health and disease, the activation phenotype of macrophages can vary greatly and is largely dependent on the surrounding microenvironment. These phenotypes can be mimicked in experimental macrophage models derived from monocytes and in conjunction with stimulatory factors, although given the complexity of *in vivo* tissue spaces these model cells are inherently imperfect. Furthermore, experimental observations generated in mice are not necessarily conserved in humans, which can hamper translational research.

The following chapter aims to provide an overview of how macrophages and their parent cell-type, monocytes, are classified, their development through the myeloid lineage, and finally, the general function of macrophages.

1. Introduction

Macrophages derived from bone marrow precursors and parent monocytes in the peripheral blood are multi-functional cells of the innate immune system that play an important role in regulating the return of host tissues to homeostasis after tissue injury or infection. They

accomplish this by engulfing and removing large particulate matter, as well as modifying the molecular and cellular makeup of their surrounding environment. In many respects they are similar to polymorphonuclear neutrophils, the most prominent phagocytic leukocyte of the peripheral blood that specializes in the clearance of extracellular pathogens, only macrophages have a greater capacity to modulate the inflammatory response and respond to a more varied complement of pathogens (***1***). Accordingly, macrophages are equipped with a broad-range of pattern-recognition receptors (PRRs), which are required for the production of an array of inflammatory and immunosuppressive cytokines, and the uptake of cellular debris and pathogenic material. To adequately perform these tasks macrophages are highly plastic cells that can rapidly shift their phenotype based on their microenvironment.

A wealth of methodology exists to study the role of monocytes and macrophages in the many facets of disease and physiology. These include techniques to isolate and identify macrophages from biological samples, as well as assays to measure the functional capacity of these cells, in particular phagocytosis, chemotaxis and cytokine secretion. Some researchers choose to take one step further and modify the inherent expression profile, and thereby function, of macrophages via knock-out mouse models and artificial expression technology to best answer their research questions. Essential to any study incorporating such methodologies is an understanding of macrophage biology. The following chapter aims to provide an overview of how macrophages and their parent cell-type, monocytes, are classified, the development of macrophages through the myeloid lineage, and finally, the general function of macrophages. Discussion will include a comparison of mouse and human monocytes/macrophages since a number of the commonly used phenotypic subset markers are not implicitly conserved across species.

2. Classification and origins of blood monocytes in mice and men

Blood monocytes are circulating phagocytic cells with the ability to perform immune effector functions and to enter tissue spaces where they can differentiate into resident macrophages or dendritic cells. While they are precursors to macrophages, the manner in which they are classified is quite distinct.

1. Murine classification of blood monocytes

In mice, circulating monocytes constitute 1.5 to 4% of the total peripheral blood leukocyte pool during the steady state (***2***). They have been classically defined as cells that express high levels of CD11b (Mac-1), an antigen known to be involved in chemotaxis via endothelial interaction, and CD115 (macrophage colony stimulation factor (M-CSF) receptor). Mouse monocytes may also express the F4/80 antigen at intermediate levels, particularly if they are in the process of differentiating into macrophages (***3***).

Murine monocytes can be subdivided based on their expression of Ly6C (***4,5***), an antigen which is involved in mediating endothelial adhesion and motility in T cells (***6***), but whose function in monocytes has yet to be determined. Ly6C exhibits a broad expression pattern on monocytes, and hence, classification is not restricted to the antigen simply being referred to as positive/present ($Ly6C^{+}$) or absent/negative ($Ly6C^{-}$/$Ly6C^{neg}$). Instead, murine monocytes can be more precisely designated as expressing Ly6C at low ($Ly6C^{low}$) and high ($Ly6C^{high}$) levels (**Fig. 1**). This designation has been suggested to be further refined using an additional cell-surface marker CD43 to offer the following subsets: "classical" $Ly6C^{high}CD43^{low}$, "intermediate" $Ly6C^{high}CD43^{high}$, and "non-classical" $Ly6C^{high}CD43^{low}$ monocytes (***7***). Expression of the Ly6C antigen is typically identified via flow cytometric analysis using either antibodies directed against epitopes specific for the Ly6C molecule, or else using the Gr-1 antibody, which recognizes an epitope present on

both Ly6C and a related protein, Ly6G. Since Ly6G is expressed on a number of myeloid lineage cells, especially neutrophils, reliance on the Gr-1 antibody as a unique identifying marker of monocyte subsets can be misleading (***8***). To complicate matters, certain rare subsets of myeloid cells (ie. myeloid derived suppressor cells (MDSCs)) have been identified that express both Ly6C and Ly6G (***9,10***). Nonetheless, the use of Ly6C has generally been proven as a useful monocyte marker. Morphological analysis can also be used to differentiate the two subsets, as the $Ly6C^{high}$ population is larger and more granulocytic than its $Ly6C^{low}$ counterpart (***3,11***). Expression of CX_3CR1 (neurotactin/fractalkine receptor) is known to correlate inversely with Ly6C expression, thus, $Ly6C^{low}$ monocytes express the highest levels (***11***). Conversely, $Ly6C^{low}$ monocytes have been identified as expressing lower levels of the monocyte chemoattractant protein-1/CCL2 (MCP-1) receptor CCR2 than their $Ly6C^{high}$ counterparts (***12***). CX_3CR1 has been shown to be important in the migration of monocytes across endothelial vessels (***13***), while CCL2 is a well-known, potent chemoattract of monocytes (***14***).

Although the characterization of mouse monocyte subsets is still in its early stages, research indicates that the murine subpopulations are functionally distinct as well. Adoptive transfer experiments have shown that $Ly6C^{high}$ monocytes are recruited to inflamed tissue where they undergo activation and act primarily in a proinflammatory capacity (***15***). In response to acute infection or injury, $Ly6C^{high}$ monocyte numbers in the blood compartment expand rapidly, likely in preparation for their accumulation at localized sites of injury (***2***). As the extent of injury/infection wanes, the circulating numbers of these monocytes decrease correspondingly (***2,15***). In states of chronic inflammation such as during atherosclerosis, $Ly6C^{high}$ monocytes have been observed to accumulate in the peripheral circulation in a progressive manner (***16***). Once recruited to peripheral tissues in response to bacterial infection $Ly6C^{high}$ monocytes release

proinflammatory mediators such as tumour necrosis factor alpha (TNF-α), inducible nitric oxide synthase (iNOS) and interleukin (IL)-12 (***17-19***).

Ly6C^{low} monocytes can crawl for long ranges along the endothelial layer of the vasculature and are generally believed to participate in tissue maintenance during homeostasis, homing to resting tissues where they can differentiate into resident macrophages (***13,20,21***). Whether their role is exclusively homeostatic is not clear as one recent report suggests that they are actively recruited to sites of inflammation and may even precede the arrival of their Ly6C^{high} counterparts in the early stages of the immune response (***13***). Consequently, they may function as effector cells in addition to being an intermediate between hematopoietic progenitors and terminally differentiated tissue macrophages.

2. Human classification of blood monocytes

Human monocytes, which comprise approximately 10% of total peripheral blood leukocytes, are less well characterized than their murine equivalents (***22***). Based on experiments conducted in the 1980s, conventional classification of human blood monocyte subclasses is centred on the expression of CD14, a lipopolysaccharide (LPS) co-receptor, and CD16, an F_C gamma receptor. Using flow cytometry these cells fall within a defined size (forward scatter) and granularity (side scatter) compartment (**Fig. 2a**) and commonly express markers such as human leukocyte antigen (HLA), CD115 and CD11b, although to varying degrees (***7,23***). Based on CD14/CD16 expression, the major subsets considered are CD14^{high}CD16^{neg} (classical), CD14^{high}CD16^{high} (intermediate), and CD14^{low}CD16^{high} (non-classical or patrolling (***21***)) (***7***) (**Fig. 2b**). The CD14^{high}CD16^{high} subset have also been referred to as 'inflammatory' given the initial observations that CD16 expressing monocytes produce TNF-α upon stimulation with pathogen associated molecular patterns

(PAMPs, ie. LPS). However recent evidence suggests that the distinction in inflammatory responses between the $CD14^{high}$ monocyte subsets is not substantial enough to warrant defining only one as 'inflammatory'. Hence, assignment as 'intermediate' monocytes may be more appropriate (***2,7***).

3. Conservation across species

Human and mouse monocyte populations share many phenotypic and functional similarities. Particularly, $CD14^{high}$ human monocytes and $Ly6C(Gr\text{-}1)^{high}$ murine monocytes are functionally similar, as are the $CD14^{low}$ human and $Ly6C(Gr\text{-}1)^{low}$ murine subsets. Although no known homolog of Ly6C has been identified in human monocytes, cross-species analysis studies indicate that the expression patterns of a number of genes is conserved between the two subsets (***21,24***). As with mouse monocytes, the expression of the chemokine receptors CX_3CR1 and CCR2 is also commonly used to further classify human subpopulations: monocytes lacking CD16 expression have elevated levels of CCR2, much like $Ly6C^{high}$ monocytes, whereas CD16 expressing human monocytes, like $Ly6C^{low}$ murine monocytes, have elevated levels of CX_3CR1 (23). Furthermore, the expression of other key monocyte markers in human and mouse subsets, including that of CD11a, CD11c, CD62L and CD43, is similarly conserved (***24***).

As in mice, human monocyte subsets exhibit varied responses upon stimulation, although the nature of the stimulant plays a governing role in this respect (***25***). Much like the $Ly6C^{high}$ murine subset, $CD14^{high}$ cells have been suggested to function in a proinflammatory manner, and as such are highly phagocytic and produce substantial amounts of proinflammatory cytokines such as IL-8 and IL-6 in response to LPS (***21***). Within this subset, classical monocytes lacking CD16 expression produce high amounts of ROS, while intermediate CD16 expressing monocytes secrete high levels of IL-1 beta and TNF-α (***21,25***,***26***). Much like their murine analogs, non-

classical CD14^{low} monocytes play an important role in local surveillance of tissues during the steady-state. These cells exhibit the ability to patrol endothelial vessels in a 'crawling' manner, and secrete proinflammatory cytokines such as IL-1 beta, TNF alpha, and IL-1 receptor agonist in response to damaged/apoptotic cells and viral antigens (***21,26***).

4. Origins and fates of mouse monocytes

The common precursor of all leukocytes is the hematopoietic stem cell (HSC) pool in the bone marrow, which can differentiate into a progeny that gradually loses its self-renewal capacity and becomes restricted to a particular lineage. Traditionally, macrophage development has been described as occurring in a stepwise manner: HSC precursors in the bone marrow can develop into monocytes, which differentiate into macrophages upon recruitment to a specific tissue site. The differentiation of HSCs gives rise to two major clonogenic progenitor classes: the common lymphoid lineage, which generates T lymphocytes, B lymphocytes and natural killer cells, and the common myeloid lineage, which generates either erythrocyte progenitors, or granulocyte/macrophage progenitors, with monocytes arising from the latter. These progenitor cell types can subsequently give rise to polymorphonuclear neutrophils, and mononuclear monocytes (**Fig. 3**). The mononuclear cell pool, or mononuclear phagocyte system, can further differentiate into plasmacytoid dendritic cells, classical dendritic cells, tissue-resident macrophages and recruited macrophages (***27***). Originally it was thought that plasmacytoid dendritic cells were derived only from the common lymphoid lineage, however recent literature suggests that these cells are derived from the myeloid lineage (***28,29***). In the differentiation process monocyte commitment is induced primarily by the presence of the growth factors macrophage colony stimulating factor (M-CSF) and granulocyte-macrophage stimulating factor (GM-CSF) (***30***).

While this paradigm of myeloid development is well supported and is still an active area of research, some investigators argue against such rigid relationships between cell-types in the myeloid lineage. Evidence for this argument includes a lack of molecular epitopes that definitively characterize a given myeloid cell-type, and striking similarities between the transcriptomes of myeloid cell-types that are considered divergent by the current dogma (***31***). Additionally, the derivation of myeloid lineages has predominantly been proven in mice, and is not necessarily conserved in humans. Although xenogeneic *in vivo* transplantation models have traversed obvious ethical barriers, offering much to our understanding of the myeloid lineage in humans, there is still a great deal of hypotheses to be experimentally verified (***29***).

Bone marrow derived monocytes are commonly classed into two major subgroups based on their expression of the cell surface marker Ly6C and functional differences, and are believed not to proliferate (***22***). From the bone marrow, monocytes are mobilized into the peripheral blood via the chemokine receptors CCR2 and CX_3CR1 (***32***) where they circulate and await further signalling to enter tissue spaces. Mobilization is a constitutive process but can be induced or repressed in response to inflammatory signals caused by infection, for example. These signals include those that are pathogen-derived, such as cell wall components, or endogenously produced by the host, such as the proinflammatory cytokines IFN gamma or TNF-α (***33***). While it was traditionally thought that monocytes were permanently fated to blood and tissue compartments upon emigrating from the bone marrow, it was recently shown that in the absence of external stimuli such as inflammation, $Ly6C^{high}$ monocytes can return to the bone marrow (***34***). It has been hypothesized that these cells may leave the circulation to provide a reservoir for the generation of $Ly6C^{low}$ monocytes, osteoclasts, or resident bone marrow dendritic cells, or to acquire antigen captured by neutrophils and B-cells also returning from the

peripheral blood ***(34)***. Recent research also suggests that precursor monocytes are not limited to the bone marrow as tissue of origin. A separate reservoir of monocytes that can be found exclusively in the spleen has been identified in mice. Splenic monocytes are distinct from the resident splenic population of macrophages, and are mobilized in response to infectious cues (***35***). In contrast to their bone marrow-derived counterparts, their deployment to the circulation occurs independent of CCR2 (***36***).

3. Classification and origins of tissue macrophages

Macrophages are professional phagocytes involved in the recycling and clearance of erythrocytes during the steady state, the removal of apoptotic cells and cellular debris, tissue remodelling and host responses to infectious disease (***37***). They are remarkably plastic cells that can rapidly shift their physiology in response to cues generated after injury or infection (***38***). The surrounding microenvironment largely determines the activation phenotype of recruited macrophages, which can most simply be classified as falling within a spectrum consisting of two opposing phenotypes: classically activated, or M1, macrophages (CAMs), and alternatively activated, or M2, macrophages (AAMs) (***39***). Additionally, there are subsets of specialized resident macrophages whose phenotypes are uniquely adapted to their location, such as brain microglia, liver Kupffer cells, bone osteoclasts and lung alveolar macrophages. These distinct cell types can also be skewed towards classical or alternative activation, although their differentiation in response to a given stimulus may not be analogous (***40, 41***).

1. Classically and alternatively activated macrophages

Stimulation with a toll-like receptor (TLR) agonist (ie. LPS) in the presence of interferon gamma (IFN-γ) promotes CAM differentiation (***38,42,43***). They have an enhanced capacity to present antigen, produce high amounts of nitric oxide (NO), secrete large amounts of chemokines and

proinflammatory cytokines, and promote the expansion of T-helper 1 (Th1) lymphocytes via interactions with major histocompatibility complex (MHC)-II and stimulation by IL-12. As such, CAMs are considered vital in the defence against bacteria, but at the same time can be damaging to the host due to collateral damage brought about by the defence mechanisms they promote (***39***). Exposure to IL-4/IL-13 produced primarily by CD4+ T-cells promotes the differentiation of AAMs. These cells are involved in the response to parasites and fungi, and express high amounts of cytosolic arginase and extracellular matrix related proteins (***44-46***). These latter two characteristics provide AAMs the ability to limit inflammation and play an important role in tissue repair; hence, the additional title as wound-healing macrophages (***38***). A third subset, the regulatory or M2b and M2c macrophage has also been described. This subset is induced by immune complexes and TLR agonists, or IL-10 and glucocorticoids, functioning to dampen immune responses and inflammation (***38***). The *in vivo* relevance of these phenotypes is an active area of research.

It should be noted that, much like blood monocytes, evidence suggests that dividing activated macrophages into rigid classes is for the most part unrealistic, and that these designations do not necessarily capture the nuances that exist between macrophage populations. Most *in situ* macrophages will lie in a more intermediate position in the activation spectrum, sharing some overlapping characteristics depending on the environmental stimuli (***38***). At the same type some macrophage subsets are not easily classified within this spectrum. Myeloid derived suppressor cells for example, named for their ability to suppress T-cell activation and proliferation, express NO, and arginase, hallmarks of both CAMs and AAMs (***47***). Macrophage foam cells generated by a dysregulated uptake of lipid compounds at sites of atherosclerotic plaques are another excellent example of a subset that exhibits an atypical phenotype with characteristics of both

activation spectrums (***48***).

Furthermore, while it may be tempting to assume that blood monocytes that are considered "inflammatory" will have a greater propensity to differentiate into M1 macrophages, and vice-versa, this phenomenon has not been conclusively verified. There is experimental evidence to support this theory (***49***), but on the other hand it is known that the activation phenotype of a macrophage can be skewed quite dramatically depending on the tissue microenvironment into which it extravasates (***50***).

2. Cross-species conservation of macrophages activation spectrums

As with all cells of the myeloid lineages, characterization of the above macrophage subsets has chiefly been performed in mice. Unfortunately, there are evident discrepancies between species that can hamper macrophage classification in human studies. In mice, the hallmark of CAM induction *in vitro* and *in vivo* is NO production. In humans, controversy arises since monocyte-macrophage cell lines cannot be readily induced to express NO, although it is seen in macrophages from tissue biopsies and in blood monocyte-derived-macrophages under certain culture conditions (***51,52***). Similarly in AAMs, the hallmark expression of cytosolic arginase in mice is not conserved in humans ***(53)***. Gene expression analysis across species has identified some conserved markers, such as HLA/MHC-II for the CAM phenotype and mannose receptor C, type 1 (MRC1) for the AAM phenotype, but it is yet to be determined if they are reliable markers under an array of conditions. Nonetheless, despite these divergences between species regarding classification markers, overall conservation of function appears to be retained for CAMs and AAMs (***44***).

3. Development of tissue macrophages from bone marrow precursors and blood monocytes

The migration of blood monocytes across the endothelial membrane initiates their differentiation into tissue macrophages (***54***). Depending on the local microenvironment, recruited monocytes have the capacity to differentiate into a macrophage subset with a tissue-specific activation state, function and phenotype (***2***). While the extravasation and differentiation of blood-borne monocytic precursors was traditionally considered the sole source of tissue-resident macrophages, two additional mechanisms involved in replenishing macrophage numbers have recently been identified. These include the self-proliferation of cells in the resident compartment, and homing/proliferation of dedicated bone marrow derived precursors to resident tissues (***27***).

Furthermore, the inflammatory state of the target tissue seems to influence the route of differentiation. Studies have shown that during the normal steady-state, the majority of adult tissue-resident macrophages, including alveolar macrophages (***55-57***), splenic macrophages (***58***) and liver Kupffer cells (***59***), are maintained through local self-renewal, independent of circulating blood monocyte populations. In contrast, during inflammation, circulating monocytic precursors, in particular the Ly6C^{high} subset, travel to inflamed tissues and make substantial contributions to the macrophage population in the respective tissue compartment (***60-62***).

4. Deriving macrophages for immunological studies

Given the obvious ethical barriers inherent to human immunological studies, employing laboratory mice to build our understanding of the myeloid cell lineage is and has been a necessary exercise. These barriers also cause us to rely on the *in vitro* manipulation of myeloid

cell types that are relatively easy to acquire as a means to bridge our murine findings to our own immune system (***53***). At the same time, many researchers working in the murine model opt to derive or elicit certain myeloid cell types due to difficulties associated with isolation and poor yields (***63***). Interestingly, some of these experimental constraints have prompted researchers to consider other mammalian species, such as the domestic pig, as models for studying myeloid cell development (***64***).

In human studies the most convenient source of macrophage precursors is the peripheral blood or umbilical cord blood. Monocytes can be isolated from blood by density-gradient centrifugation and differentiated into macrophages by allowing adherence to tissue culture plastic in the presence of serum, M-CSF or GM-CSF (***65***). It should be noted that macrophages derived in the presence of these supplements, while similar, are not phenotypically or functionally equal. Macrophages derived in GM-CSF as compared to M-CSF differ in cell-surface marker and endogenous gene expression profiles as well as their ability to control HIV-1 viral replication (***66-68***), and both are inferior to those derived in human serum with regards to TNF-α secretion (***69***).

For murine studies, macrophages are commonly derived from *ex vivo* extracted bone marrow precursor cells using a combination of M-CSF and tissue culture grade plastic adherence, or harvested from the peritoneal cavities (ie. resident peritoneal macrophages) or lungs (ie. alveolar macrophages). To substantially increase yields it is a common practice to induce the recruitment of macrophages to the peritoneum of naive mice using a sterile inflammatory agent (ie. elicited macrophages) such as Bio-gel, polyacrylamide beads or thioglycollate broth, a complex mixture of yeast components (***70***). While all of these macrophage subtypes are suitable models for experimental studies, they do differ in phenotype and may not respond analogously

after stimulation. For example, elicited macrophages are highly phagocytic and generate large amounts of ROS, while resident peritoneal macrophages do not produce detectable levels of MHC or reactive oxygen species (ROS) (***71,72***).

Some have proposed adding additional factors to culture in order to promote a model phenotype for a particular macrophage subset using the derived or elicited cells described above. Although it is unlikely these cells are homologous to their *in vivo* counterparts as it is impossible to replicate the complex cytokine milieu tissue microenvironment and the physical association between cells, they are the best experimental model that researchers have at their disposal (***63***).

4. An overview of macrophage function

The macrophage is a fascinating cell type in that its primary role is maintaining homeostasis. This includes host defence against foreign invaders, the clearance of necrotic and apoptotic debris and tissue remodelling following injury. It performs these roles via four basic innate functions: sensing, chemotaxis, phagocytosis and repair, and adaptive stimulation. Although macrophages have the ability to promote adaptive immune responses, they are considered innate effector cells since they do not require previous exposure to a given antigen in order to initiate a response.

1. Sensing

Macrophages use intracellular and cell-surface PRRs to sense their local environment. When bound to a given ligand these receptors generate signals that direct the macrophage response. Unlike the antigen-specific receptor found on T and B lymphocytes for example, these innate receptors can recognize molecular patterns that may be common across a number of species.

They can generally be broken down into two sensing groups: pathogen and danger signals (exogenous), and modified host proteins and lipids and necrotic/apoptotic cellular debris (endogenous). While some of the receptors falling into either of these groups are considered markers of a particular activation phenotype (for example MRC1 and dectin-1 with respect to AAMs (***44,73***)), they are not mutually exclusive to either spectrum and are likely expressed on most macrophage subsets, albeit at relatively low concentrations.

The TLR family is one of the predominant pathogen sensing group of molecules and currently includes 14 members (***74***). Some of these include cell-surface TLR-2 and -4, which bind the PAMPs lipoteichoic acid (LTA) and LPS, respectively, and intracellular TLR-3 and -9, which bind viral and bacterial derived oligonucleotides, respectively (***75***). These receptors collectively promote proinflammatory signalling including the expression of cytokines such as IL-6, TNF-α, and IL-12 (***39,39,44,75***). As opposed to the TLRs that promote proinflammatory activities, dectin-1 and MRC1 are two commonly expressed PRRs that promote anti-inflammatory activities. Dectin-1 recognizes β-glucan polysaccharides found on fungi and some bacteria, and signals the inhibition of TNF-α and/or IL-12, and the induction of IL-10 (***73,76)***, whereas MRC1 binds mannose and fucose polysaccharides commonly found on fungi, bacteria and viruses, and signals the inhibition of IL-12 secretion (***73,77***). Additionally, macrophages express cell-surface F_c receptors such as F_c gamma (CD16, CD32, and CD64) and F_c epsilon (CD23). These receptors bind circulating antibodies that are themselves bound to foreign antigens, leading to macrophage phagocytosis (***78***).

Macrophages use their ability to sense endogenous molecules to facilitate the clearance of modified host proteins and lipids and apoptotic and necrotic cell debris after a disruptive event.

An example of a PRR that is integral for this function is the Tyro3, Axl and Mer (TAM) receptor family, which bind the phosphatidylserine associated proteins Gas6 and ProS, which are associated with the recognition and uptake of apoptotic cells (***79***). Recognition of apoptotic or necrotic debris generally leads to phagocytosis, but can also stimulate the secretion of anti-inflammatory and immunoregulatory cytokines such as IL-10 and transforming growth factor (TGF) beta and modulate TLR signalling, all in an independent manner (***80***). In addition, scavenger receptors (e.g scavenger receptor class A (SRA) and CD36) recognize both exogenous ligands (e.g. bacterial cell wall components) and endogenous ligands (e.g. oxidized low-density lipoproteins) (***44,81,82***).

2. Chemotaxis

Upon sensing an antigen belonging to a potentially harmful foreigner invader, macrophages stimulate the expansion of activated T cells and secrete chemokines that function to recruit appropriate effector cells to aid in their neutralization and clearance. Classically activated macrophages, who play a dominant role in anti-bacterial defence and promoting Th1-type responses, commonly secrete CCL-3 (MIP1 alpha), CCL-4 (MIP1 beta), CCL-5 (RANTES), CXCL-9 (MIG), CXCL-10 (IP-10) and CXCL-11 (I-TAC), which are all potent chemoattractants for monocyte/macrophages, Th1 lymphocytes and natural killer cells. They also secrete CXCL-8 (IL-8), a potent chemokine for the recruitment of neutrophils, which are crucial for the resolution of many types of acute infections (***39,83***). Consistent with their role in the defence against parasitic and fungal infections, AAMs commonly secrete the chemokines CCL-17 (TARC) and CCL-22 (MDC), which attracts Th2 lymphocytes and natural killer cells, and CCL-24 (Eotaxin-2), which attracts eosinophils and basophils (***39,83***).

3. Phagocytosis and tissue repair

To return a tissue to homeostasis after a disruptive event, the phagocytic clearance of damaged and redundant material is essential. Examples include inflammatory events triggered by infection or injury and physiological changes within the host (ie. embryonic development and postpartum mammary gland involution (***84,85***)), during which a great deal of tissue remodelling occurs. Using cell-surface receptors to identify its targets, as described above, macrophages engulf unwanted material, sequestering it within a phagosomal compartment. This compartment subsequently fuses with a lysosomal compartment, which contains a number of highly reactive and toxic molecules that facilitate the destruction of the phagosomal contents. Although ROS such as hydrogen peroxide and oxide anions play a major role in the destruction of engulfed material, NO is a major immunomodulator of this process. Nitric oxide regulates the levels and reactivity of ROS and can itself interact with ROS to produce toxic reactive nitrogen species (RNS). As a whole, NO and ROS is constitutively produced by macrophages, but upon signalling by cell-surface receptors and/or proinflammatory molecules the production of NO is highly induced through its parent enzyme inducible nitric oxide synthase (iNOS) (***86***). Returning host tissues to a homeostatic state also requires the repair and remodelling of the local environment. Returning host tissues to a homeostatic state also requires the repair and remodelling of the local environment. Alternatively activated macrophages are primarily responsible for this task, promoting extracellular matrix remodelling, cell growth, collagen production and angiogenesis (***78***).

4. Adaptive stimulation

The phagocytosis and subsequent destruction of foreign material by macrophages also provide a means to generate antigenic peptide sequences for presentation to T lymphocytes by way of cell-surface MHC class II receptors. Given suitable additional signalling, IL-12 or IL-4 for example, this interaction will lead to the expansion of antigen specific T lymphocytes, and thus, promote an adaptive immune response ***(87)***. However, unlike dendritic cells, most tissue macrophages can only present antigen and stimulate the expansion of activated T lymphocytes (***88,89***).

5. Summary

To understand the innate immune response to its fullest extent, it is necessary to recognize the importance of macrophages, and thus, their parent cell types. Although we have made impressive strides developing a framework to understand the relationship between tissue macrophages and their progenitors, especially peripheral blood and bone marrow monocytes, it is not currently possible to conclude at which point a given phenotype is terminal. Organizing these cells into rigid classes, although appealing as a framework, it is likely inaccurate as they represent a dynamic phenotype, one that is as unique as the microenvironment in which they lie. Furthermore, classifications developed in mice are not necessarily conserved in humans. Hence, conclusions that are made regarding the particular phenotype of a monocyte or macrophage in an experimental mouse model can often lead to confusion when attempting to translate it to humans. Transcriptional profiling, systems biology and increasingly elegant functional experiments have contributed to resolving these issues (***21,90***), and will undoubtedly continue to do so in the future.

Acknowledgements

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Figures and Figure Legends

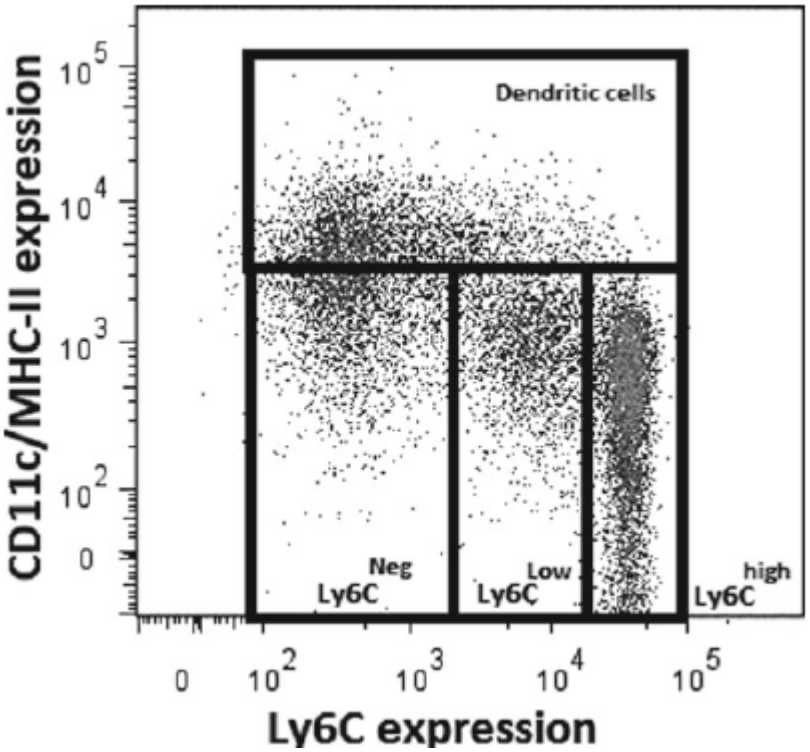

Figure 1: Characterization of mouse peripheral blood monocytes by flow cytometry. Monocytes can be distinguished using the cell-surface markers CD11c and MHC-II, and separated into three subsets based on expression of Ly6C. Note: Cells identified as expressing CD11c/MHC-II at high levels are considered dendritic cells, and as such are not included in the monocyte subsets.

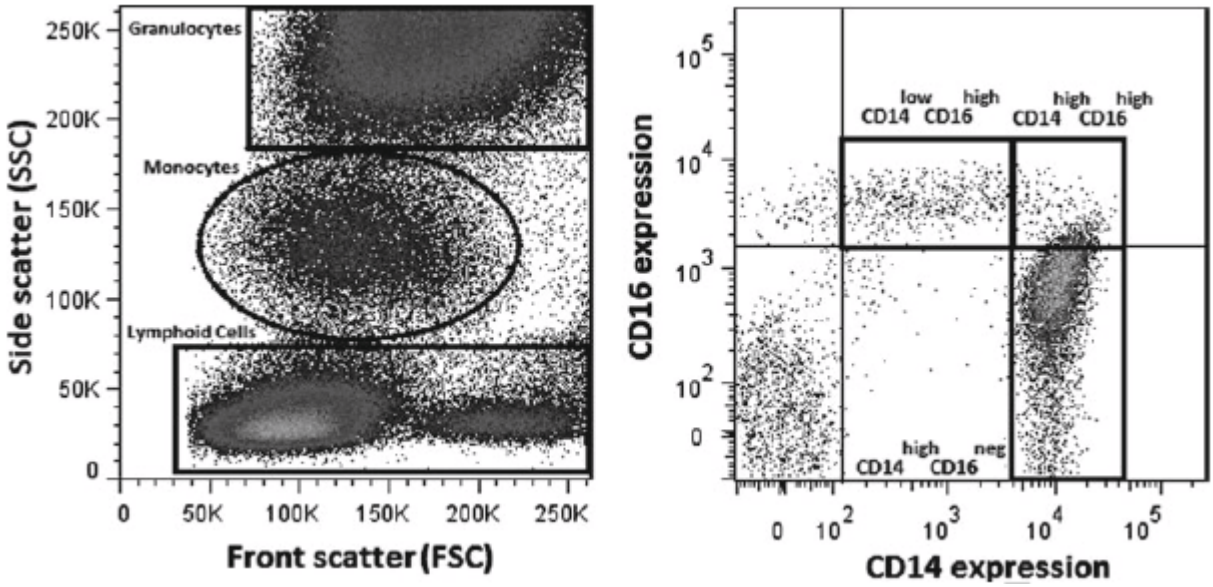

Figure 2: Characterization of human peripheral blood monocytes by flow cytometry. Monocytes can be distinguished amongst other blood leukocytes by size (front scatter, FSC) and granularity (side scatter, SSC) (A), and separated into three subsets based on the expression of the cell-surface markers CD14 and CD16 (B).

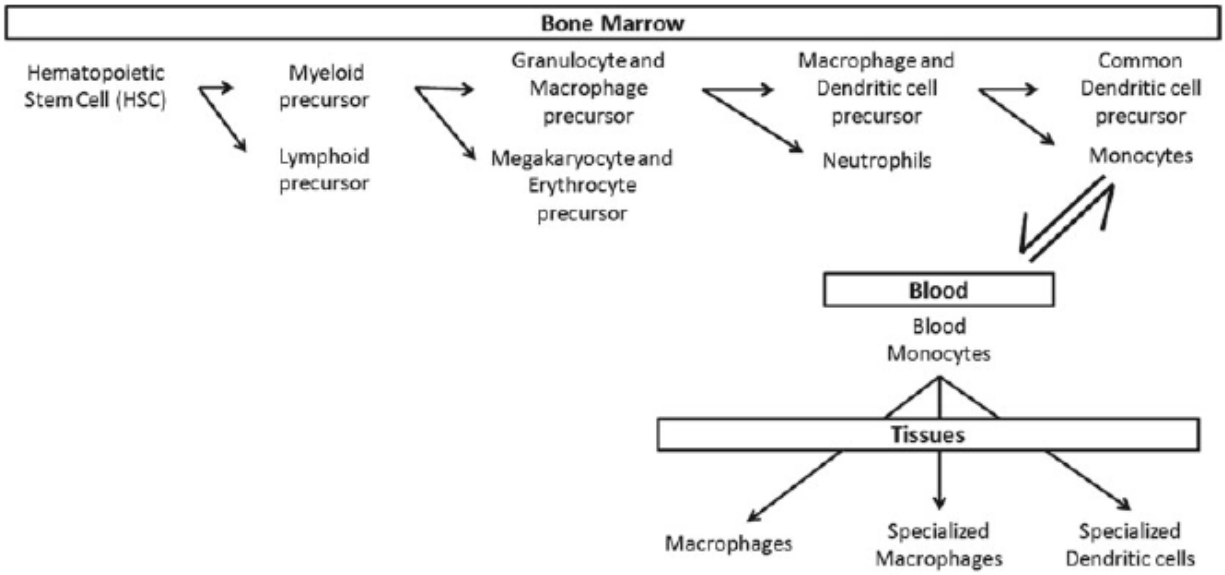

Figure 3: An overview of the development of the myeloid lineage.

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