MACROPHAGE-MEDIATED IMMUNITY TO PNEUMOCOCCAL COLONIZATION

THE ROLE OF MACROPHAGE RECEPTORS IN THE PROTECTION OF THE MURINE NASOPHARYNX FROM *STREPTOCOCCUS PNEUMONIAE*

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Lay Abstract

The bacterium *Streptococcus pneumoniae* is one of the most dangerous pathogens in the world, accounting for more one million deaths every year worldwide. This bacterium is also very common, with approximately one third of all people having some *S. pneumoniae* in their noses at any given time. The goal of this thesis is to provide a better understanding of how our immune cells interact with *S. pneumoniae* when it first enters our noses and how these initial interactions prevent healthy people from becoming sick. I have found that white blood cells called macrophages are crucial to these interactions. Macrophages are able to 'eat' the bacteria using a specialized protein called MARCO to grab onto them. This information will be vital in trying to develop new vaccines and treatments for *S. pneumoniae*-related diseases like bacterial pneumonia (lung infection) and meningitis (brain infection).

Abstract

Streptococcus pneumoniae (the pneumococcus) is one of the leading causes of death due to infectious disease in the world, with over one million deaths being attributed to this bacterium each year. While the majority of these deaths occur in children in developing nations, significant morbidity and mortality in the developed world, especially in the elderly, can be attributed to pneumococcal diseases such as bacterial pneumonia and meningitis. This is despite the near-universal use of anti-pneumococcal vaccines in these parts of the world. The work presented in this thesis describes the ways in which resident nasal macrophages respond to nasopharyngeal pneumococcal colonization, allowing for the protection of immunocompetent individuals from these diseases. This thesis describes the role of the macrophage scavenger receptor MARCO in recognizing the bacterium upon colonization, and the chain of events that are subsequently established. I have found that MARCO is vital in orchestrating the clearance of pneumococci from the nasopharynx in an expedient manner, as well as preventing the swift spread of bacteria to other tissues of the body early on in colonization. I also outline a role for regulatory micro-RNAs present in macrophages in the mounting of this anti-pneumococcal response via the induction of specific T cell populations. The collection of data found herein is an important resource for those attempting to understand the complex narrative that takes place between the pneumococcus and the innate immune system during a colonizing event and will lead to further discovery on how healthy individuals escape fatal pneumococcal disease.

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The existence of this thesis and, by extension, my scientific career would never be were it not for the courage, support, intelligence, and determination of my mentor Dr. Dawn Bowdish. Having little more than a gut feeling and need to fill a graduate student position quickly, Dawn accepted me into her lab despite my inexperience, lousy grades, and lack of appropriate references. To say this was a gamble is the understatement of my lifetime. I am forever indebted to Dawn for this opportunity. Dawn is the consummate leader - leading by example she instills in her students a love of science that will last a lifetime and is always there to hear outlandish ideas (and temper them), support life decisions, and provide advice. I thank Dawn from the bottom of my heart for providing me with the attention and ideas I needed to get through these five years and allow me to excel where no-one else could.

I would also never be here without my second, more unofficial, mentor Dr. Mark McDermott. Mark ensured I had the best opportunity possible in coming to McMaster to learn under Dawn's tutelage. He had the foresight to see that we could do excellent work together and was supportive the whole way through. Whether it was help with writing, navigating the minefield of 'the school of graduate studies', or providing us with a space to celebrate our achievements, Mark was always ready and willing to go the extra mile. I know my father regarded Mark as one of his closest colleagues and friends, and I can see why. Mark and his wife Marilyn opened their home and their lives to my wife Laura and I and we will be forever grateful (and willing to drink tequila with them).

A number of other great scientists have helped me with the work you will find within these pages. Drs. Martin Stampfli, Mike Surette, and Alba Guarné were instrumental in keeping me on track throughout my time at McMaster, giving me excellent advice on my experiments as well as in my writing. Drs. Karen Mossman and Ali Ashkar both provided me with mice and other materials needed for my experiments without so much as a second thought. In fact, the entirety of the McMaster Immunology Research Centre were so open and generous with their time, expertise, and reagents - I can't think of a better environment in which to learn how to become a scientist. I will take the attitudes you have instilled in me wherever I end up.

I have been lucky in having some of the best lab-mates I can imagine throughout my time in the Bowdish Lab. From my first day on I was totally comfortable thanks to the welcome I got from people like Dr. Alicja Puchta, Dr. Chris Verschoor, and Fiona Whelan. Fiona, I thank you especially for all of the science (and non-science) talks over coffees in the morning and for your infectious excitement about the work we were doing. Later additions to the team like Kyle Novakowski, Dessi Loukov, Avee Naidoo, Netusha Thevaranjan, and Mohammad Malik were always willing to help out with experiments, take a minute to talk over an idea, or celebrate my achievements with me at the Phoenix. Pat Schenck, my only wish is that you would have come and joined this team sooner. You're always welcome to come over for dinner down in DC (I'm sure Laura will let us talk about science). Thank you all for making the past five years enjoyable (even when nothing was working).

To my family, thank you for the support you have given me throughout my education and especially in this past half-decade. Thank you to my mom for talking

science with me and to Geoff for putting up with us speaking a different language at the dinner table. Thank you to my siblings for always being available for a dinner or drink when we needed it, and for keeping things fun for us in Hamilton.

Last but not least, thank you to my wonderful wife Laura. Coming home everyday to such a supporting environment has made all the difference since we came together. Thank you for forcing me to not be 'science-y' all the time and enjoy Hamilton to its fullest. Thank you for dealing with stupid companies on the phone and getting a dog (hi Hudson) and forcing me to not be lazy. Thank you for all the love you give to me everyday. This document, though it would bore you to tears, is a testament to that love and support.

This thesis is dedicated to the memory of Dr. Keith J. Dorrington, who wrote one of these himself back in the day. I know you'd be proud.

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List of Figures and Tables

There are no figures or tables in this version of the thesis outside of those associated with each of the three manuscripts presented in Chapters 3, 4, and 5. Please consult those chapters for Figure legends.

List of Abbreviations

Ab	Antibody	mRNA	Messenger RNA
Ag	Antigen	MyD88	Myeloid differentiation primary response gene 88
BIC	B cell integration cluster	Mø	Macrophage
C3	Complement protein 3	NALT	Nasal-associated lymphoid tissue
CD	Cluster of differentiation	NLR	NOD-like receptor
CFU	Colony-forming unit	NOD	Nucleotide-binding oligomerization domain-containing
CNS	Central nervous system	pafR	Platelet-activating factor receptor
CPS	Capsular polysaccharide	pIgR	Polymeric immunoglobulin receptor
CSR	Cellular stress response	PRC	Polycomb repressive complex
CytoD	Cytochalasin D	PRR	Pattern recognition receptor
DC	Dendritic cell	RA	Rheumatoid arthritis
DYN	Dynasore hydrate	RISC	RNA-induced silencing complex
EAE	Experimental autoimmune encephalomyelitis	RLR	RIG-I-like receptor
FACS	Fluorescence-activation cell sorting	SHIP	Src homology 2 domain-containing inositol 5-phosphate
HBSS	Hank's balanced salt solution	siRNA	Small interfering RNA
IFN	Interferon	SOCS	Suppressor of cytokine signaling
IFNAR	Type I IFN α/β receptor	SP	*Streptococcus pneumoniae*
IL	Interleukin	SR	Scavenger receptor
IPD	Invasive pneumococcal disease	SR-A	Scavenger receptor A
IRF	IFN regulatory factor	SRCL	SR with C-type lectin domain
ISG	IFN-stimulated gene	SRCR	SR cysteine-rich
JNK	c-Jun N-terminal kinase	ST	*Salmonella enterica* serovar Typhimurium
LPS	Lipopolysaccharide	STAT	Signal transducer and activator of transcription

MAPK	Mitogen-activated protein kinase	STING	Stimulator of IFN genes
MARCO	Macrophage receptor with collagenous structure	TLR	Toll-like receptor
MCP	Macrophage chemoattractant protein	TNF	Tumour necrosis factor
MDP	Muramyl dipeptide	TRAF	TNF receptor-associated factor
miR	Micro-RNA	TRAM	TRIF-related adaptor molecule
MMP	Matrix metalloprotease	TRIF	TIR-domain-containing adaptor inducing IFNβ
MOG	myelin oligodendrocyte glycoprotein	VSV	Vesicular stomatitis virus
MOI	Multiplicity of infection	WT	Wild-type
MR	Mannose receptor		

Declaration of Academic Achievement

This document is an accurate reflection of my experimental achievements over the course of my time at McMaster University - first as an M.Sc. candidate and then as a Doctoral candidate.

Chapters 1, 2, and 6, along with all of the preliminary pages in this thesis, were written entirely by myself, with editing input from my supervisor Dr. Dawn Bowdish and colleague L. Patrick Schenck. These chapters reflect what I believe to be a comprehensive background of previous work performed in my field as well as an informed discussion of the relevance and importance of my work within the context of that background.

Chapters 3, 4, and 5 are three related works that have either been published in reputable journals or are to be submitted for publication. The work found in these three manuscripts is a reflection of a series of collaborations with scientists both at McMaster and other institutions. My contribution to each of these works is outlined below:

Chapter 3 takes the form of a published manuscript that appears in the January 1st, 2013 edition of the *Journal of Immunology* and is the culmination of my studies as an M.Sc. candidate. I share authorship on this paper with Dr. Aoife Roche, a post-doctoral fellow who was under the tutelage of Dr. Jeffrey Weiser at the University of Pennsylvania at the time. Dr. Roche and myself planned and performed all of the experiments, analyzed the data, and wrote the manuscript. I was responsible for all of the MARCO$^{-/-}$ and SRA$^{-/-}$ mouse data along with the *in vitro* assays found in the publication. Dr. Roche performed the C3$^{-/-}$, MAC-1$^{-/-}$, and MR$^{-/-}$ mouse data and all experiments using the bacterial strain TIGR4 as well as the microscopy. Sarah Chauvin

and Zhongyuan Tu performed the extensive optimization needed for the luciferase and interferon protection assays, respectively. Dr. Karen Mossman provided the expertise and methods for the interferon-based assays. Dr. Dawn Bowdish oversaw the project and edited the manuscript.

Chapter 4 consists of a manuscript that is currently being prepared for submission to the *Journal of Immunology* and will be submitted in November of 2015. I designed and performed all experiments, analyzed and interpreted the data as well as wrote and prepared the manuscript for publication. Experimental assistance was provided by undergraduate student Mohammad Malik. Dr. Dawn Bowdish oversaw the project and edited the manuscript. Experiments that remain to be performed will be done by myself with the help of Kyle Novakowski and Avee Naidoo.

Chapter 5 is a manuscript that was published in November, 2014 in the journal *Infection and Immunity* and is a co-authored paper with Dr. Chris Verschoor, a post-doctoral fellow in Dr. Dawn Bowdish's laboratory. Dr. Verschoor and I designed all of the experiments, analyzed and interpreted the data as well as wrote and prepared the manuscript for publication. I designed and performed all animal experiments as well as cytokine and macrophage killing assays. Dr. Verschoor performed all T cell- and antibody-based experiments along with the flow cytometry. Experimental help was provided by Julie Kaiser, Varun Anipindi, and Kyle Novakowski. Sputum samples were induced by Katherine Radford under the supervision of Param Nair. Dr. Nair, Dr. Charu Kaushic and Dr. Michael Surette provided guidance and materials for experiments. Dr. Dawn Bowdish oversaw the project and edited the manuscript.

Chapter 1
Introduction

Despite recent (and not-so recent) medical advances, bacterial infections remain some of the most common causes of death in the world today. One of the most common pathogenic bacteria worldwide is *Streptococcus pneumoniae* (also known as the pneumococcus), which causes bacterial pneumonia, meningitis, and other potentially fatal diseases (1). There are currently two vaccines that protect against a limited subset of pneumococcal serotypes, which have decreased pneumococcal disease to a significant degree in developed nations (2). However, invasive pneumococcal disease (IPD) still accounts for more that one million deaths each year and are a significant source of morbidity and mortality in both developed and developing countries (3). The populations most affected by IPD are children under the age of five and the elderly (4). This chapter will introduce the pneumococcus and why it remains one of the most prevalent bacteria in the world today, as well as the natural immune responses that protect healthy individuals from IPD. This will be followed by an in-depth review of the immune mediators that are further studied in the three works presented as the bulk of this thesis.

The pneumococcus

The pneumococcus is a Gram-positive, aerotolerant, anaerobic diplococcus that commonly colonizes the human nasal passages. Its genome consists of a closed circular DNA structure with approximately 2.1 million basepairs forming 1500 genes. There are greater than 90 different serotypes of the pneumococcus based on the structure of the polysaccharide capsule that envelops the bacteria (5). This species of

bacteria is known to very frequently undertake homologous recombination in which regions of DNA are replaced by corresponding sequences from neighbouring donors. This typically happens between members of the same species and serogroup, but sometimes occurs between members of two different species, thus allowing for novel gene sequences to occur in the pneumococcus (6). Events such as this have led to a sharp increase in antibiotic (most commonly β-lactam) resistance within various strains of the pneumococcus (7). This decreasing efficacy in treatment strategies for bacterial infection dictates a need to develop preventative strategies.

IPD is preceded by nasopharyngeal colonization in humans (8). The pneumococcus must first gain a foothold in the nasal passages where it can grow as a colony and switch from a colonizing to an invasive phenotype through phase variation (see below). If, at this point, the immune response has not been able to clear the bacteria from the body, it is possible that it will gain access to further tissues such as the lungs and cause life-threatening disease. The average healthy individual will be periodically colonized with pneumococci and clear the bacteria in three to five weeks (9). During this timespan, innate immunity will keep bacterial numbers in check and adaptive immunity will clear the colonization. The prerequisite colonization stage before invasive disease provides us with a window in which prophylactic measures can successfully protect the host. This, coupled with the high rate of mutation in pneumococcal colonies, means preventative measures, such as vaccines, are the most promising avenue by which to reduce pneumococcus-related deaths significantly.

Pneumococcal vaccination

Prophylactic strategies are predominantly based on the stimulation of an adaptive immune response through vaccination. Traditional vaccines employ the use of known antigens to stimulate antigen presentation and subsequent expansion of effector and memory T and B cell populations. The memory cells created in the process can be activated by a subsequent infection, which will be rapidly cleared before any symptoms occur. While this strategy has been invaluable in decreasing infectious disease around the world, it is ineffective in many cases - including in the protection against the pneumococcus.

There are currently two pneumococcal vaccines in widespread use in the developed world. Both vaccines induce immunoglobulin responses that target the capsular polysaccharide, which is different for each serotype of the bacteria. Thus, only serotype-specific protection can be elicited (10). The first vaccine is a 23-valent polysaccharide vaccine and is meant to be effective against approximately 90% of the disease-causing strains common in North America and Europe. Due to a lack of a protein carrier, immune activation in the context of this vaccine is T cell-independent, thus making it is poorly immunogenic in children. The second vaccine is a protein conjugate vaccine, with 13 polysaccharides conjugated to a diphtheria toxoid carrier. The conjugate vaccine is much more effective in children but only protects against 13 serotypes. While these vaccines have been effective in reducing IPD in the developed world, they fail to replicate natural immunity to the pneumococcus, which is T cell-dependent and serotype-independent (11). Both vaccines protect against a limited number of serotypes of the bacteria and it has been shown that eliminating these serotypes from the population through vaccination often creates an environment for

previously less common serotypes to infect humans, a phenomenon termed 'serotype replacement' (12). It has been suggested that these replacement serotypes could potentially be more pathogenic than those we are protected against. The vaccines are also costly to manufacture, making them unlikely to ever gain widespread use in the developing world (13). The obvious shortcomings of the current vaccines warrant research into novel vaccination methods as well as gaining a better understanding of how the body and the pneumococcus interact in pneumococcal disease.

Virulence factors

The pneumococcus has various virulence factors that help it evade the host immune response [reviewed in (5)]. While an in-depth description of all of these factors is beyond the scope of this introduction, I will outline some of the more important below. These include the polysaccharide capsule, the pore-forming toxin pneumolysin, and the IgA1 protease.

Perhaps the most important factor in regards to subverting immune responses in the human nasopharynx is the polysaccharide capsule that envelops each bacterium. The capsular polysaccharide (CPS) structures making up the capsule are the primary antigenic determinants during colonization, with over 90 different serotypes of the bacteria being distinguished by these capsule types (14). The vast majority of pneumococci that are isolated from infected individuals are encapsulated and capsule mutants are severely attenuated in animal models (15). The capsule allows the bacteria to avoid becoming trapped in the mucous of the nasal passage, permitting them to gain access to the nasal epithelium (16). With the help of the molecule PspA, the capsule inhibits complement-mediated opsonization, by preventing the binding of C3b (17), as

well as antibody-mediated opsonization by preventing the binding of the F_c portion of IgG to the bacterial surface (18). The capsule is also important for obscuring surface features of the bacterial cell wall and associated proteins that would be recognized by resident phagocytes in the nasopharynx.

While a thick polysaccharide capsule is beneficial for the development of colonization in the human nasopharynx, the shift from a colonizing phenotype to an invasive one is predicated on a reduction in capsule thickness (19). Individual pneumococci within a colony will vary in how thick their capsules are in a phenomenon termed 'phase variation'. The ability to control the amount of CPS being produced allows the pneumococcal colony to survive in a variety of environments within the host (20). In general, high-CPS pneumococci are adept at inter-individual transmission, evading immune responses, and colonizing the nasopharynx while low-CPS pneumococci are better able to adhere to epithelia in the lungs and cause IPD (21). Understanding the mechanisms by which phase variation occurs is an important endeavour as preventing it could preclude the transition from pneumococcal colonization to IPD.

The pneumococcus produces one toxin, termed pneumolysin - a protein that forms a large transmembrane pore in target cells when oligomerized. Pneumolysin activity results in various cell modulatory effects, such as induction of cytokine production via interaction with toll-like receptor- (TLR-)4, inhibition of phagocyte respiratory burst, and $CD4^+$ T cell activation (22). Pneumolysin is also responsible for the recruitment of neutrophils to regions of infection, effectively promoting an inflammatory response (23). It is believed that this occurs to enhance transmission of the

bacteria from host-to-host via contact with nasal secretions, even though it can lead to an accelerated clearance of the bacteria (5).

Like many upper airway colonizers, pneumococci produce an IgA-specific protease that cleaves the hinge region of human IgA1, the most plentiful immunoglobulin at mucosal surfaces (24). Cleavage of IgA limits its effectiveness by leaving pneumococci coated in F_{ab} fragments, eliminating the F_c receptor-mediated mechanisms of the antibody, including opsonization and complement fixation. As a result, the production of significant levels of IgG is more beneficial to clearance. Indeed, the effectiveness of the conjugate vaccine described above has largely been attributed to its ability to induce capsule-specific IgGs instead of IgA. The effectiveness of the IgA protease is further substantiated by the fact that individuals incapable of producing IgA are no more likely to suffer from IPD than those who are immunocompetent (25).

The pneumococcus is a highly-evolved human pathogen, subverting our immune responses while it gains purchase in the nasopharynx before moving on to further tissues. However, the vast majority of healthy people between the ages of five and sixty-five are periodically colonized with pneumococci with no detriment to their health. What allows these people to clear the bacteria despite its plethora of virulence factors? To answer this, we must closely examine what immune responses are initiated during a colonization event.

The immune response to the pneumococcus

Primary colonization is characterized by a brisk, yet ineffective, innate immune response followed by an adaptive response that usually clears the bacterial burden in three to five weeks (26). As soon as the bacterium enters the nasal cavity, it comes into

contact with mucous secretions, which should prevent adherence of bacteria to the nasal epithelium. However, the hydrophobic capsule around the bacterium allows it to repel the mucous and attach itself to the epithelium, establishing what is termed the 'carrier state' (16). Once colonization is successful, innate immunity begins to mount a response. Starting approximately one day after exposure to the pathogen, there is an influx of neutrophils into the nasopharyngeal passage, which attempt to clear the carrier state (27). The neutrophils are attracted by chemokines released by the epithelial cells to which the bacteria adhere. The bacteria form pores in the epithelial cells using pneumolysin and the resulting osmotic stress activates the p38 mitogen-activated protein kinase (MAPK) pathway leading to an increase in these chemokines (5). The resulting influx of neutrophils is able to facilitate the transition to an adaptive immune response by a pneumolysin-dependent mechanism (28). However, the neutrophils are ineffective at clearing the infection and the pneumococcal population persists even after the neutrophils leave the site of colonization.

The resolution of the carrier state requires the recruitment of monocytes from the blood into the nasal interstitial spaces where they differentiate into macrophages (29). Macrophages are large phagocytic cells and can be distinguished from other cells by surface markers including F4/80 (in mice), FMR1 (in humans), CD64, Ly6C, and MAC-1. Macrophages have many roles in innate and adaptive immune responses, as well as in tissue remodeling/repair and debris removal. In the context of this study, it is important to understand the macrophages' roles in the immune response to extracellular bacteria such as the pneumococcus. Resident and recruited macrophage populations are activated by a combination of interferon- (IFN-)γ and tumour necrosis factor (TNF), in that order (30). The IFN-γ in a pneumococcal response is likely provided by afflicted

epithelial cells after the bacteria gain access to them. These macrophages then produce TNF themselves (22). These activated macrophages are then able to engulf bacteria via a process known as phagocytosis.

Phagocytosis is the binding and internalization of insoluble debris or bacteria in a receptor-mediated fashion. Normally, phagocytosis is facilitated by opsonization, a process in which a pathogen is bound by serum components, such as immunoglobulin or complement cleavage products, so that they can be recognized by phagocytes such as neutrophils and macrophages (31). In an innate response, the primary opsonins are proteins that are members of the complement system. This system consists of a complex cascade of molecules acting together to either influence the activity of immune cells or directly kill pathogens. Complement can be activated one of three ways. In defense against the pneumococcus it appears that the classical pathways, wherein complement protein C1q interact with antigen-antibody complexes, is the primary pathway (32). In a primary infection, a non-specific complement response will lead to opsonization of pathogens. The primary opsonizing protein of the complement system is C3b, which is a cleavage product of the molecule C3. Macrophages are able to bind to C3b via the MAC-1 receptor and then internalize the bacterium to which the C3b molecule is bound.

Opsonization can also be performed by antibodies (Abs). This is an aspect of humoral adaptive immunity. Abs that are specific for an epitope on the antigen will attach to the epitope via the paratope formed by their Fab portion. Once such binding occurs, the opposite end of the Ab, known as the F_c portion, can then be bound by the F_c receptor expressed by phagocytes such as neutrophils and macrophages. This will, again, lead to internalization and destruction of the pathogen. However, work done by ours and Jeffrey Weiser's groups have shown that pneumococcal clearance is not

affected when Ab, C3, and MAC-1 are removed from a system of infection one-at-a-time (33-36). These findings suggest that humoral immunity is not the primary way in which the immune system clears primary pneumococcal infections.

A number of macrophage proteins have been shown to be important for the clearance of pneumococcal colonization. Innate immune receptors such as TLR-2 and nucleotide-binding oligomerization domain-containing receptor- (NOD-)2 are vital to the production of pro-inflammatory cytokines and chemokines such as interleukin- (IL-) 6, TNF, IL-1β, and the macrophage chemoattractant proteins (MCPs) (29, 35, 37, 38). TLR-2, a cell-surface pattern recognition receptor (PRR), specifically recognizes both the lipoteichoic acid and cell wall peptidoglycan expressed by Gram-positive bacteria like the pneumococcus. Numerous groups have shown that TLR-2-deficient mice are more susceptible to pneumococcal meningitis, with these mice having reduced cellular recruitment to the brain early in infection, followed by hyper-inflammation leading to fatal tissue damage (39). In models of pneumonia, however, TLR-2-deficient mice have no increase in mortality or morbidity, showing that tissue-specific responses are important. In addition, TLR-2 (but not TLR-4) has been shown to be vital for the timely clearance of pneumococcal colonization from the murine nasopharynx (40).

NOD-2 is a cytoplasmic macrophage pattern recognition receptor (PRR) that recognizes muramyl dipeptide (MDP), a peptidoglycan constituent that is released when the human muramidase lysozyme cleaves it from the bacterial cell wall. Lysozyme is found at high concentrations in nasal secretions, producing ample amounts of MDP for resident macrophages, as well as those recruited later on in the colonization. Previous studies have shown that the sensing of pneumococcal MDP by NOD-2 in macrophages is vital for the production of MCP-1 (also known as CCL2) (29). MCP-1 acts as a

chemoattractant for monocytes expressing its receptor CCR2, bringing these cells to the site of colonization where they can differentiate into macrophages and act to clear the bacteria. It is unclear how the MDP cleaved by lysozyme enters the cytoplasm to activate NOD-2, but it has been suggested that pore formation by pneumolysin might be involved (41). Mice lacking NOD-2 have reduced pneumococcal clearance from the nasopharynx that coincides with a paucity of MCP-1 production leading to less monocyte/macrophage recruitment than wild-type (WT) mice (29).

Studies performed using $CD4^+$ T cell-depleted mice have shown a reduction in the host's ability to generate an appropriate antibacterial response to the pneumococcus (11, 26, 40). More specifically, a reduction in IL-17 expressing T-cells, known as Th17 cells, was shown to cause a significant reduction in clearance of pneumococci as well as a reduction in the recruitment of both neutrophils and monocytes/macrophages. Th17 cells have been insufficiently characterized in the short time since they were discovered, but they are highly inflammatory cells that produce inflammatory cytokines including IL-17, which can act as a chemoattractant for monocytes and macrophages (42). These data indicate that pathogen-specific Th17 cells are necessary for the recruitment of phagocytes into the nasal lumen where they are then able to clear the bacterial burden. This is done in a TLR2-dependent fashion, as shown by results from Dr. Weiser's group (26).

The model of how the immune system reacts to pneumococcal colonization can be summarized as follows: bacterial colonization induces a rapid, yet ineffective, neutrophil response which initiates a transition to an adaptive, $CD4^+$IL-17^+ T-cell-mediated response. These Th17 cells are then able to recruit a sustained number of monocytes/macrophages into the nasal lumen where they are able to phagocytose and

eliminate the bacteria. With this model in place, it is now important to analyze how the macrophages accomplish this without the required use of opsonins.

MARCO

The fact that macrophage activity, but not opsonization, is vital to the clearance of the pneumococcus from the nasopharynx, raises an important question: how do macrophages recognize and successfully phagocytose bacteria without the aid of opsonins? The answer likely lies in the macrophage's unique ability to phagocytose cells and debris that are unopsonized (43). This phenomenon occurs in the lungs where it is performed by human alveolar macrophages. Recent studies have shown that a scavenger receptor (SR) known as macrophage receptor with collagenous structure (MARCO), which is present on the macrophage cell surface, is involved in the uptake of unopsonized particles and bacteria in the lungs (44, 45).

MARCO (also known as scavenger receptor A-6) is a member of the class A SR family (46). Also included in this family are the splice variants scavenger receptor-A (SR-A)I and SR-AII, scavenger receptor class A, member 3 (SCARA3)/CSR (Cellular Stress Response), SCARA4/SRCL (Scavenger Receptor with C-type lectin domain), and SCARA5. The SRs, in general, are a diverse group of receptors originally grouped together due to their ability to bind and internalize acetylated low-density lipoprotein (47). The class A SRs form a structurally-similar subgroup that, through bioinformatics, have been shown to be genetically related (48). Each of these proteins is a homotrimeric type II transmembrane protein with a ligand-binding domain at the extracellular C-terminus. In the case of MARCO (as well as SR-AI and SCARA5), this binding domain is the scavenger receptor cysteine-rich (SRCR) domain, an evolutionarily-ancient

protein module associated with innate immunity (49). SRCR domains are very common, existing in all levels of the animal kingdom, from sponges to humans (50). Despite this, there is not one unifying function and the SRCR domain has not been studied in-depth in the class A SR family. The work that has been done, however, points to the SRCR domain being the primary ligand binding domain of MARCO (51).

The class A scavenger receptors are known for their broad ligand specificity and actions as phagocytic cell receptors. Class A scavenger receptors are able to bind Gram-positive and Gram-negative bacteria via modified lipoproteins, polyribonucleotides, and polysaccharides (45). While most research to date has highlighted a redundant function between SR-AI/II and MARCO, based on their similar structure, more recent findings found that these receptors often have certain opposing functions and are regulated by disparate immune responses (52). MARCO expression is upregulated in Th1-mediated adaptive reponses while SR-AI/II are upregulated during Th2 responses. However, the relationship between these receptors and Th17 cells has yet to be studied.

Initial interest in MARCO as an important receptor for antibacterial mechanisms came from the fact that it is only constitutively expressed on a select portion of macrophages including alveolar macrophages, decidual macrophages, peritoneal macrophages, and the marginal zone macrophages of the spleen (53, 54). All of these locations are where the immune system can come into direct contact with invading infectious bacteria on a regular basis, which makes MARCO a good candidate for antibacterial activity. MARCO is also expressed on macrophages and dendritic cells (DCs) at sites of inflammation as a result of bacterial infection, thus reinforcing the idea that it is important in immune defense. In contrast, SR-AI/II are expressed on most, if not all, macrophages as well as other cell types, such as endothelial cells (55).

Previous studies of MARCO have uncovered a variety of roles for the receptor in innate immunity, especially associated with alveolar macrophages. The bulk of MARCO's effect on immune responses revolve around its capacity as a phagocytic receptor (56). MARCO recognizes a wide variety of polyanionic ligands using its SRCR domain. This is likely due to interactions between the negatively charges ligands and the positively charged arginine residues found in the SRCR domain (57). This allows MARCO-expressing alveolar macrophages to bind to and internalize modified lipoproteins, environmental particulates, and apoptotic cells, removing them from the lungs where they can cause damage (45). Interestingly, MARCO deficiency has been linked in both mice and humans to systemic lupus erythematosus, likely due to apoptotic cells not being cleared efficiently in the lungs and spleen (58). These largely homeostatic mechanisms are shared by SR-AI, which is capable of binding the same ligands, though it seems SR-AI does so via its collagenous domain. As such, much of the early work on SR-AI and MARCO has focussed on maintaining a healthy lung environment and not on host defense against pathogens. However, MARCO appears to be especially capable of dealing with foreign invaders.

MARCO in immune responses

A large proportion of MARCO-related literature focusses on MARCO's interactions with *Mycobacterium tuberculosis* in the lung. *M. tuberculosis* is the causative agent in tuberculosis infection and affects more than a third of the world's population (59). A study from Siamon Gordon's laboratory showed that MARCO is vital to the production of pro-inflammatory cytokines in response to mycobacterial trehalose dimycolate, a glycolipid found in the bacteria's cell wall (60). MARCO-deficient cells

were less capable of producing TNF in response to both purified trehalose dimycolate and whole *M. tuberculosis*. This was shown to also require TLR2 and CD14, providing precedent for MARCO working in conjunction with these other receptors. A more recent study has shown that certain polymorphisms in the *marco* gene are associated with susceptibility to pulmonary tuberculosis in humans, thus showing that MARCO's importance is not constrained to rodents (51). Indeed, a study performed in zebrafish (a member of the minnow family) showed that MARCO was the primary phagocytic receptor for *M. marinum,* a close relative of *M. tuberculosis* (61). This work also highlighted MARCO's contributions to pro-inflammatory cytokine responses, a common theme in MARCO-related research.

Other studies have focussed on MARCO's contributions to immunity using other bacteria, such as *Neisseria meningitidis* and *Clostridium sordellii*, two very important human pathogens. Work on *N. meningitidis* from Siamon Gordon's laboratory showed that deleting the MARCO gene did not have a significant effect on bacterial uptake and cytokine production in a model of sterile peritonitis (62). However, deleting both SR-AI/II and MARCO genes together increased TNF and IL-6 production while decreasing bacterial uptake in this model. The study went on to show that MARCO deficiency leads to hyporesponsiveness in macrophages stimulated with the NOD-2 ligand MDP, which produce less TNF and IL-6. These data together suggest that MARCO increases intracellular PRR activation at the expense of extracellular PRR signaling. Therefore, MARCO can modulate innate immune responses depending on its ability to take up a particular bacteria.

MARCO is also the primary phagocytic receptor for *C. sordellii*, a pathogen that causes lethal infections of the female reproductive tract (63). Decidual macrophages

from MARCO-deficient mice were significantly deficient in their ability to phagocytose these bacteria, and these mice were also at greater risk of death due to the infection. While this particular study did not measure cytokine production or investigate inflammation in any way, it does highlight the importance of MARCO-mediated phagocytosis in an *in vivo* model of bacterial infection. MARCO is also capable of binding to *Staphylococcus aureus* and *Escherichia coli*, though there have been no studies as to whether MARCO deficiency leads to increased mortality in infections with either of these pathogens (45).

Most importantly to this study, MARCO has been shown to be a phagocytic receptor for pneumococci. Lester Kobzik's group showed in 2004 that mice lacking MARCO were more susceptible to succumbing to pneumococcal pneumonia in a model of direct bacterial lung instillation (56). The study showed that MARCO$^{-/-}$ mice had increased TNF and CXCL-2 four and 20 hours after bacterial infection compared to WT mice. This correlated with increased neutrophil recruitment to the lungs despite which bacterial colony-forming units (CFUs) were significantly higher in the MARCO$^{-/-}$ mice. This study establishes a role for MARCO in the recognition of *S. pneumoniae* by alveolar macrophages in a murine pneumonia model. However, the mechanisms by which MARCO aids the anti-pneumococcal response in the nasopharynx remain a mystery, establishing the major question behind this project.

Type I Interferon

The type I IFNs are a family of cytokines made up of multiple IFN-α subtypes and single IFN-β, ε, κ, and ω species as well as the δ and τ subtypes found in pigs and sheep, respectively (64). All of these cytokines function by binding to the type I IFNα/β

receptor (IFNAR), which is expressed nearly ubiquitously throughout the body. The type I IFNs were discovered nearly 60 years ago when a secreted factor was shown to 'interfere' with viral replication in chicken eggs. For the next 50 years, the vast majority of interferon-related research focussed on its effects during viral infections and in cancer patients. More recently, there has been a growing consensus that these cytokines are important for determining the outcome of bacterial infections as well (65). Before one can fully appreciate the role of type I IFNs in bacterial infection, it is first important to understand where these cytokines come from and their known effects on immune cells.

Traditional thinking states that type I IFNs are predominantly produced by epithelial cells that have come into contact with an infectious organism or other stimulus, which could include IFNs themselves. However, evidence has been uncovered that other cell types, including macrophages and DCs, are also capable of producing appreciable amounts of type I IFN (66). A large number of PRRs are upstream of type I IFN responses, including TLRs, RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs). Macrophages and DCs are well-equipped with these receptors as well as the downstream signalling molecules that lead to type I IFN production. These molecules include scaffolding proteins such as TIR-domain-containing adaptor-inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and TNF receptor associated factor 6 (TRAF-6), as well as the transcription factors known as the interferon response factors (IRF)-3 and 7 - all important for the induction of these cytokines (67). As such, these cells could theoretically respond to pathogens in the same way that epithelial cells can.

Previous studies have shown that in certain infection models, loss of macrophages can lead to a significant decrease in type I IFN production, leading to detrimental outcomes. Indeed, alveolar macrophages appear to be the primary producers

of type I IFN during lung infections (68). This can have wide-ranging effects as IFN works in both an autocrine and paracrine fashion, restricting the replication and dissemination of virus particles in both the macrophage and surrounding epithelium. Macrophages responding to type I IFN are also important for the recruitment of immune cells to areas of inflammation, as IFN can upregulate CCL-2 production while decreasing CXCR-2 ligands (69). This brings in monocytes, which then differentiate into macrophages, at the expense of neutrophils, which are often associated with increased pathology and mortality in animal models of lung infection.

The ligation of type I IFN by IFNAR triggers a complex set of cellular reactions centred around the IFN-stimulated genes (ISGs) (70). The ISGs are a diverse group of hundreds of genes, encoding for proteins a wide variety of functions to do with anti-viral responses. For example, IRF-7 is a potent inducer of type I IFN responses that is itself regulated by IFNAR ligation. This allows for a positive feedback loop to exist wherein IFN stimulation leads to that cell becoming even more responsive to IFN-producing stimuli. Other examples include ISG-56 and signal transducer and activator of transcription (STAT)-2, which are capable of suppressing viral replication and promoting viral clearance, respectively (71, 72). While the role of a number of these genes is known in the context of viral infection, comparatively little is known about the role of ISGs in battling pathogenic bacteria.

Type I IFN and bacterial infection

The importance of the type I IFN response to bacteria is remarkably convoluted. Studies have shown that IFN production can restrict the growth of some intracellular bacteria, such as *Chlamydia sp.* and *Legionella pneumophila* (73, 74). Conversely, type I

IFN production in the context of *Listeria monocytogenes* and *M. tuberculosis* infections is associated with poor outcomes (*75*, *76*). In fact, both of these bacteria actively induce type I IFN production in infected macrophages with IRF-3-deficient mice being resistant to infection. *Salmonella enterica* serovar Typhimurium (ST) has been shown to be capable of inducing type I IFN in an attempt to restrict a wide range of TLR and NLR responses (*77*). Interestingly, pre-treatment of macrophages with IFN-β renders them hyporesponsive to various bacterial ligands, including lipopolysaccharide (LPS), especially in the production of neutrophil chemokines and IL-1β. This type I IFN production also leads to a cytokine cascade in macrophages wherein type I IFN stimulates the cells to produce IL-27, which acts in an autocrine fashion on the macrophage, causing it to produce large amounts of IL-10 (*78*). These phenomena are linked, in that IL-10 production can directly inhibit TLR responses. Therefore, it appears that some intracellular bacteria have evolved to stimulate the IFN response in order to subvert PRR signaling by promoting IL-10 production. While this is not necessarily restricted to intracellular bacteria, there has been much less work on the effects of type I IFN on extracellular bacteria like *S. pneumoniae*.

The production of type I IFN in response to extracellular bacteria can occur through the stimulation of TLRs, responding to either LPS or other cell wall components, or cytosolic receptors that recognize endocytosed or secreted bacterial cell wall components and nucleic acids (*67*). Most commonly, LPS from Gram-negative bacteria stimulate the endocytosis of TLR-4 in early endosomes, where the receptor signals through the TRIF/TRAM pathway causing *ifnb* transcription by IRF-3 (*79*). IFN production can also be induced by TLR-7, 8, or 9 using the myeloid differentiation primary response gene-88 (MyD88) pathway as well as non-TLR receptors such as

RIG-I and NOD-2 (64). In general, type I IFN production is elicited from receptors that are either in the cytoplasm or have been endocytosed after coming into contact with their ligand. Despite this, there have been no links thus-far between scavenger receptor-related uptake of bacteria with type I IFN production.

The pneumococcus has been shown to induce *ifnb* gene transcription in both the nasal-associated lymphoid tissues (NALT) and the lungs of mice (80, 81). There is some disagreement as to whether this IFN production is protective or detrimental to the host during pneumococcal infection. An early study on the effects of IFN-β production in bacterial infection showed that giving mice an antibody against IFN-β prior to lung instillation of pneumococci increased the chance of death compared to mice given isotype antibodies (82). Pneumococcal colonization experiments in $IFNAR^{-/-}$ mice showed increased bacterial burden in the nasal passages compared to WT mice. Type I IFN has also been shown to reduce the transmigration of pneumococci across the lung epithelium by informing epithelial cell responses (81). $IFNAR^{-/-}$ mice are more susceptible to IPD in a model of direct pneumococcal lung instillation compared to WT mice. These mice were lacking in tight junction upregulation, allowing the bacteria to escape the lung into the blood. These data show that type I IFN is vital to containing pneumococci at mucosal surfaces early in infection.

However, *S. pneumoniae* is also a common cause of post-influenza bacterial pneumonia. Mice previously infected with influenza virus have increased bacterial load in their lungs five days after pneumococcus instillation, unless they are deficient in IFNAR (83, 84). The type I IFN produced in response to the virus appears to diminish neutrophil recruitment post-bacterial inoculation. Therefore, a type I IFN-dependent response to the virus inhibits the appropriate neutrophil response to the bacteria.

Nasopharyngeal pneumococcal colonization is also affected by previous influenza infection. WT mice infected with influenza and subsequently colonized with pneumococci exhibited increased bacterial load, decreased *ccl2* gene transcription, and less macrophage recruitment than their IFNAR$^{-/-}$ counterparts seven days post-pneumococcal colonization (85). The seeming contradiction between these studies and those in the previous paragraph highlight the need for more rigorous experimentation concerning how type I IFNs are elicited in reaction to extracellular bacteria and how these cytokines determine the outcome of these infections.

Macrophages produce type I IFN in response to pneumococcal stimulation. It appears this can occur by cytosolic pneumococcal DNA sensing by the stimulator of IFN genes (STING) (41, 86). STING-mediated IFN production depends on pneumolysin expression by the bacteria as well as IRF-3 expression by the macrophage. STING interacts with pneumococcal DNA, which likely enters the cytoplasm via a pneumolysin-dependent mechanism after the phagocytosis of the bacterium (41). How the bacterium is phagocytosed in this context has yet to be elucidated. *S. pneumoniae* can trigger inflammatory responses by macrophages via both TLR-2 and TLR-4 and these receptors have been directly linked to type I IFN production, but it has yet to be seen whether pneumococci can induce IFN production via a TLR-specific mechanism (87).

MicroRNA-155

While the primary objective of the studies found in this thesis was to determine the role of MARCO in anti-pneumococcal responses, it is also important to understand how MARCO's expression on macrophages is regulated. Protein expression can be regulated at the transcriptional and post-transcriptional levels. One way in which post-

transcriptional regulation occurs is through RNA interference wherein evolutionarily ancient, small, non-coding RNAs interrupt the translation of mRNAs to proteins (88). Two examples of these RNAs are the micro-RNAs (miRNAs or miRs) and the small interfering RNAs (siRNAs), both of which are found in all eukaryotic cells and some DNA viruses. Our work focusses on a particular miRNA and, as such, these will be introduced here. miRNAs function by binding, via complementary sequences to messenger RNAs (mRNAs), thus eliminating their ability to initiate translation. This effectively silences the mRNA translation and prevents the production of the protein encoded by that mRNA (89).

The production of a miRNA begins with the transcription of a 'primary' (or pri-) miRNA, which consists of a stem-loop structure, by RNA polymerase II. The pri-miRNA is then processed similarly to a mRNA, including splicing and poly-adenylation. Before it can exit the nucleus, the pri-miRNA is first cleaved by the enzyme Drosha, which releases the stem-loop from any flanking RNA sequences creating a 'precursor' miRNA (pre-miRNA). Once the pre-miRNA is exported, the enzyme Dicer cleaves the loop portion, releasing the mature miRNA. The miRNA is then recruited to the RNA-induced silencing complex (RISC) which includes a member of the Argonaute protein family - the active components in RNA interference. The miRNA can then direct the associated Argonaute protein to a specific target mRNA through sequence complementarity (90).

There are three main mechanisms by which the binding of a miRNA to an mRNA disrupts translation (88). The most simple mechanism is steric hindrance. When the miRNA is bound to the single-stranded mRNA, it is impossible for that mRNA to interact with a ribosome due to the altered structure of the mRNA. This occurs when

the miRNA is not 100% complementary to the mRNA sequence. Incomplete complementarity can also result in mRNA modification through deadenylation and decapping, followed by natural degradation by exoribonuclease-1. When there is complete complementarity between the miRNA and mRNA sequences, the mRNA becomes 'tagged' for destruction by the RISC itself. Binding of the miRNA to its complementary mRNA sequence activates endonuclease activity in Argonaute, leading to cleavage of the mRNA molecule rendering it unable to initiate translation. In these ways, miRNAs are able to very efficiently and specifically silence protein expression.

In the past 15 years, over 2500 miRNAs encoded in the human genome have been discovered, each having multiple target mRNAs, and it is currently thought that these RNAs can regulate more than one third of all genes in humans (91). As such, miRNAs are associated with nearly every biological process from development to metabolism to host defense. miRNAs have also been significantly associated with cancers [reviewed in (92)]. One half of all *miR* genes are located at chromosomal sites associated with amplification, deletion, or translocation in cancer. Altered expression of miRNAs has been tied directly to colon, breast, and lung tumours. The study of many miRNAs has also found links to various neurologic, inflammatory, and infectious diseases - including miR-155.

In **Chapter 5**, our work focusses on miR-155, a microRNA that has predominantly been tied to hematopoiesis, inflammation, and immune responses (93). The *miR-155* gene is located within the B cell integration cluster (BIC) on chromosome 21 and is highly expressed in lymphoid organs across the animal kingdom. miR-155 has hundreds of potential target genes based on its sequence. Hematopoietically, miR-155 is important for limiting the differentiation of hematopoietic stem cells into myeloid and

erythroid progenitors as stem cells transduced with miR-155 produce a fraction of the colonies produced by unaffected cells and these colonies are significantly smaller (94, 95). Mice lacking BIC have normal levels of T and B cell subset numbers and distributions in the steady state, but have significantly reduced natural killer cell numbers in their spleens (96). Inflammatory mediators such as LPS, IL-1β, and TNF can upregulate miR-155 expression in human monocytes through the c-Jun N-terminal kinase (JNK) and mitogen-activated protein kinase (MAPK) pathways (97). It has been hypothesized that this could be a protective function associated with diseases such as rheumatoid arthritis (RA) as miR-155 expression is negatively correlated with the production of matrix metalloproteinases (MMPs) in the synovial fluid of RA patients. Indeed, miR-155 levels in $CD14^+$ cells in the joints of RA patients are significantly higher than patients with osteoarthritis (98).

Expression of miR-155 (and the entire BIC) is highest in activated B and T cells as well as macrophages and DCs (96). The effects in these cells types are myriad. miR-155-deficient mice have fewer T and B cells and are unable to produce significant amounts of antibody, IL-2, or IFN-γ in response to immunization with tetanus toxin fragments. These mice also have impaired antigen-presenting function by DCs. However, these mice have increased IL-10 responses, possibly signalling a skewing towards Th2 responses. There are also a number of B cell-intrinsic effects of miR-155 deficiency (99). Mice lacking this microRNA have reduced extrafollicular and germinal centre B cell responses and are incapable of producing high-affinity IgG1 antibodies, likely due to overexpression of PU.1, an important B cell differentiation factor. Normally miR-155 would bind to PU.1 mRNA, limiting its expression in B cells

undergoing activation and class switching and allowing for the production of IgG1 antibodies (100).

Relationships between miR-155 and Th17 cell differentiation and activity has been a heavily-researched topic in recent years. This work began when a group attempted to immunize miR-155-deficient mice with myelin oligodendrocyte glycoprotein (MOG) in order to study miR-155's role in experimental autoimmune encephalomyelitis (EAE - a common model of multiple sclerosis) (101). miR-155 mice were protected from EAE and it was subsequently found that this was due to a lack of Th17 cell in both the spleen and lymph nodes of these mice. Interestingly, the authors also saw a reduction in regulatory T cells in the miR-155$^{-/-}$ mice. Later studies showed that Th17 cells lacking miR-155 cannot respond to IL-23/IL-23R ligation and have significantly reduced IL-17 production (102). It has been hypothesized that these effects are related to miR-155's regulation of suppressor of cytokine signalling (SOCS-1), which has been linked to the IL-2 and IL-6 signalling pathways via signal transducer and activator of transcription 5 (STAT-5).

How miR-155 expression affects innate immune responses has been decidedly understudied compared to adaptive responses. As mentioned above, miR-155 expression can be upregulated following stimulation of monocytic cells with TLR ligands and pro-inflammatory cytokines such as IL-1β and TNF. Studies have shown that macrophage responses to *Helicobacter pylori* are aided by miR-155's repression of SOCS-1 and resulting increases in inflammatory cytokines, including type I IFNs (103). miR-155 also inhibits Src homology 2 domain-containing inositol 5-phosphatase-1 (SHIP-1), promoting immunity to both *Staphylococcus aureus* (104) and *Francisella tularensis* (105), but negatively impacting responses to *Mycobacterium tuberculosis* (106). Perhaps most

importantly for the studies that follow, it has been shown that the gene encoding for SR-AI (*msr1*) has a target sequence for miR-155 and that miR-155 can regulate SR-AI expression on alveolar macrophages (107). Interestingly, murine recipients of bone marrow transplants had increased SR-AI expression on their alveolar macrophages associated with decreased levels of miR-155 expression when compared to naïve mice. It was also shown that MARCO expression was decreased on macrophages in these mice, but this was not directly linked to miR-155 expression. Instead, it was hypothesized that it was the expression of SR-AI that negatively regulated MARCO expression - though this was not fully tested. Since MARCO is a phagocytic receptor for *S. pneumoniae*, it is possible that deleting the miR-155 gene would affect clearance of pneumococcal colonization from the nasopharynx by reducing the levels of MARCO on nasal macrophages in these mice. This formulates the basis for the hypotheses to be tested in **Chapter 5**.

Chapter 2
Hypothesis & Aims

Hypothesis

I hypothesize that the class A SR MARCO is an integral part of the clearance of the pneumococcus from the murine nasopharynx. MARCO's role will be directly related to the uptake of the bacterium, leading to the production of pro-inflammatory cytokines by macrophages as well as bacterial killing. MARCO's phagocytic capabilities will lead to the production of type I IFNs, decreasing bacterial dissemination into the bloodstream from the nasal passages. miR-155 also plays a role in bacterial clearance through the regulation of MARCO expression on the surface of murine nasal macrophages.

Study Aims

1. Establish a role for MARCO in the clearance of pneumococcal colonization from the murine nasopharynx. Link MARCO expression to cytokine and chemokine production in nasal macrophages.
2. Show that type I IFN is necessary for protecting mice from IPD and that MARCO expression is vital to this IFN production by nasal macrophages.
3. Show that miR-155 positively regulates MARCO expression in the murine nasal passages, increasing clearance of pneumococcal colonization.

Chapter 3
MARCO Is Required for TLR2- and Nod2-Mediated Responses to Streptococcus pneumoniae *and Clearance of Pneumococcal Colonization in the Murine Nasopharynx*

Preface

This chapter consists of a published manuscript from the *Journal of Immunology**. The goal of the manuscript was to establish a role for MARCO in the clearance of pneumococcal colonization from the murine nasopharynx. Previous to this paper's publication, it was known that macrophages, TLR-2, NOD-2, Th17 cells, IL-17, and CCL-2 were all important for this clearance while specific antibodies production were not. In this manuscript, we expanded on this by showing that MARCO is integral for macrophages to recognize and phagocytose the bacteria, leading to the production of many pro-inflammatory mediators and that this process did not require any opsonization of the bacteria.

The importance of this work lies in the fact that MARCO appears to be important for the clearance of many pneumococcal serotypes and does not require the aid of opsonization. These types of strain-independent responses to pneumococci are important to understand so that we can begin to generate serotype-independent prophylactics to bacterial colonization. These therapies would be an improvement over the current pneumococcal vaccines as they would protect against all pneumococcal serotypes, making them more effective while likely producing less economic strain on healthcare systems as well.

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MARCO Is Required for TLR2- and Nod2-Mediated Responses to *Streptococcus pneumoniae* and Clearance of Pneumococcal Colonization in the Murine Nasopharynx

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***Streptococcus pneumoniae* is a common human pathogen that accounts for >1 million deaths every year. Colonization of the nasopharynx by *S. pneumoniae* precedes pulmonary and other invasive diseases and, therefore, is a promising target for intervention. Because the receptors scavenger receptor A (SRA), macrophage receptor with collagenous structure (MARCO), and mannose receptor (MR) have been identified as nonopsonic receptors for *S. pneumoniae* in the lung, we used scavenger receptor knockout mice to study the roles of these receptors in the clearance of *S. pneumoniae* from the nasopharynx. MARCO$^{-/-}$, but not SRA$^{-/-}$ or MR$^{-/-}$, mice had significantly impaired clearance of *S. pneumoniae* from the nasopharynx. In addition to impairment in bacterial clearance, MARCO$^{-/-}$ mice had abrogated cytokine production and cellular recruitment to the nasopharynx following colonization. Furthermore, macrophages from MARCO$^{-/-}$ mice were deficient in cytokine and chemokine production, including type I IFNs, in response to *S. pneumoniae*. MARCO was required for maximal TLR2- and nucleotide-binding oligomerization domain–containing (Nod)2-dependent NF-κB activation and signaling that ultimately resulted in clearance. Thus, MARCO is an important component of anti-*S. pneumoniae* responses in the murine nasopharynx during colonization. *The Journal of Immunology*, 2013, 190: 250–258.**

Streptococcus pneumoniae (the pneumococcus) is one of the most prevalent human pathogens and causes >1 million deaths each year, most of which are young children (1). The bulk of these deaths occurs as the result of pneumococcal pneumonia, in which bacteria spread from their preferred niche, the nasopharynx, to the lungs. Pneumococci are armed with a plethora of colonization factors that allow them to establish a carrier state in the nasopharynx of ∼10% of the adult population at any given time, with higher rates in children <5 y of age (2). Colonization events are sequential, and each lasts days to weeks before clearance. Despite the use of antibiotics and the introduction of polysaccharide-based vaccines, antibiotic resistance and serotype replacement have resulted in continued challenges in the management of this pathogen (3–6).

The initial immune response to pneumococcal colonization is characterized by a brisk, yet ineffective, neutrophil response (7). The resolution of the carrier state appears to require the subsequent recruitment of monocytes from the blood into the nasal interstitial spaces, where they differentiate into macrophages, which are then thought to phagocytose and destroy the bacteria. These monocytes are attracted by the chemokine CCL2, which is produced in response to the recognition of bacterial peptidoglycan by nucleotide-binding oligomerization domain–containing (Nod)2 (8). Signaling through Nod2 also drives the expression of type I IFNs, which contributes to clearance (9). Additionally, the influx of monocytes is affected by TLR2, although the mechanism of this interaction has not been fully elucidated (7). These innate pathways involved in clearance of pneumococcal colonization all require prior recognition of the pneumococcus by resident cells, yet this initial interaction remains elusive.

It also was shown that the pneumococcus, through the expression of its capsular polysaccharide and other mechanisms, is able to avoid opsonization by Abs (10, 11), as well as complement components (12–14). Although it is not known which macrophage receptors recognize the pneumococcus in the low-opsonic environment of the nasopharynx, previous studies showed that mannose receptor (MR) (15), SIGNR1 (16), and the class A scavenger receptors (SRs), known as scavenger receptor A (SRA) (17) and macrophage receptor with collagenous structure (MARCO) (18), are capable of binding to *S. pneumoniae* via nonopsonic mechanisms.

The class A SRs are known for their broad ligand specificity and phagocytic functions (19). They were also shown to directly and indirectly modulate TLR and NLR signaling (20, 21). MARCO is constitutively expressed only on specific subsets of macrophages (22); however, its expression can also be induced on macrophages at sites of inflammation as a result of bacterial infection, thus reinforcing the notion that it is important in immune defense. In contrast, SRA is constitutively expressed on most, if not all, macrophages, as well as other cells, such as endothelial cells. MARCO and

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Abbreviations used in this article: GFP-VSV, GFP-expressing vesicular stomatitis virus; MΦ, BioGel-elicited macrophage; MARCO, macrophage receptor with collagenous structure; MDP, muramyl dipeptide; MOI, multiplicity of infection; MR, mannose receptor; Nod, nucleotide-binding oligomerization domain–containing receptor; SR, scavenger receptor; SRA, scavenger receptor A; URT, upper respiratory tract; WT, wild-type.

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SRA were shown to directly bind and phagocytose *S. pneumoniae* in a murine pneumonia model; however, the bacterial ligands were not identified (23, 24). The role of SRs in colonization is not known and is the focus of this study.

In the current study, we show that MARCO is a key component in the macrophage response to *S. pneumoniae* during colonization. We demonstrate that MARCO is involved in the timely clearance of pneumococcal colonization from the nasopharynx of mice by augmenting the production of proinflammatory cytokines and chemokines. In vitro analyses confirm MARCO's role in the TLR2- and Nod2-signaling pathways that lead to the production of these soluble mediators. To our knowledge, we also show, for the first time, a connection between SR activity and the production of type I IFNs in response to extracellular bacteria.

Materials and Methods

Mice

C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) and The Jackson Laboratory (Bar Harbor, ME) at 6–8 wk of age. Original MARCO$^{-/-}$ and SRA$^{-/-}$ mice were generously provided by the laboratory of Professor Siamon Gordon (University of Oxford, Oxford, U.K.) and bred at the McMaster Central Animal Facility. MR$^{-/-}$ mice were from University of Oxford, C3$^{-/-}$ mice by John Lambris (University of Pennsylvania), and MAC1$^{-/-}$ mice were purchased from The Jackson Laboratory. All animals were used at either 9–11 wk of age or when they reached 20 g in weight, whichever occurred first. Wild-type (WT) mice were sex matched to the knockout groups. All procedures were performed in accordance with the McMaster Animal Research Ethics Board guidelines and Institutional Animal Care and Use Committee protocols at the University of Pennsylvania.

Bacterial strains and culture conditions

S. pneumoniae strain P1121 (a clinical isolate, serotype 23F) (25) was used for all assays and inoculations. TIGR4 (a clinical isolate, serotype 4) was also used for colonization studies of SR knockout mice. Bacteria were propagated in tryptic soy broth (Life Technologies) at 37˚C and 5% CO_2 until cultures reached log phase, OD_{600} between 0.45 and 0.50. In vitro and ex vivo experiments were performed with lysozyme-digested bacteria, because this enzyme is abundant on the mucosa and during macrophage processing. Lysozyme treatment does not lyse the pneumococcus, but it releases peptidoglycan fragments that promote innate-immune signaling (8). Lysozyme-digested pneumococcus was prepared as follows: bacteria were heat-killed by incubation at 65˚C for 10 min, followed by incubation for 18 h in the presence of 0.5 μg/ml recombinant human lysozyme (Cedarlane, Burlington, ON, Canada) at room temperature, with vortexing every few hours.

Murine model of pneumococcal colonization

Pneumococcus was grown to log phase, concentrated 10-fold in PBS, and stored on ice. Unanesthetized mice were inoculated intranasally with 10 μl bacterial suspension containing ~1×10^7 CFU. At the time indicated, mice were sacrificed, the trachea was cannulated, and 200 μl PBS was instilled. Lavage fluid was collected from the nares, serially diluted in PBS, and plated on tryptic soy plates containing 5% sheep's blood and neomycin (5 μg/ml for TIGR4 and 10 μg/ml for P1121). Colonies were counted after overnight incubation at 37˚C and 5% CO_2.

Macrophage culture and stimulation

All macrophages were cultured in a humidified environment at 37˚C with 5% CO_2 in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 mM L-glutamine. For assays involving stimulation of cultured cells, lysozyme-digested P1121 was added in RPMI 1640 containing 1% FCS and L-glutamine. To study recruited or "elicited" macrophages, BioGel elicitation was performed. Mice were injected i.p. with 1 ml 2% (w/v) BioGel P100 45–90-μm-diameter microbeads (Bio-Rad). Peritoneal lavages were performed 5 d later with 10 ml ice-cold PBS. Cells were then washed once with RPMI 1640 and resuspended in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 mM L-glutamine and incubated in 24-well tissue culture plates at a concentration of 5×10^5 cells/well in 1 ml medium. Cells were allowed to adhere for 2 h and then nonadherent cells were removed by washing with warm media, and adherent cells were incubated at 37˚C with 5% CO_2 overnight. The following day, cells were stimulated with 20 μg/ml recombinant murine IFN-γ (PeproTech) for 24 h to upregulate Nod2 expression. Elicited macrophages were then stimulated with lysozyme-digested *S. pneumoniae* at a multiplicity of infection (MOI) of 25, 10 ng/ml LPS (Sigma) plus 5 μg/ml muramyl dipeptide (MDP; Sigma), or media alone for 16 h. Supernatants were collected and either used immediately for cytokine ELISA or stored at −80˚C for later use.

RNA extraction and RT-PCR

Total RNA was extracted from nasal lavages collected with RLT Lysis Buffer (QIAGEN) containing 0.7% 2-ME. An RNA extraction Micro kit (Ambion) was used following the manufacturer's directions. cDNA was synthesized using MMLV reverse transcriptase (Invitrogen), following the manufacturer's directions. Quantitative real-time PCR was performed on cDNA with SYBR green (Promega), following the manufacturer's directions, and results were compared with a GAPDH control gene. The primers used for each gene can be found in Table I.

Immunofluorescent staining

Nasal lavage fluid was applied to ColorFrost Plus Microscope Glass Slides using the Shandon Cytospin 3 cytocentrifuge (Thermo Scientific) at 230 × *g* for 10 min. The cytospin preparations were air-dried briefly before fixing in acetone. Samples were blocked with 10% donkey serum before the addition of primary Ab. Signal was detected with Cy2- or Cy3-conjugated species-specific secondary Ab (Jackson ImmunoResearch, West Grove, PA) incubated at 1:500 dilution in block for 2 h at room temperature. After washing with PBS followed by distilled H_2O, sections were counterstained with DAPI (Molecular Probes, Invitrogen, Carlsbad, CA) diluted 1:10,000 in distilled H_2O. All image analysis was carried out using iVision-Mac (BioVision Technologies, Exton, PA).

For positive control of SR staining, spleens were dissected from naive mice and fresh-frozen in Tissue-Tek OCT embedding medium (Miles) in a Tissue-Tek Cryomold. Five-micrometer-thick sections were cut and stored at −80˚C. Tissue sections were stained as above.

Flow cytometry

Nasal lavage samples were stained with the following fluorescent Abs: Ly6C:FITC, Ly6G:PE, F4/80:allophycocyanin, and CD45:Pacific Blue (eBioscience). Staining was completed by incubating cells with the fluorophore-conjugated Abs after blocking with the 2.4G2 Ab (eBioscience) at 4˚C. Cells were then fixed with 1% paraformaldehyde and assayed with a BD LSRII flow cytometer the following day. Data were gathered using FACSDiva software (BD) and analyzed using FlowJo software (TreeStar).

Cytokine ELISA

ELISAs for TNF-α, IL-1β, CCL2, and IL-6 were performed as per the manufacturer's directions (eBioscience). Plates were read on a Safire plate reader within 20 min of the addition of H_2SO_4.

IFN bioassay

Elicited macrophages were prepared as described above and stimulated with lysozyme-digested P1121 at an MOI of 25 for 24 h. Supernatants were removed from the macrophages, serially diluted, and added to a confluent monolayer of L929 cells overnight at 37˚C and 5% CO_2. IFN-α was used as a concentration standard. The next day, the medium was removed and replaced with 30 μl 1×10^5 PFU/ml a GFP-expressing vesicular stomatitis virus (GFP-VSV) in serum-free media for 24 h, as described (26). If the supernatants contained any type I IFN (i.e., if the macrophages produced IFN in response to the bacterial stimulation) then the virus would not be able to replicate and no fluorescence would be seen. The fluorescence signal given off by GFP was measured the next day using a Typhoon Trio variable mode imager and quantified using ImageQuant software (ImageMaster).

NF-κB luciferase assay

Human TLR2, CD14, TLR9, and NF-κB luciferase plasmids were provided by Dr. Cynthia Leifer (Cornell University, Ithaca, NY), human Nod2 plasmid was provided by Dr. Dana Philpott (University of Toronto, Toronto, ON, Canada), and β-galactosidase plasmid was provided by Dr. Brian Lichty (McMaster University). All plasmids were amplified by *Escherichia coli* DH5-α cells and purified using a HiPure Plasmid Filter Midiprep Kit (Invitrogen).

Low-passage ($n \leq 4$) HEK293T cells were seeded at 5×10^5 cells/well in 3 ml DMEM/well in a six-well plate overnight. HEK293T cells were transfected with NF-κB luciferase (100 ng), β-galactosidase (100 ng), and optimal combinations of human MARCO (300 ng), TLR2 (30 ng), CD14 (30 ng), and Nod2 (50 ng). The total amount of DNA was brought to 1 μg by transfecting empty pcDNA3.1 vector. Transfections were performed

using GeneJuice transfection reagent, as per the manufacturer's instructions (Novagen). At 5 h posttransfection, serum-free DMEM media was replaced with complete DMEM media. Twenty-four hours later, transfected cells were stimulated with lysozyme-digested P1121 (MOI 25) in 3 ml serum-free DMEM media. After 48 h, the lysates were collected using Reporter Lysis Buffer (Agilent) and were analyzed for luciferase (Agilent) and β-galactosidase (Clontech) activity using a luminometer (Turner Biosystems).

Cell association/internalization assay

BioGel-elicited macrophages (MΦs) or HEK293T cells were suspended in 1 ml HBSS at a concentration of 1×10^6 cells/ml. Live P1121 was added at an MOI of 10, and the solution was mixed on a nutating mixer at 37°C for 1 h. The cells were then separated from unbound bacteria by centrifuging at 1500 rpm for 5 min. To measure cell association, cells were washed once in HBSS and then lysed in H_2O. Serial dilutions were performed in H_2O and plated on sheep's blood agar supplemented with 10 μg/ml neomycin. Colonies were counted the next day. To measure uptake directly, extracellular bacteria were removed by adding 25 μg/ml gentamicin for 10 min at 37°C. Cells were then washed with HBSS and lysed in H_2O.

Statistics

Statistical analyses were carried out using the unpaired Student *t* test (GraphPad), except where indicated. Results were considered statistically significant if $p \leq 0.05$.

Results

Clearance of S. pneumoniae *colonization does not require opsonins*

Although Abs have been shown to be dispensable for pneumococcal clearance in the nasopharynx (7, 10), it is not clear whether other opsonins, such as complement, play a role in the clearance of pneumococcal colonization. To address this, mice lacking complement receptor 3 (also known as MAC-1) or complement component 3 (C3) were colonized intranasally with *S. pneumoniae* isolate P1121. The bacterial burden in the nasopharynx was measured in WT, MAC-$1^{-/-}$, and $C3^{-/-}$ mice at 21 d postinoculation (Fig. 1A). The clearance of *S. pneumoniae* was not significantly different among the three genotypes, demonstrating that complement opsonization is not necessary for the clearance of pneumococcal colonization.

MARCO and SRA were shown to be opsonin-independent phagocytic receptors important in innate-immune pneumococcal surveillance (17, 18). MR was also shown to bind to pneumococcal polysaccharides (27). To determine the importance of these receptors in the clearance of P1121 from the nasopharyngeal passage, we inoculated mice with 10^7 CFU intranasally and assessed bacterial numbers at various time points postinoculation. We compared pneumococcal CFU in the nasal lavages of WT, MARCO$^{-/-}$, SRA$^{-/-}$, and MR$^{-/-}$ mice. MARCO$^{-/-}$ mice, but not SRA$^{-/-}$ or MR$^{-/-}$ mice, were significantly impaired in clearing the bacteria beginning at day 14, with an even greater deficit seen at day 21 postinoculation, at which point clearance was completed in WT mice (Fig. 1B, 1C). The role of MARCO in the clearance of pneumococcal colonization was confirmed using TIGR4, an isolate that expresses a different capsular polysaccharide (Fig. 1D), thus demonstrating that the effect of MARCO is likely conserved across pneumococcal serotypes.

RNA transcripts of SIGNR1, an additional nonopsonic receptor that has been implicated in antipneumococcal immune responses (16), were undetectable in colonized nasal lavages (data not shown), effectively ruling it out as an important receptor in our model.

Expression of class A SRs in the nasopharynx

To determine the cell population expressing MARCO in the upper respiratory tract (URT), nasal lavages were analyzed for MARCO transcripts at 30 min, as well as days 3 and 7, when the effector monocytes/macrophages reach a maximum level in the URT. All primers used for mRNA transcript analysis can be found in Table I. CD45 transcript increased at days 3 and 7, correlating with the influx of effector cells (Fig. 2A). However, although MARCO transcript was detected in nasal lavages at all time points, the amount of transcript did not increase (Fig. 2A). This suggests that MARCO is present on resident cells in the nasopharynx but not the recruited effector cells. To further confirm this, we stained cytospin preparations of nasal lavages from colonized mice at the same time points. Although SRs CD68 and MR were both detected on recruited lumenal cells, MARCO was not detected on this population (Fig. 2B).

Cellular recruitment to the URT is hindered in MARCO$^{-/-}$ mice

It was shown that effector cells are recruited to the nasopharynx after pneumococcal colonization and are required for clearance (7). Thus, we examined whether MARCO$^{-/-}$ mice were deficient in

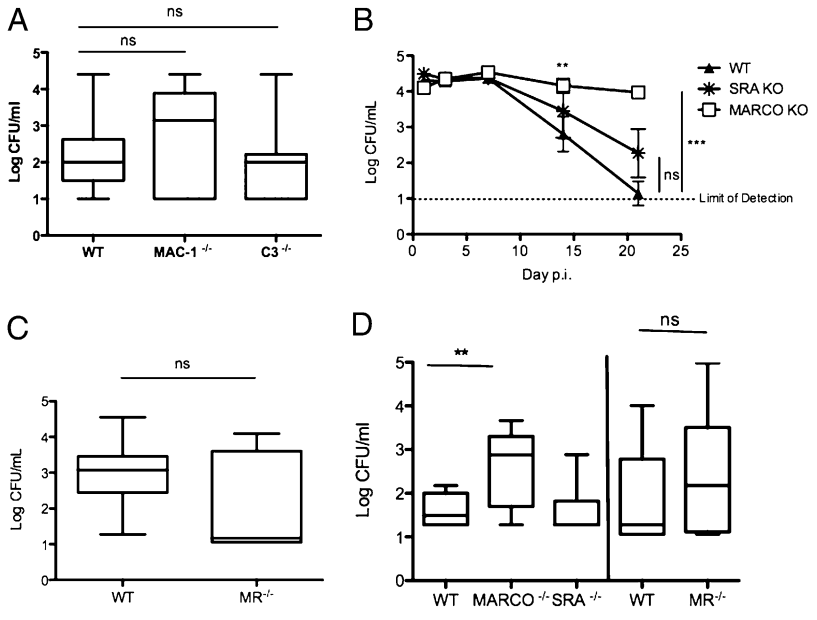

FIGURE 1. Clearance of pneumococcal colonization is complement independent and MARCO dependent. Mice were inoculated intranasally with *S. pneumoniae*, and colonization was assessed at the indicated time points. (**A**) WT, $C3^{-/-}$, and MAC-$1^{-/-}$ mice were colonized with P1121 for 21 d ($n \geq 10$/group), and bacterial burden in nasal lavages was determined. (**B**) Bacterial burden in nasal lavages was determined for WT, MARCO$^{-/-}$, and SRA$^{-/-}$ mice colonized with P1121 for 1, 3, 7, 14, and 21 d postinoculation (p.i.) ($n \geq 6$/group). Data are mean ± SEM. (**C**) WT and MR$^{-/-}$ mice were colonized with P1121 for 21 d ($n \geq 7$/group), and bacterial burden in nasal lavages was determined. (**D**) WT, MARCO$^{-/-}$, SRA$^{-/-}$, and MR$^{-/-}$ mice were colonized with TIGR4 for 21 d ($n \geq 7$/group), and bacterial burden was determined in nasal lavages. Box-and-whisker plots indicate high and low values, median, and interquartile ranges. $^{**}p \leq 0.005$, $^{***}p \leq 0.001$, WT versus MARCO$^{-/-}$ mice, by Mann–Whitney *U* test. ns, Not significant.

Table I. RT-PCR primer sequences

Gene	Sense Primer (5′–3′)	Antisense Primer (5′–3′)
marco	GGCACCAAGGGAGACAAA	TCCCTTCATGCCCATGTC
cd45	CAGAGCATTCCACGGGTATT	GGACCCTGCATCTCCATTTA
ccl2	GTCTGTGCTGACCCCAAGAAG	TGGTTCCGATCCAGGTTTTTA
il6	ATACCACTTCACAAGTCGGAGGC	CTCCAGAAGACCAGAGGAAATTTTC
tnfa	CAAAGGGAGAGTGGTCAGGT	ATTGCACCTCAGGGAAGAGT
il1b	GCCTCGTGCTGTCGGACCCATA	GATCCACACTCTCCAGCTGCAGG
ifnb	GCACTGGGTGGAATGAGACT	AGTGGAGAGCAGTTGAGGACA
gapdh	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA

neutrophil, macrophage, and monocyte recruitment to the nasopharynx during colonization. We used a highly sensitive flow cytometry assay to quantitate the low numbers of cells recruited to the murine nasopharynx during pneumococcal colonization. Neutrophil ($Ly6G^+Ly6C^-$) counts remain high in the WT lavages until day 21, when the majority of bacteria have been cleared, whereas neutrophil levels remain low in $MARCO^{-/-}$ mice. Monocyte ($Ly6C^+Ly6G^-$) recruitment also remains at basal levels throughout colonization in $MARCO^{-/-}$ mice, whereas WT mice show robust levels of monocytes at day 14. Interestingly, there is an influx of recruited macrophages ($F4/80^+$) by day 14 postinoculation in WT mice, whereas this recruitment is delayed in $MARCO^{-/-}$ mice, possibly accounting for the lack of bacterial clearance in these mice at this time point (Fig. 3C). In summary, $MARCO^{-/-}$ mice have delayed recruitment of leukocytes in response to pneumococcal colonization.

MARCO enhances S. pneumoniae*–induced chemokine and cytokine responses*

During the course of pneumococcal colonization, macrophage-mediated detection of bacteria or bacterial components drives an inflammatory response that is required for clearance. It was shown that class A SRs are able to modulate cytokine production (20). To determine whether MARCO contributes to the production of inflammatory cytokines, we examined the expression of TNF-α, IL-1β, and IL-6 mRNA in the nasopharynx of WT and $MARCO^{-/-}$ mice during the course of colonization (Fig. 4A), as well as cytokine production resulting from stimulation of MΦs with *S. pneumoniae* ex vivo (Fig. 4B). $MARCO^{-/-}$ mice colonized with P1121 exhibited significantly delayed TNF-α, IL-6, and IL-1β mRNA transcription compared with WT mice throughout colonization, with the largest differences seen at day 7. Correspondingly, MΦs from $MARCO^{-/-}$ mice produced significantly less of all three cytokines compared with WT MΦs when stimulated with bacteria ex vivo. In fact, in most cases, MARCO-deficient macrophages did not produce any cytokines when stimulated with *S. pneumoniae* compared with stimulation with media alone.

The macrophage-chemotactic protein CCL2 (also known as MCP-1) was shown to be of primary importance for the recruitment of monocytes/macrophages to the nasopharynx during *S. pneumoniae* colonization (8). We investigated CCL2 expression at the RNA level in nasal lavages during colonization of WT and $MARCO^{-/-}$ mice (Fig. 4A). $MARCO^{-/-}$ mice colonized with P1121 had significantly delayed CCL2 transcription. At days 1 and 3, there were no significant differences in CCL2 transcription; however, by day 7 there was a significant reduction in CCL2 transcription in the $MARCO^{-/-}$ mice compared with WT mice. When MΦs from $MARCO^{-/-}$ mice were stimulated ex vivo with

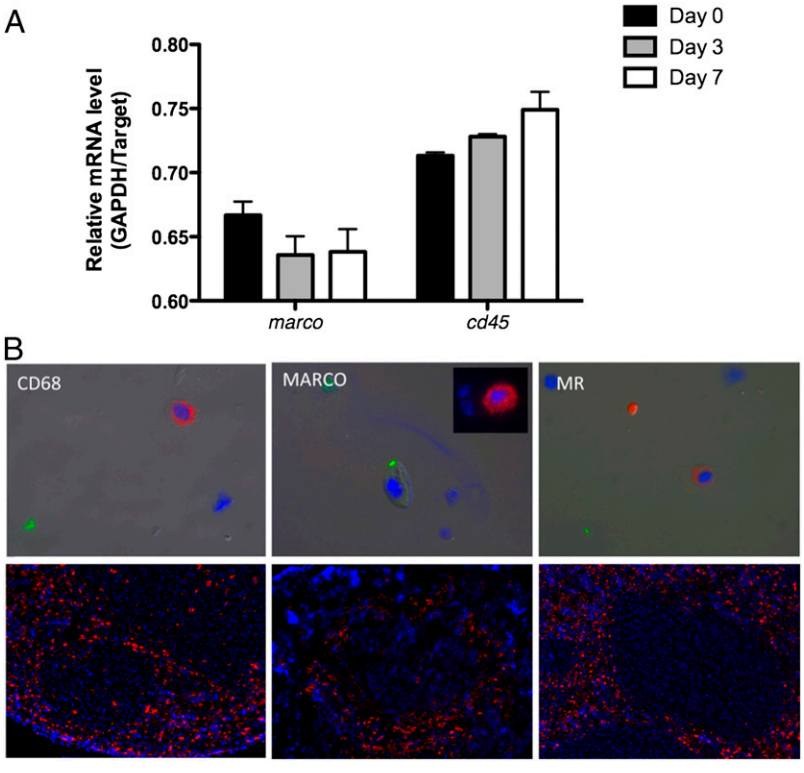

FIGURE 2. MARCO is not expressed on the effector cells recruited to the nasopharynx. (**A**) Quantitative RT-PCR on RNA lysis buffer nasal lavages at 30 min, 3 d, and 7 d postinoculation with P1121. Bars represent relative mRNA levels (GAPDH/Target) ($n \geq 3$). (**B**) Immunofluorescent staining of cytospin preparations from nasal lavages (*upper panels*). Representative images from day 3 (original magnification ×400). *Inset* in MARCO panel demonstrates positive staining in alveolar macrophages (original magnification ×400). Fresh-frozen spleen sections stained as positive controls for SR Abs (*lower panels*, original magnification ×100).

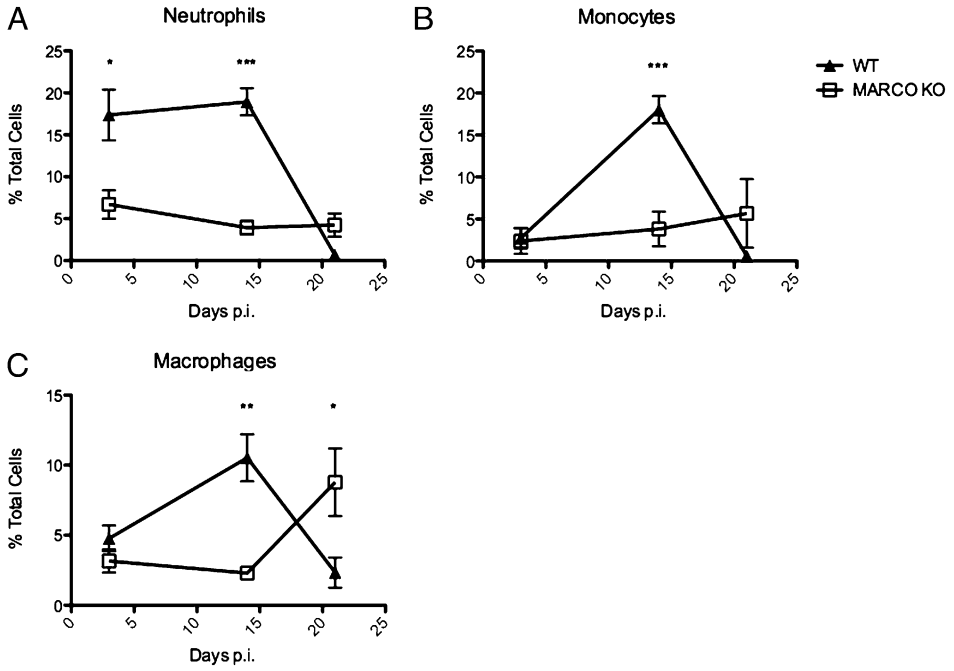

FIGURE 3. Recruitment of leukocytes to the nasopharynx is impaired in MARCO$^{-/-}$ mice. WT and MARCO$^{-/-}$ mice were colonized with *S. pneumoniae* for 3, 14, and 21 d postinoculation (p.i.). Nasal lavages were stained and analyzed for cell surface markers by flow cytometry. These markers were used to determine the number of neutrophils (Ly6G^{+}Ly6C^{-}) (**A**), monocytes (Ly6C^{+}Ly6G^{-}) (**B**), and macrophages (F4/80^{+}) (**C**) in each population ($n \geq 5$/group). Data are mean percentage of total cells ± SEM. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.001$.

S. pneumoniae, the level of CCL2 production did not exceed background levels; however, their WT counterparts produced a robust CCL2 response (Fig. 4B). This reduction in CCL2 production by macrophages was consistent with the diminished macrophage influx seen in MARCO$^{-/-}$ mice.

Together, these results demonstrate that MARCO-mediated recognition drives the inflammatory response during pneumococcal colonization.

MARCO modulates type I IFN production in macrophages

We recently showed that pneumococcal colonization of the nasopharynx leads to an increase in type I IFN production (9). To test whether MARCO was involved in type I IFN production, we first analyzed nasal lavages from WT and MARCO$^{-/-}$ mice for IFN-β mRNA content. MARCO$^{-/-}$ mice had significantly less IFN-β mRNA than did their WT counterparts at days 1, 3, and 7 postinoculation (Fig. 5A). We then stimulated MΦs with *S. pneumoniae* for 24 h, after which the cell supernatants were used in a standard bioassay to measure type I IFN production. Supernatants from WT and MARCO$^{-/-}$ MΦs stimulated with P1121 were transferred to L929 cells that were subsequently infected with a GFP-VSV. Cell supernatants from WT MΦs treated with *S. pneumoniae* protected L929 cells from GFP-VSV infection, indicating pneumococcal IFN stimulation, as measured by low levels of viral GFP fluorescence. Conversely, supernatants from MARCO$^{-/-}$ MΦs yielded less protection from viral infection, indicating that they produced significantly less type I IFN (Fig. 5B). Thus, MARCO has a role in the production of type I IFNs in response to pneumococcal stimulation.

Type I IFN signaling was shown to require uptake of the bacteria and subsequent intracellular signaling (28). To determine whether MARCO's role in type I IFN production was related to its capacity to bind and internalize the bacteria, we performed a bacterial cell–association assay with WT and MARCO$^{-/-}$ MΦs (Fig. 5C). Upon stimulation with live P1121 for 1 h, bacterial association was reduced by ~50% in MARCO-deficient MΦs

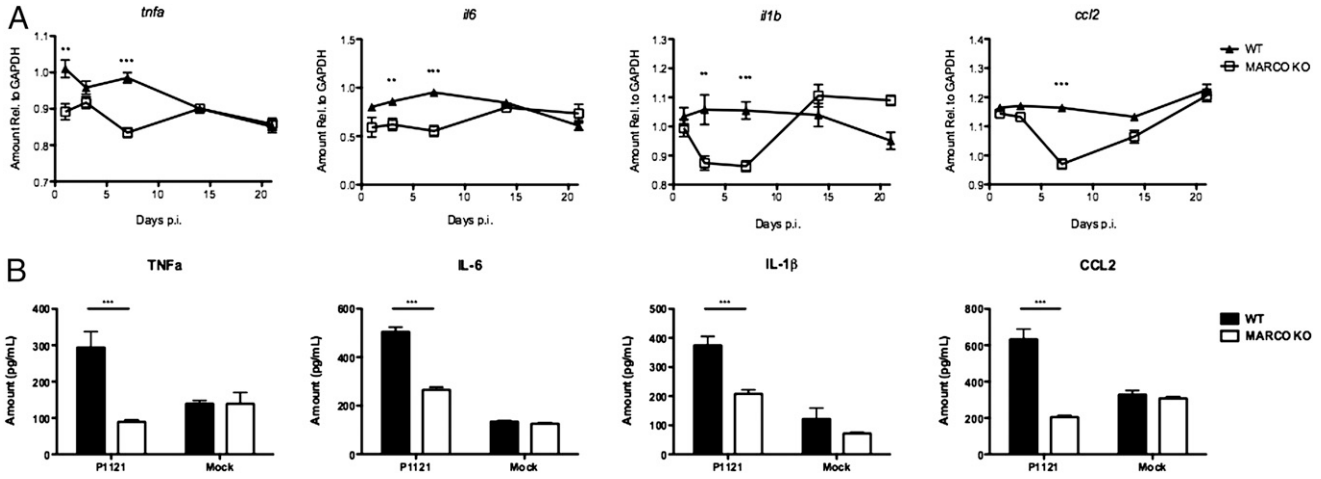

FIGURE 4. MARCO enhances the production of cytokines and chemokines. (**A**) WT and MARCO$^{-/-}$ mice were inoculated intranasally with *S. pneumoniae* and sacrificed at days 1, 3, 7, 14, and 21 postinoculation (p.i.). RNA was isolated by intratracheal nasal lavage. cDNA was analyzed by semiquantitative RT-PCR ($n \geq 3$/time point). (**B**) MΦs were isolated from WT and MARCO$^{-/-}$ mice and stimulated with lysozyme-digested *S. pneumoniae* preparations and controls ex vivo. Cytokine production was measured by ELISA ($n \geq 6$/group). Data are mean ± SEM. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.001$.

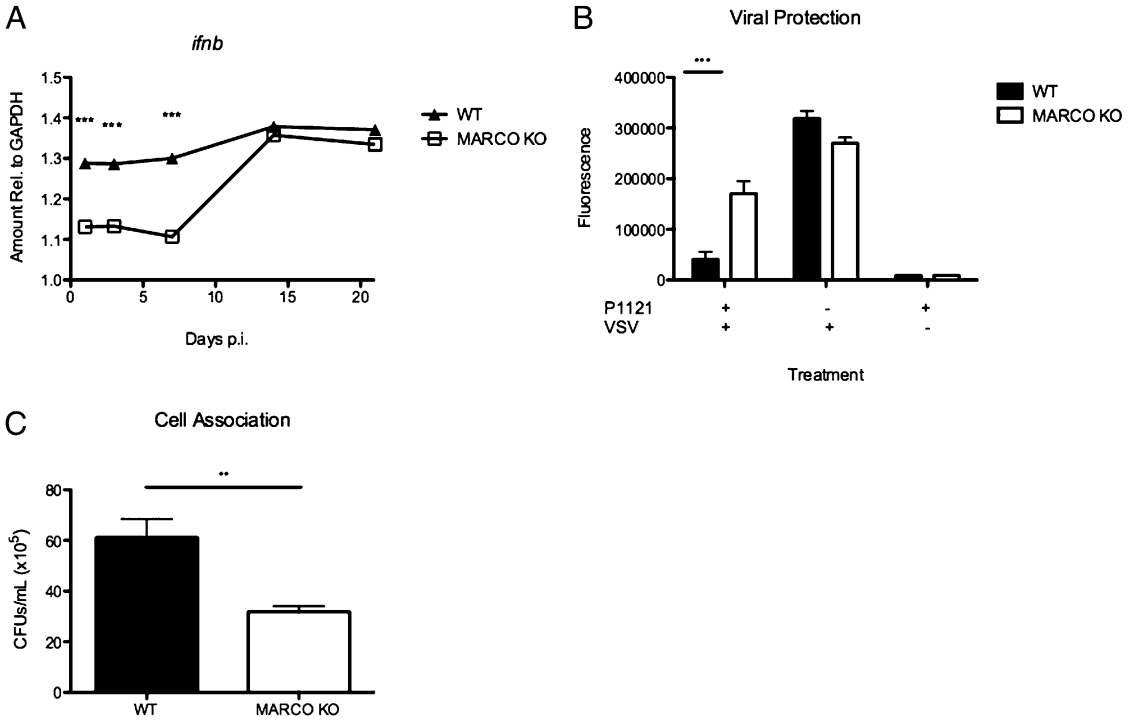

FIGURE 5. MARCO modulates the production of type I IFNs. (**A**) IFN-β mRNA from nasal lavages of colonized mice. RNA from WT and MARCO$^{-/-}$ mice was examined by RT-PCR. (**B**) MΦs from WT and MARCO$^{-/-}$ mice were isolated and stimulated with lysozyme-digested *S. pneumoniae*. Resulting supernatants were then added to L929 cells in culture. VSV-GFP was used to infect L929 cells, and cell infection was measured by GFP fluorescence. (**C**) Bacterial association with MΦs from WT and MARCO$^{-/-}$ mice was measured by cellular lysis after 1 h of stimulation. GFP fluorescence data, indicating viral infection, are shown as mean fluorescence units across replicates ($n = 3$) ± SEM. $^{**}p \leq 0.005$, $^{***}p \leq 0.001$.

compared with WT MΦs, providing us with a possible mechanism for MARCO's role in type I IFN production.

MARCO contributes to TLR- and NLR-signaling pathways

Although MARCO itself has no known signaling capacity, it was shown to enhance TLR signaling in response to certain bacteria or their components (29). Therefore, we hypothesized that MARCO may be required for bacterial recognition by pattern recognition receptors, such as TLR2 and Nod2, to enhance *S. pneumoniae*–specific responses. We used an NF-κB luciferase assay to discern MARCO's role in the signaling capacities of these receptors. Cells transfected with MARCO and Nod2 demonstrated significantly more NF-κB activation upon stimulation with P1121 than did cells transfected with Nod2 alone (Fig. 6A). The same was true for cells transfected with MARCO, TLR2, and CD14 versus those transfected with just TLR2 and CD14 (Fig. 6B). Interestingly, cells transfected with TLR2, CD14, and SRA showed a decrease in NF-κB activation compared with cells transfected with TLR2 and CD14 alone. Cells transfected with MARCO or CD14 alone did not show any NF-κB activation, nor did MARCO enhance NF-κB activation upon stimulation with the TLR2 ligand Pam3CSK4 or the Nod2 ligand MDP (data not shown). In addition, when this assay was performed using plasmids expressing TLR4 or TLR9, MARCO had no effect on NF-κB activation (data not shown).

To determine whether the forced expression of MARCO by the HEK293T cells led to an increased ability of the cells to bind and internalize the bacteria, we performed a bacterial cell–association assay with HEK293T cells transfected with a MARCO-expressing plasmid or an empty vector. When these cells were incubated with live P1121 for 1 h, there was no significant difference in total cell association (i.e., binding and uptake) of the bacteria (Fig. 6C). However, when all extracellular bacteria were eliminated by the addition of gentamicin, the MARCO-expressing cells were better able to internalize the bacteria (Fig. 6D), although the total bacterial numbers were quite low for both sets of cells. Together, these results demonstrate that MARCO enhances some, but not all, TLR and NLR responses to pneumococci, which may be due to its phagocytic capabilities.

Discussion

Because nasopharyngeal colonization precedes pneumococcal disease, it is an attractive therapeutic target; thus, it is important to understand host defense at this site, which requires interactions between the effector cells and the bacteria. It was reported that macrophages recruited to the nasal mucosa are important effector cells in the clearance of colonization and that they are able to recognize the bacteria without the aid of opsonizing Abs (10). In this study, we showed that clearance of pneumococcal colonization does not require complement C3 or its cognate receptor, supporting the idea that macrophages are able to act without the aid of opsonins (Fig. 1A). Although this had been shown in the lung, we are now able to extend the role of nonopsonic receptors in pneumococcal clearance from the nasopharynx.

MARCO, MR, and SRA were shown to be important for clearance of pneumococci in the lungs and the CNS (15, 17, 18, 27). This led us to investigate their role in clearance from the nasopharynx. Interestingly, the SRs appear to play distinct roles in upper versus lower respiratory tract clearance. In the lungs, MARCO and SRA play redundant roles in the recognition, uptake, and subsequent clearance of pneumococci. We can only hypothesize about the importance of this redundancy, although it is likely rooted in the need to overcome the abundance of virulence factors expressed by the pneumococcus in the environment of the lung. Both MARCO and SRA, which are constitutively expressed on alveolar macrophages, were shown to directly recognize the bacteria and trigger their engulfment by these cells (17, 23). MR

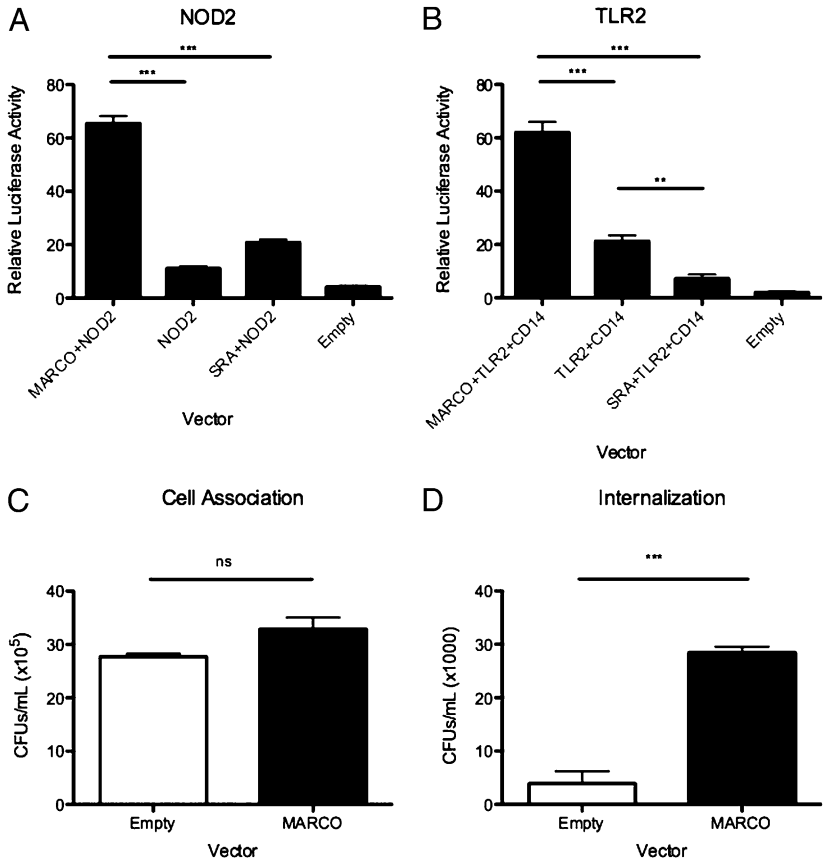

FIGURE 6. MARCO affects Nod2 and TLR2 responses to the pneumococcus. HEK293T cells were cotransfected with various combinations of plasmids expressing Nod2, SRA, and MARCO or empty vector (**A**) or TLR2, CD14, SRA, and MARCO or empty vector (**B**). 24 h posttransfection, cells were infected with lysozyme-digested *S. pneumoniae* at an MOI of 25. Twenty-four hours postinfection, luciferase activity was measured ($n \geq 6$/group). This assay was normalized for transfection efficiency by dividing the luciferase activity by the β-galactosidase activity. Average of three independent experiments ± SEM. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, one-way ANOVA with the Bonferroni posttest. Bacterial association with HEK293T cells transfected with MARCO plasmid or empty vector was measured by cell lysis before (**C**) or after (**D**) the addition of gentamicin to kill extracellular bacteria. $^{***}p \leq 0.001$. ns, Not significant.

was also shown to directly recognize pneumococci (27). Conversely, despite MARCO, MR, and SRA (data not shown) being expressed in the nasopharynx, only MARCO enhances clearance of pneumococcal colonization from the nasopharynx (Fig. 1). Also, although MARCO is required for efficient clearance of nasal colonization, we did not find it on the recruited effector cells in WT mice. This leads us to believe that MARCO's role may be linked to resident cells in the nasopharyngeal mucosa, which function in immune surveillance (Fig. 2). These cells likely act as indirect mediators of the immune response to *S. pneumoniae* by contributing to the complex cytokine and chemokine milieu at the site of colonization. The receptor(s) on the effector cells responsible for recognizing the pneumococcus remains unknown. These differences in the role of SRs could explain why pneumococci are able to asymptomatically colonize the nasopharynx but induce a violent inflammatory state once they gain access to the lungs.

The chemokine CCL2 was shown to be vital to the recruitment of monocytes/macrophages to the nasopharynx during pneumococcal colonization in a TLR2- and Nod2-dependent manner (8). We showed a significant deficiency in the transcription of CCL2 mRNA in the nasopharynx of MARCO$^{-/-}$ mice at early time points in colonization compared with WT mice (Fig. 4A). We also showed that CCL2 production by macrophages from MARCO$^{-/-}$ mice in response to pneumococcal stimulation is severely impaired (Fig. 4B). This defect in CCL2 production is likely responsible for the impaired recruitment of myeloid cells, and especially macrophages, to the nasopharynx throughout colonization (Fig. 3). The increased recruitment of macrophages to the nasopharynx in WT mice correlated with decreased bacterial load, which began between days 7 and 14 postinoculation (Fig. 1B). Neutrophil recruitment was also impaired in MARCO$^{-/-}$ mice; however, prior studies showed that, although these cells are robustly recruited early during colonization, they are not sufficient to clear the pneumococcus (7). It is important to note that the macrophages recruited to the nasopharynx were CD11c^{-}MHCII^{-} and CD11b^{hi} (data not shown) and, therefore, were more similar to recently recruited monocytes in the process of differentiation to macrophages than they were to alveolar macrophages.

A consequence of pneumococcal activation of TLR2 and Nod2 is a robust proinflammatory response. In accordance with this, we showed that MARCO is important in the pathway leading to Nod2- and TLR2-dependent NF-κB activation, which leads to the production of proinflammatory cytokines and chemokines. Nasal lavages from MARCO$^{-/-}$ mice had significantly lower levels of TNF-α, IL-1β, and IL-6 mRNA at day 7 postinoculation compared with WT mice (Fig. 4A). This time point coincides with the beginning of bacterial clearance in WT mice (Fig. 1B). Also, MΦs from MARCO$^{-/-}$ mice were unable to produce these proteins following stimulation with pneumococci (Fig. 4B). This is consistent with other studies demonstrating MARCO's contribution to the proinflammatory response by macrophages when stimulated with other pathogenic organisms (18, 23, 29, 30). Although it is unclear whether this MARCO-dependent production of proinflammatory cytokines is a TLR2- or a Nod2-specific phenomenon, we have provided evidence that MARCO enhances both TLR2- and Nod2-mediated NF-κB activation. Both of these receptors were shown to be important in pneumococcal clearance in previous studies (8, 31). We hypothesize that either TLR2 or Nod2, or both, is the primary signaling receptor involved in the production of TNF-α, IL-1β, and IL-6 and that MARCO is responsible for enhancing this response during colonization. Interestingly, we showed that MARCO is also involved in the production of type I IFNs by MΦs. Two studies showed a protective effect for IFN-β in pneumococcal disease (32, 33), whereas another showed the opposite in the setting of concurrent influenza A infection (9). Thus, the production of type I IFNs during pneumococcal colonization

has profound effects on the disease outcome and is linked to MARCO-mediated signaling.

The mechanism by which MARCO enhances TLR2 and Nod2 signaling has yet to be elucidated. MARCO appears to be essential for, and possibly upstream of, Nod2 and TLR2 signaling in response to the pneumococcus but not to monomeric ligands, such as MDP and Pam_3CSK_4 (data not shown). Likewise, many studies showed that Nod2- and some TLR2-signaling pathways require internalization of ligands (34, 35). Our data showing an increased capacity of MARCO-expressing cells to internalize the bacteria (Figs. 5C, 6D) provide evidence that MARCO can increase signaling by intracellular receptors, such as Nod2, by facilitating the transition of the bacteria into the cell. This is especially evident in the NF-κB luciferase assays in which HEK293T cells not expressing MARCO were almost completely unable to internalize live pneumococci. However, because overall binding of the bacteria to HEK293T cells is not significantly affected by the expression of MARCO, we do not believe that MARCO's role in TLR2 signaling is confined to its binding and uptake capacities. Also consistent with the hypothesis that MARCO lies upstream of TLR2 and Nod2, the degree to which the bacteria were able to persist in $MARCO^{-/-}$ mice was greater than shown in previous studies on $Nod2^{-/-}$ (8) and $TLR2^{-/-}$ (10) mice. Previous studies, as well as our work, showed an inhibitory effect of SRA on TLR2 signaling (Fig. 6) (18, 23, 29, 30). A recent report presented a mechanism for TLR4 inhibition by SRA, which involves direct contact between SRA and the signaling machinery of TLR4 (21). It is possible that a similar mechanism occurs with MARCO, where it can directly bind the TLR2-signaling machinery but, in this case, to enhance signaling.

The production of type I IFNs in response to extracellular bacteria is also reliant on the ability of the cell to internalize bacterial ligands via endocytosis (28). Therefore, it is probable that MARCO's role in type I IFN production is linked to its ability to internalize the bacteria, as shown in Fig. 5C. This does not preclude roles for other molecules formerly established in other laboratories. For example, it is possible that the uptake of live bacteria into a cell could lead to pneumolysin-dependent rupture of endosomes, leading to bacterial ligands reaching the cytoplasm to be sensed by cytosolic or transmembrane receptors (as proposed in Refs. 32, 36).

In summary, we showed that MARCO is vital to the clearance of *S. pneumoniae* colonization from the murine nasopharynx. This is due to its role in nonopsonic recognition of the bacteria, which leads to increased Nod2- and TLR2-dependent chemokine and cytokine production and, ultimately, the recruitment of effector monocytes/macrophages. To the best of our knowledge, this is the first demonstration of MARCO-mediated collaboration with pattern recognition receptor signaling contributing to the clearance of the pneumococcus. Our hope is that targeting Ags to the MARCO-mediated response will provide us with novel, serotype-independent vaccination strategies against the pneumococcus.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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Chapter 4
MARCO-dependent type I IFN production by macrophages protects mice from invasive pneumococcal disease

Preface

This chapter consists of a manuscript that is currently being prepared for submission to the *Journal of Immunology*. This study delves into the role of MARCO in preventing the transition from pneumococcal colonization to active disease. The current body of literature on pneumococcal disease either focuses on the establishment and subsequent clearance of colonization *or* the immune response to an acute infection through directly introducing bacteria into the lungs, blood, or peritoneal cavity. However, there is very little known about the mechanisms by which pneumococci escape the nasal passages and enter the lungs or blood. This study shows that type I IFN production in the nasal passages is important for restricting pneumococci to the nasal passages and that this IFN production is dependent on MARCO expression. MARCO's ability to phagocytose the bacteria leads to macrophage-driven type I IFN production and, subsequently, the upregulation of cell junction proteins in the nasal epithelium.

This work shows another role for MARCO in protecting mice from pneumococcal disease while also highlighting an emerging role for the type I IFNs in anti-bacterial immune responses. These cytokines, once thought to be purely anti-viral in mechanism, are clearly important for protection against extracellular bacteria as well. This work is an important step to a better understanding of type I IFN's role in bacterial colonization - a research question with vast translational potential to the medical field.

MARCO-dependent type I IFN production by macrophages protects mice from invasive pneumococcal disease

The role of the scavenger receptor MARCO in the production of type I interferons by macrophages in the nose and how this protects mice from lung and blood infections.

Running Title: MARCO protects mice from pneumococcal disease

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Abstract

Streptococcus pneumoniae (the pneumococcus) is a common human pathogen that periodically colonizes the nasal passages of humans. The bacterium can cause fatal disease when it escapes this niche and moves to the lungs, blood, or central nervous system. Control of pneumococcal colonization is dependent on macrophages expressing the class A scavenger receptor MARCO (macrophage receptor with collagenous structure). However, little is known about the role of macrophages in preventing the transition from colonization to invasive pneumococcal disease (IPD). In this study, we colonized mice with an invasive strain of the pneumococcus and measured invasiveness in the lungs and blood. $MARCO^{-/-}$ mice were more susceptible to bacterial invasiveness, corresponding with decreased induction of type I interferon (IFN) and cellular junctions in the nasopharynx. We also show that macrophages from these mice require MARCO, TLR2, and bacterial uptake in order to produce type I IFN. The IPD phenotype in $MARCO^{-/-}$ mice could be rescued by pre-treating the mice with poly(I:C), inducing a type I IFN response in a MARCO-independent manner. In conclusion, MARCO expression is vital to the production of type I IFN, which act on the nasal epithelium to decrease permissiveness to bacterial invasion of the lungs and blood.

Introduction

Streptococcus pneumoniae (also known as the pneumococcus) is a common human pathogen that is a major contributor to worldwide morbidity and mortality, especially in young children and the elderly (1). While the bulk of pneumococcus-related deaths occur in the developing world, *S. pneumoniae* remains one of the largest contributors to health-adjusted life years lost in the developed world as well (2). The bacterium is a periodic asymptomatic colonizer of the nasal passages, where it must gain a foothold before it is able to move further into the body's tissues and cause disease (3). This spread, termed invasive pneumococcal disease (IPD), is most commonly to the lungs or middle ear, causing pneumonia or otitis media, respectively. However, the bacteria can also spread to the blood or meninges, causing life-threatening disease. IPD is one of the most prevalent and deadly infectious diseases in the world, making it a high priority for prophylactic treatment strategies (4).

The fact that nasal colonization must be established before the bacteria can spread provides us with a defined window in which prophylaxis is required to work. However, protection against the pneumococcus is difficult due to its many virulence factors and the fact that the *S. pneumoniae* species is made up of greater than 90 serotypes, each antigenically unique (5). Natural immunity to the pneumococcus improves over the course of childhood in a seemingly serotype-independent manner, alluding to immune responses that can protect against all serotypes (6). Consequently, recent studies on anti-pneumococcal responses have focussed on innate immunity. Investigating these responses could lead to improved prophylactic measures against the bacteria and, therefore, a significant decrease in global mortality.

A successful primary immune response to pneumococcal colonization is predicated on a complex cocktail of cells and proteins working together. The response begins when the

bacterium is recognized by resident nasal macrophages, which establish an inflammatory state by producing IL-1β, IL-6, TNF, and the chemokines CXCL-1, and CCL-2 that recruit neutrophils and monocytes respectively (7-9). While the neutrophil response is unable to control the colonization, monocytes differentiating into macrophages are capable of limiting the colonization to the nasopharynx while a T cell response is mounted (10). IL-17 production by Th17 cells further recruits macrophages that eventually clear the colonizing bacteria without them disseminating to further tissues. Our previous work has shown that many of these early events are dependent on resident macrophages expressing class A scavenger receptors at the time of colonization (11).

The class A scavenger receptors (SRs) are a group of phagocytic receptors known for their ability to bind polyanionic ligands (12). Our previous work has shown that macrophage receptor with collagenous structure (MARCO, also known as SR-A6), but not it's close relative Scavenger Receptor A-1 (SRA, also known as SR-A1.1), is vital to clearance of primary colonization with various strains of the pneumococcus and is capable of performing its actions without the need for opsonizing specific antibodies or complement molecules [see (11)]. MARCO is necessary for appropriate cytokine and chemokine production, with macrophage recruitment to the nasopharynx lacking in MARCO-deficient mice. However, it remains unknown how MARCO expression in the nasal passages can affect the ability of *S. pneumoniae* to spread to other tissues and cause IPD.

While type I interferon (IFN) is predominantly associated with viral infections, recent studies have shown that it can be equally important in responses to extracellular bacteria (13-15). In fact, type I IFN is important in regulating pneumococcal colonization (16). However, in the context of post-influenza bacterial pneumonia, type I IFN has been shown to be detrimental to

CCL-2-mediated macrophage recruitment (17). What is not known is how much of this IFN is produced by macrophages in the nasopharynx, and what role MARCO may play in this production. We have previously shown that MARCO$^{-/-}$ mice have less type I IFN production in their nasopharynges upon pneumococcal colonization and that macrophages from these mice produce less type I IFN upon *ex vivo* stimulation with whole bacteria (11). In this study, we have set out to understand if it is MARCO's phagocytic capacity that allows for the triggering of intravesicular receptors such as TLR-2, leading to the activation of interferon regulatory factors (IRFs) and subsequent type I IFN production. Perhaps more importantly, we have been able to uncover the importance for this type I IFN production in the nasopharynges of colonized mice. These cytokines are vital to informing the epithelium's reaction to the bacterium, causing an upregulation in tight junction formation, blocking bacterial migration to the lungs and blood soon after inoculation.

Materials & Methods

Mice

C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbour, ME) at 6-8 weeks of age or else bred in-house. Original $MARCO^{-/-}$ and $SRA^{-/-}$ mice were generously provided by the laboratory of Professor Siamon Gordon (University of Oxford, UK) and bred at the McMaster Central Animal Facility. All animals were used at either 9-11 weeks of age or when they reached 20g in weight, whichever occurred first. WT mice were sexed to match the knock out groups. All procedures performed were done so in accordance with McMaster Animal Research Ethics Board guidelines.

Bacterial Strains and Culture Conditions

S. pneumoniae strain P1547 (a clinical isolate, serotype 6A) was used for all assays and inoculations. Bacteria were propagated in tryptic soy broth (Gibco) at 37˚C and 5% CO_2 until cultures reached log phase, OD_{600} between 0.45 and 0.50. *In vitro* and *ex vivo* experiments were performed with either live or heat-killed then lysozyme-digested bacteria as this enzyme is abundant on the mucosa and during macrophage processing. Lysozyme treatment does not lyse the pneumococcus but releases peptidoglycan fragments that promote innate immune signaling (9). Lysozyme-digested pneumococcus was prepared as in (9). Briefly, bacteria were heat killed by incubation at 65˚C for 10 min, followed by incubation for 18h in the presence of 0.5µg/mL recombinant human lysozyme (Cedarlane, Burlington, ON, Canada) at room temperature, nutating throughout.

Murine Model of Pneumococcal Colonization

Pneumococci were grown to log phase, concentrated 10 fold in PBS and stored on ice. Unanaesthetized mice were inoculated intranasally with 10 µL of bacterial suspension containing ~$1x10^7$ CFUs. At the time indicated, mice were sacrificed, the trachea was cannulated and 300 µL PBS was instilled. Lavage fluid was collected from the nares and serially diluted in PBS and plated on tryptic soy plates containing 5% sheep's blood and neomycin (10 µg/mL). Colonies were counted after overnight incubation at 37˚C and 5% CO_2.

Macrophage Culture and Stimulation

All macrophages were cultured in a humidified environment at 37˚C with 5% CO_2 in RPMI 1640 supplemented with 10% FCS, penicillin (100U/mL), streptomycin (100 µg/mL), and 10 mM L-glutamine. For assays involving stimulation of cultured cells, lysozyme-digested P1547 was added in RPMI 1640 containing L-glutamine. In order to study primary murine macrophage responses, BioGel elicitation was performed. Mice were injected intraperitoneally with 1 mL of 2% (w/v) BioGel P100 45-90 µm diameter microbeads (Bio-Rad). Peritoneal lavages were performed 5 days later with 10 mL of ice-cold PBS supplemented with 10% FCS. Cells were then washed once with RPMI 1640 and resuspended in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100 µg/mL), and 10 mM L-glutamine and incubated in 6-well tissue culture plates at a concentration of $1x10^6$ cells per well in 3 mL of media. Cells were allowed to adhere for 2 hours then non-adherent cells were removed by washing with warm PBS and adherent cells were incubated at 37˚C with 5% CO_2 overnight in complete media. For inhibitor studies, inhibitors were added to cultures 30 minutes prior to

bacterial stimulation. Elicited macrophages were then stimulated with either lysozyme-digested *S. pneumoniae* or live bacteria at an MOI of 25 in serum and antibiotic-free RPMI. For live bacterial stimulations, 50μg/mL of gentamycin was added to cultures after two hours (Assuming the stimulation time was longer than this) to prevent bacterial replication and cell death. Supernatants were collected at the appropriate time points and either used immediately for cytokine ELISA or stored at -80˚C for later use.

Detroit-562 Cell Culture

The Detroit-562 (ATCC CCL-138) cell line was purchased from the ATCC. Cells were cultured in DMEM supplemented with 10% FCS, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10 mM L-glutamine in 12-well tissue culture plates. Cells were allowed to reach confluence, as determined by light microscope viewing. Before RNA analysis or pneumococcal stimulation, cells were washed twice with warm PBS, then antibiotic-free media was added. Cells were treated with carrier-free IFN-β (BioLegend) at 1000U/mL or a PBS control. After 20h, cells were either lysed for RNA analysis, or were used for the bacterial association assay (see below).

RNA Extraction & RT-PCR

Total RNA was extracted from nasal lavages collected with RLT Lysis Buffer (Qiagen) containing 0.7% 2-mercaptoethanol. An RNA extraction Micro kit (Ambion) was used following the manufacturer's directions. Complementary DNA was synthesized using MMLV reverse transcriptase (Invitrogen) following manufacturer's directions. Quantitative real time PCR was performed on cDNA with SYBR green (Promega) following manufacturer's directions and results were compared to a GAPDH control gene. For cell cultures, RNA lysis buffer (Ambion) was added to each well then plates

were frozen at -80˚C to achieve complete lysis, then allowed to thaw. RNA was then extracted using the extraction kit mentioned above. The primers used for each gene can be found in Table 1.

Bacterial Association Assay

Detroit-562 cells were cultured as above. After IFN-β or PBS treatment, $1x10^6$ live P1547 bacteria were added to each well. Plates were centrifuged at 2000rpm for 1 minute to allow bacteria to settle onto the monolayers. After 30 minutes, media was removed and cells were washed vigorously with warm PBS, removing any unbound bacteria. Cells were then lysed by repetitive pipetting with 500μL of water. To measure pneumococcal CFUs, lysates were serially diluted in PBS then plated on sheep's blood agar, as above.

Cytokine ELISA

ELISAs for IL-6 were performed as per manufacturer's directions (eBioscience). Plates were read using a SpectraMax i3 plate reader (Molecular Devices, California) within 20 minutes of the addition of H_2SO_4.

Interferon Bioassay

Elicited macrophages were prepared as described above and were stimulated with lysozyme-digested P1547 at an MOI of 25 for 24h. For inhibitor studies, inhibitors were added 30 minutes prior to stimulation. Supernatants were removed from the macrophages, serially diluted, and added to a confluent monolayer of L929 cells overnight at 37˚C and 5% CO_2. The next day the media was removed and replaced with 30 μL of $1x10^5$ pfu/mL of a GFP-expressing vesicular stomatitis virus in serum-free media for 24h as in (18). If the supernatants contained any type I IFN (ie. if the macrophages produced IFN in response to the bacterial stimulation) then the virus would

not be able to replicate and no fluorescence would be seen. The fluorescence signal given off by GFP was measured the next day using a Typhoon Trio variable mode imager and quantified using ImageQuant software (ImageMaster).

Bacterial Internalization Assay

BioGel-elicited macrophages were suspended in 1mL of Hank's Balanced Salt Solution (HBSS) at a concentration of $1x10^6$ cells/mL. Live P1547 was added at an MOI of 10 and the solution was mixed on a nutating mixer at 37˚C for 1h. The cells were then separated from unbound bacteria by centrifuging at 1500rpm for 5min. In order to measure uptake directly, extracellular bacteria were removed by adding 50µg/mL gentamycin for 10min at 37˚C. Cells were then washed with HBSS three times and lysed in H_2O. Serial dilutions were performed in HBSS and plated on sheep's blood agar supplemented with 10µg/mL neomycin. Colonies were counted the next day.

Statistics

Statistical analyses were carried out using the unpaired Student's t-test (GraphPad) except where indicated. Results were considered statistically significant if $p \leq 0.05$, as indicated in the figure legends.

Results

MARCO protects mice from invasive pneumococcal disease

Previous studies on *S. pneumoniae* infection have shown that type I IFN responses are important in both the context of colonization as well as invasive pulmonary disease (16, 19). In our previous work, we uncovered evidence that MARCO expression in the nasopharynx is necessary for early expression of IFN-β mRNA (11). $MARCO^{-/-}$ macrophages also produce significantly less type I IFN than their WT counterparts when stimulated with colonizing strains of *S. pneumoniae*. In order to gain a better understanding of MARCO's role in the protective response against IPD, as well as the importance of type I IFN in these mice, we performed one day colonizations of WT, $MARCO^{-/-}$, and $SRA^{-/-}$ mice with the invasive pneumococcal strain P1547. As seen in Figure 1, $MARCO^{-/-}$ mice are more likely to suffer from IPD than their WT counterparts, despite similar amounts of bacteria in their nasal washes [Fig. 1A]. The $MARCO^{-/-}$ mice, on average, had more bacteria in their lungs [Fig. 1B] and spleens [Fig. 1C] when compared to their WT counterparts. Perhaps more importantly, the proportion of $MARCO^{-/-}$ mice (100%) compared to WT mice (55%) that had bacteria in either their lungs or spleens (or both) was higher [Fig. 1D]. While no mice had any adverse reactions to the infection at day one post-inoculation, previous work has shown that bacteria in the lungs or spleen can lead to a rapid decrease in health ending in death in approximately two-to-three days (data in review).

$MARCO^{-/-}$ mice produce less type I IFN, leading to dysregulated nasal epithelia

In order to determine whether a paucity of type I IFN production in the nasopharynges of $MARCO^{-/-}$ mice could be implicated in their susceptibility to invasive disease, we first looked at *ifnb* as well as IFN-stimulated gene transcription in nasal lavages from P1547-colonized mice.

MARCO$^{-/-}$ mice had significantly less *ifnb*, *isg15*, and *irf7* gene transcription in their nasal passages one day post-inoculation (determined by fold-change compared to naïve mice) [Fig. 2A-C]. The work of LeMessurier et al. has shown that, in the lung, type I IFN can reduce spread of pneumococci to the blood by upregulating tight junction proteins in the lung epithelium (19). We tested whether this was possible in the nasal passages by measuring mRNA levels of various epithelial junction proteins in our nasal lavages. MARCO$^{-/-}$ mice had significantly less *tjp1* (ZO-1) and *cdh1* (E-cadherin), but not *cldn5* (Claudin-5), transcription when compared to WT mice [Fig. 2D-F]. Because pneumococci often invade through epithelial barriers, this could account for the increased invasiveness of the bacteria in our MARCO$^{-/-}$ mice. The geography of this response also provides some context as to how some of our mice had bacterial dissemination to the blood, but not the lungs. Lack of cell junction gene induction provides context as to how bacteria could move directly from the nose to the bloodstream without first having to colonize the more highly vascularized lungs.

To provide a more direct analysis of the effects of type I IFN on nasal epithelia, we employed the human pharyngeal cell line Detroit-562 (ATCC CCL-138) which grows into functional monolayers. To test whether IFN stimulation of these cells affects monolayer integrity, we treated the cells with 1000U/mL of recombinant IFN-β by adding the protein or a PBS control to the wells. We then lysed the cells and extracted RNA. As seen in Figure 3, IFN-treated Detroit cells had increased transcriptional levels of the junction proteins *tjp1*, *cdh1*, and *cldn5* [Fig. 3A]. We also tested how well pneumococci were able to bind to the apical surface of these cells using a simple bacterial binding assay. Live P1547 were added to cultures of Detroit-562 cells and then spun down so that all of the bacteria would reach the monolayer surface at the same time. After 30 minutes, the cells were washed multiple times to remove unbound bacteria.

The cells were then lysed and bacterial CFUs were determined by plating the lysate and allowing colonies to grow. There was no significant difference between the binding capacity of *S. pneumoniae* on IFN-treated and untreated cells [Fig. 3B]. This infers that pneumococcal binding receptors are not changed due to IFN-β treatment.

Macrophages require MARCO, uptake, and TLR2 to produce type I IFN

Type I IFNs are traditionally viewed as epithelial cell cytokines, being produced once these cells are infected with virus. However, it has become well-accepted recently that mobile immune cells, such as macrophages, can be significant contributors to type I IFN production in the context of bacterial infection (20, 21). In order to ascertain whether or not macrophages could be contributing to the $MARCO^{-/-}$ phenotype, we first extracted macrophages from WT and $MARCO^{-/-}$ mice and stimulated them with live P1547. As seen in Figure 4A, *ifnb* gene transcription is significantly higher in WT macrophages when compared to $MARCO^{-/-}$ macrophages. Type I IFN protein production is also abrogated in $MARCO^{-/-}$ mice, as shown by the lack of antiviral protection that supernatants from these cells confer upon L929 cells infected with VSV [Fig. 4B]. Adding to the evidence for MARCO's role is the fact that $MARCO^{-/-}$ macrophages are less capable of internalizing P1547, as shown by the gentamicin-protection assay in Figure 4C.

Type I IFN production by macrophages can be induced through various pathways, most of which involve internalization of bacteria and subsequent activation of TLRs (15). In order to delve deeper into how *S. pneumoniae* stimulation leads to type I IFN production by macrophages, we stimulated cells from WT mice with live P1547 after pre-treating them with the inhibitors dynasore hydrate (blocking endocytosis), cytochalasin D (preventing phagocytosis), or

CU-CPT22 (blocking TLR2/1 activation) and then performed RNA analysis as well as an interferon protection assay, as described above. Both endocytosis and TLR2/1 heterodimers appear to be important in the production of type I IFNs, as cells treated with dynasore or CU-CPT22 had significant less *ifnb* transcription than untreated cells [Fig. 5A]. This was not the case for cytochalasin D-treated cells, inferring that phagocytosis is not necessary for IFN-β production. L929 cells treated with supernatants from cells pretreated with dynasore or CU-CPT22 were also less protected from viral infection compared to those treated with supernatants from mock-treated cells or those pre-treated with cytochalasin D [Fig. 5B].

Our previous studies, and those of others, have shown that MARCO expression is important for the production of many pro-inflammatory cytokines in response to stimulation with pneumococci and other bacteria (11, 22). These included IL-6 and TNF. To better understand MARCO's role in this, we performed the same inhibitor studies as above but looked at IL-6 and TNF protein production by ELISA. Both of these cytokines were positively regulated by MARCO expression in (), where we stimulated WT and MARCO$^{-/-}$ cells with lysozyme-digested P1121, a non-invasive strain of pneumococcus. When stimulating WT macrophages with lysozyme-digested P1547, we saw that blocking endocytosis or TLR-2 signaling, but not phagocytosis, significantly reduced IL-6 production [Fig. 5C], while only the blocking of TLR-2 reduced TNF production [Fig. 5D]. These experiments suggest that MARCO-mediated type I IFN and IL-6 production by macrophages is linked to the endocytosis of pneumococci as well as TLR-2 signaling. On the other hand, TNF production is not altered by bacterial uptake, but is at least partially linked to TLR-2 signaling. Because MARCO expression has a positive effect on TNF production, it is possible that MARCO's role as a 'tethering' receptor for TLR-2 is not entirely dependent on endocytosis.

Inducing type I IFN in a MARCO-independent manner protects against IPD

If a paucity of type I IFN production is responsible for the increased invasiveness of P1547 in MARCO$^{-/-}$ mice, then it should be possible to 'rescue' this phenotype by inducing type I IFN production in a MARCO-independent manner. To this end, we pre-treated MARCO$^{-/-}$ mice intranasally with polyinosinic:polycytidylic acid (poly(i:c)), an analogue of double-stranded RNA that signals through TLR3 and does not require MARCO (23). We then colonized the mice as in Figure 1 and looked at bacterial CFUs after one day. While MARCO$^{-/-}$ mice were more likely to have pneumococci in their lungs and spleens when compared to WT mice, this phenotype was reversed when pre-treated with poly(i:c), showing that stimulation of TLR3 was enough to protect these mice from IPD [Fig. 6A-C]. Indeed, these mice had significantly higher levels of *ifnb* mRNA transcription when compared to non-treated MARCO$^{-/-}$ mice as well as higher levels of *tjp1* and *cdh1* transcription [Fig. 6D-F]. These data suggest that type I IFN production independent of MARCO expression upregulated tight junction protein expression, blocking invasion of pneumococci from the nasopharynx to the lungs and blood.

Discussion

The immune response to pneumococcal colonization is multi-faceted and temporally varied, with many cell types, receptors, and transcriptional regulators being important for protecting the host against progression to invasive disease (24). While type I IFNs are traditional contributors to anti-viral and intracellular bacterial responses, there is a growing consensus that these proteins are important regulators of immune responses to extracellular bacteria as well (15). Many previous studies have highlighted the negative consequences of type I IFN production in bacterial pneumonia secondary to influenza infection (17, 25, 26). In contrast, we have shown that type I IFN is vital to containing the pneumococcus to the nasopharynx during primary bacterial colonization.

The current literature concerning type I IFNs and *S. pneumoniae* is complicated by the use of many different strains of the bacteria, mouse lines, and cell types for *in vitro* assays. Most of the data that exists today comes from models of pneumonia wherein invasive bacteria are directly instilled to the lungs of mice (19) or *in vitro* assays using epithelial cell lines (27). Our experiments focus on type I IFN production by macrophages *ex vivo* and the anti-colonization response using our model of pneumococcal carriage. The data we have accrued here is in agreement with many aspects of other studies, including Vanessa Redecke's work showing how type I IFN is important for informing the epithelial response to the bacteria, with an increase in cellular junction proteins leading to less bacterial invasiveness from the lung to the blood (19). Interestingly, this is also true in the nasopharynx, where type I IFN is important for protection from bacterial invasiveness both to the lungs and directly into the blood. This second point is important in that there is no established model for occult bacteraemia - that is, dissemination of pneumococci directly from the nasal passages to the blood. Occult bacteraemia is not uncommon

in children, but is very rarely seen in mice colonized intranasally with *S. pneumoniae* and, as such, there are no current studies on the mechanisms behind this phenomenon (28). Our work suggests that a paucity of type I IFN production may be an underlying cause of occult bacteraemia.

Work performed by numerous groups has shown a negative correlation between type I IFN responses to viral infections and the ability of the immune system to control secondary bacterial pneumonia. These studies show that the large amounts of type I IFN produced during viral infection can have negative influences on neutrophil recruitment (by reducing CXCL-1 and CXCL-2 expression) (25), γδ-T cell activity (especially IL-17 production) (26), and macrophage recruitment (by reducing CCL-2 production) (17). It is important to note that, unlike the current study, these works all focus on the effect of IFN well before pneumococci are introduced to the research animal and in most cases the bacteria are directly instilled to the lungs. In contrast, we have shown that type I IFN produced at the time of nasopharyngeal colonization is beneficial in that it protects mice from the bacteria becoming invasive before the innate immune response is able to control it. While these studies seem to be contradictory, we hypothesize that the temporal differences between their methods can account for this discrepancy. This is because other groups have shown that type IFN can lead to the production of significant levels of IL-10 by an indirect mechanism, possibly leading to the 'pro-bacterial' phenotype seen by our colleagues (20).

Despite type I IFN being considered a predominantly epithelial cytokine, macrophages are also capable of producing significant amounts of these cytokines in response to bacteria. Previous studies have shown that macrophages can produce type I IFN via the ligation of various TLRs including TLR-2, TLR-3, TLR-4, TLR-7, and TLR-9 (15). Macrophage-mediated IFN production is also dependent on stimulator of interferon genes (STING) and, depending on the

signaling pathway, can be controlled by IRF-1, IRF-3, or IRF-7(29, 30). We have shown here that in order to produce IFN, macrophages must also take up the bacteria via a MARCO-mediated mechanism followed by signaling via TLR-2. Surprisingly, TLR-4 does not appear to contribute to MARCO-mediated type I IFN production (data not shown). Therefore, it appears that pneumococci induce IFN production by macrophages after recognition and uptake of the bacteria by MARCO followed by TLR-2 signaling from the endolysosome. This IFN then induces increased epithelial integrity *in vivo*.

MARCO's role in cytokine production does not end with type I IFNs. We show here that MARCO-mediated uptake of pneumococci leads to increased production of IL-6, but not TNF. This is interesting due to parallels and contradictions with previous work performed in Siamon Gordon's laboratory (22). This study showed that MARCO reduced pro-inflammatory signaling from surface-bound receptors, such as TLR-4, by scavenging ligands from the surface of the cell while increasing signaling through intracellular receptors like TLR-3. We can gather from this study and the data shown here that TNF production is likely coming from the signaling of surface receptors, while IL-6 production is the result of intracellular receptors. However, TLR-2 blockade significantly reduces IL-6 production, leading us to believe that IL-6 is being produced as a result of TLR-2 signaling from the endolysosome in a fashion similar to that of type I IFN. Interestingly, we had shown previously that MARCO expression does increase TNF production in macrophages (31). Our data leads us to believe that MARCO's role in this production is not endocytic in nature. Therefore, it is possible that MARCO expression at the cell surface can also lead to increased inflammatory signaling through surface-bound receptors.

None of our experiments, whether they involve the $MARCO^{-/-}$ mice or chemical inhibitors, show a complete abrogation of cytokine production. This is likely due to redundancies in the

pathways leading to bacterial uptake as well as in the cytokine signaling pathways. However, our *in vivo* data show that the reduction we see in MARCO-mediated type I IFN production is enough to produce a significant phenotype in our mice. This is recapitulated in the fact that establishing type I IFN production independent of MARCO, using poly (I:C), rescues the phenotype completely.

Pneumococcal invasion across epithelial barriers can happen in two ways: receptor-mediated transcytosis of the bacteria (32, 33), or non-specific pericellular movement through a disruption of cellular junctions (34, 35). Transcytosis of the bacteria usually occurs via bacterial binding to platelet-activating factor receptor (pafR) (36) or the polymeric immunoglobulin receptor (pIgR) (32). Unlike another study, we did not find that type I IFN affected the expression of either of these receptors in the nasopharynx (data not shown). This leads us to believe that type I IFN's role in the nasopharynx is protecting the epithelium from pericellular invasion by upregulation cell junction proteins. This may account for the 55% of WT mice that we saw with bacteria in their lungs and/or spleens, as the bacteria still have access to their transcytotic pathway of invasion.

The timing of our experiments suggests that it is resident, and not recruited, macrophages that are responsible for steeling the nasopharyngeal epithelium from bacterial invasion. Previous studies, performed by ourselves and others, have shown that macrophage recruitment does not begin until day seven post-inoculation and that macrophage numbers peak at day 14 (9, 11, 37). The only cells that have begun to arrive at the nasopharynx after one day of colonization are neutrophils. Neutrophils do not express MARCO and, as such, are unlikely to be involved in this phenomenon. Our previous studies also showed that MARCO expression in the nasopharynxx did not significantly rise with the influx of macrophages during colonization. These data argue

that resident nasal macrophages are the predominant expressers of MARCO in the nasopharynx and that these cells are significant producers of type I IFN in response to pneumococcal colonization.

The significance of this work is two-fold. Firstly, while it is important that we gain a better understanding of the innate immune processes that inform the adaptive response that eventually clears pneumococcal colonization, it is equally important to understand how invasive strains of bacteria are held in the nasopharynx by this innate response thereby preventing serious disease in the period before adaptive immunity can be established. Resident macrophages in the nose play a vital role due to their ability to recognize these bacteria and establish an 'anti-invasive' state by informing the behaviour of the epithelium. Secondly, we have been able to show that type I IFN is crucial to the protection of animals from invasive pneumococcal disease that spreads from the natural reservoir of *S. pneumoniae* as opposed to forced invasiveness from the lung. This is an important distinction, as human pneumococcal diseases have been shown to always be preceded by a colonizing event in the nasopharynx (3). This makes the nasopharynx an important anatomical site to study if we are going to gain a better understanding of how the human body deals with this periodic colonization. Further study into how this site is protected, and the immune responses that are initiated upon colonization, will better inform how to boost these responses in at-risk populations.

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Figure Legends

Figure 1. MARCO protects mice from invasive pneumococcal disease. WT and MARCO$^{-/-}$ mice were inoculated with 10^7 CFUs of P1547. After one day mice were sacrificed and nasal washes [A], lungs [B], and spleens [C] were collected and tested for bacterial CFUs. [D] shows the same data but as a ratio of mice with no invasive disease versus those who had bacterial dissemination to the lungs, spleen, or both. * = $p \leq 0.05$, by Mann-Whitney U test.

Figure 2. MARCO-deficient mice have diminished type I IFN responses leading to a dysregulated epithelium. Mice were inoculated with P1547 as in Figure 1. After one day, mice were sacrificed and nasal lavages were performed using an RNA lysis buffer. RT-PCR was performed to examine IFN and IFN-stimulated genes [A-C] as well as cellular junction proteins [D-F]. Data shown is fold change over uncolonized mice using *gapdh* as the control gene. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ by Student's t-test.

Figure 3. Pharyngeal epithelia react to type I IFN by increasing barrier integrity. Detroit-562 cells were grown to confluence on tissue plastic. Cells were treated with either recombinant IFN-β or PBS control. [A] After 20h, cells were lysed and RT-PCR was performed on RNA samples. Data shown is fold change of IFN-β treated vs. PBS-treated cells with *gapdh* as the control gene. [B] Live P1547 were added to Detroit-562 cell cultures after IFN-β or PBS treatment. After 30 minutes cells were washed vigorously then lysed and bacterial CFUs were counted. ** = $p \leq 0.01$ by Student's t-test.

Figure 4. MARCO contributes to type I IFN responses in macrophages. [A] RT-PCR was

performed on macrophage lysates after with one hour or 12 hour stimulation with live P1547. [B] Macrophages from WT and MARCO$^{-/-}$ mice were isolated and stimulated with live P1547. Resulting supernatants were then added to L929 cells in culture. VSV-GFP was used to infect L929 cells and cell infection was measured by GFP fluorescence. [C] Live P1547 were added to nutating macrophage cultures for one hour. Bacterial uptake was measured by killing extracellular bacteria with gentamycin, washing, and then lysing cells and plating for CFUs. * = $p \leq 0.05$, *** = $p \leq 0.001$ by Student's t-test.

Figure 5. MARCO-mediated uptake modulates type I IFN and IL-6, but not TNF, production. WT macrophages were pre-treated with inhibitors and then stimulated with lysozyme-digested P1547. [A] Cells were lysed and RNA was measured by RT-PCR. Data shown as fold change vs. unstimulated cells with *gapdh* as the control gene. [B] Macrophage supernatants were used in L929 protection assay, as in Figure 4. [C-D] Macrophage supernatants were used for IL-6 and TNF ELISAs. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ by Student's t-test.

Figure 6. Boosting type I IFN responses prior to colonization protects MARCO-/- mice from IPD. Mice were colonized with P1547 as in Figure 1, but half of mice were pre-treated with intranasal instillations of poly(I:C). After one day mice were sacrificed and nasal washes [A], lungs [B], and spleens [C] were collected and tested for bacterial CFUs. [D-F] RNA levels were measured in RNA lysis buffer nasal lavages, as in Figure 2. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ by Student's t-test.

Tables

Table 1: Primers used for RT-PCR

Gene	Sense Primer	Antisense Primer
gapdh	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA
ifnb	GCACTGGGTGGAATGAGACT	AGTGGAGAGCAGTTGAGGACA
isg15	GAGACTGGCTATTGGGGGAG	GGTGTCCGTGACTAACTCCAT
irf7	GACCGAAATGCTTCCAGGG	TGGAAAGGGTAAGACCGTCCT
tjp1	CGAGGCATCATCCCTAATAAGAAC	TCCAGAAGTCTGCCCGATCAC
cdh1	CAGGTCTCCTCATGGCTTTGC	CTTCCGAAAAGAAGGCTGTCC
cldn5	GCAAGGTGTATGAATCTGTGCT	GTCAAGGTAACAAAGAGTGCCA

Figures

Figure 1

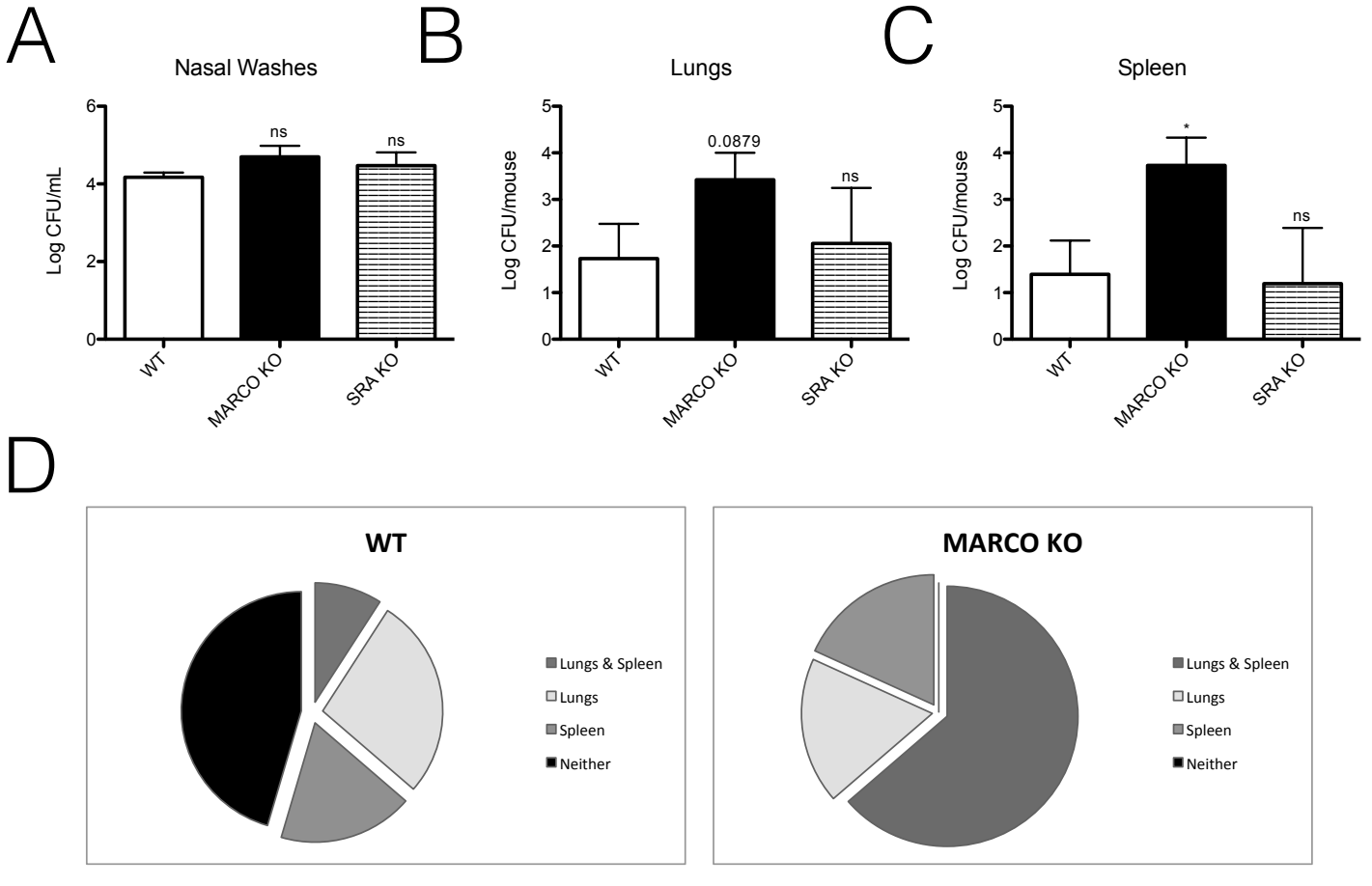


Figure 2

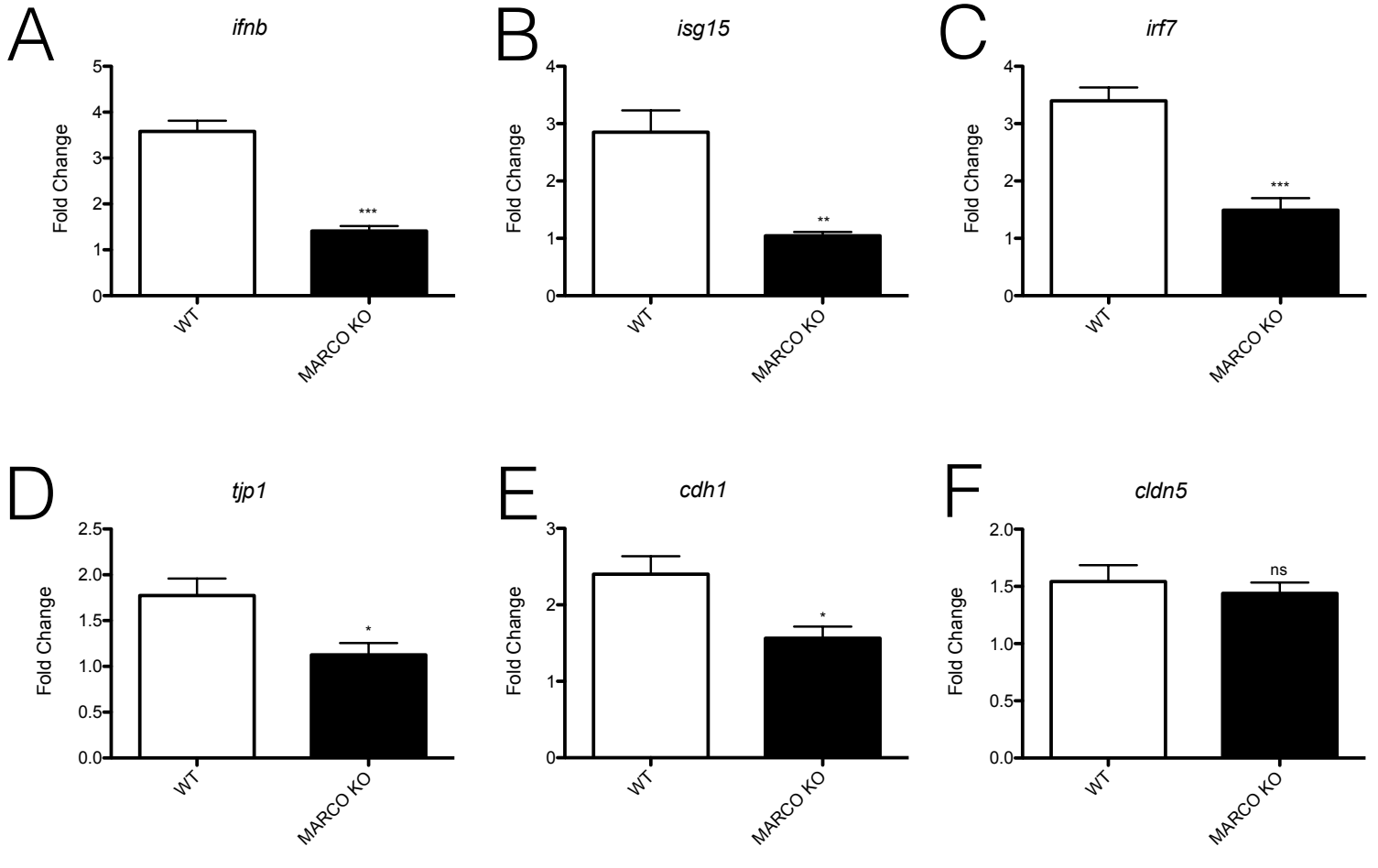

A
ifnb
Fold Change
0
1
2
3
4
5
***
WT
MARCO KO
B
isg15
Fold Change
0
1
2
3
4
**
WT
MARCO KO
C
irf7
Fold Change
0
1
2
3
4
***
WT
MARCO KO
D
tjp1
Fold Change
0.0
0.5
1.0
1.5
2.0
2.5
*
WT
MARCO KO
E
cdh1
Fold Change
0
1
2
3
*
WT
MARCO KO
F
cldn5
Fold Change
0.0
0.5
1.0
1.5
2.0
ns
WT
MARCO KO

Figure 3

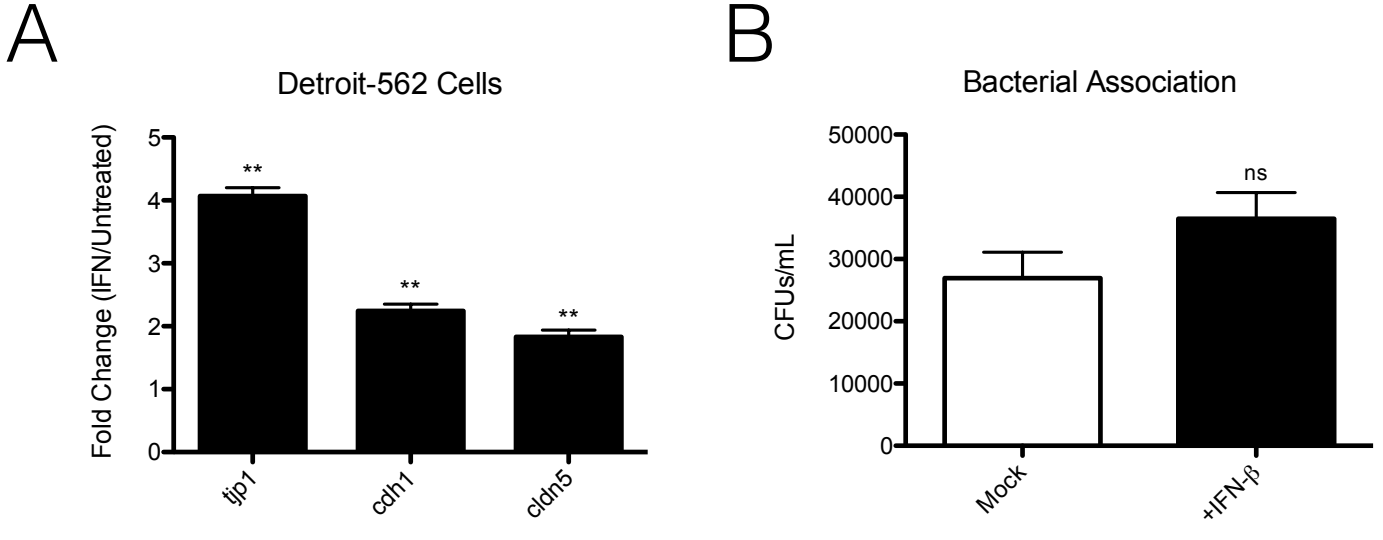
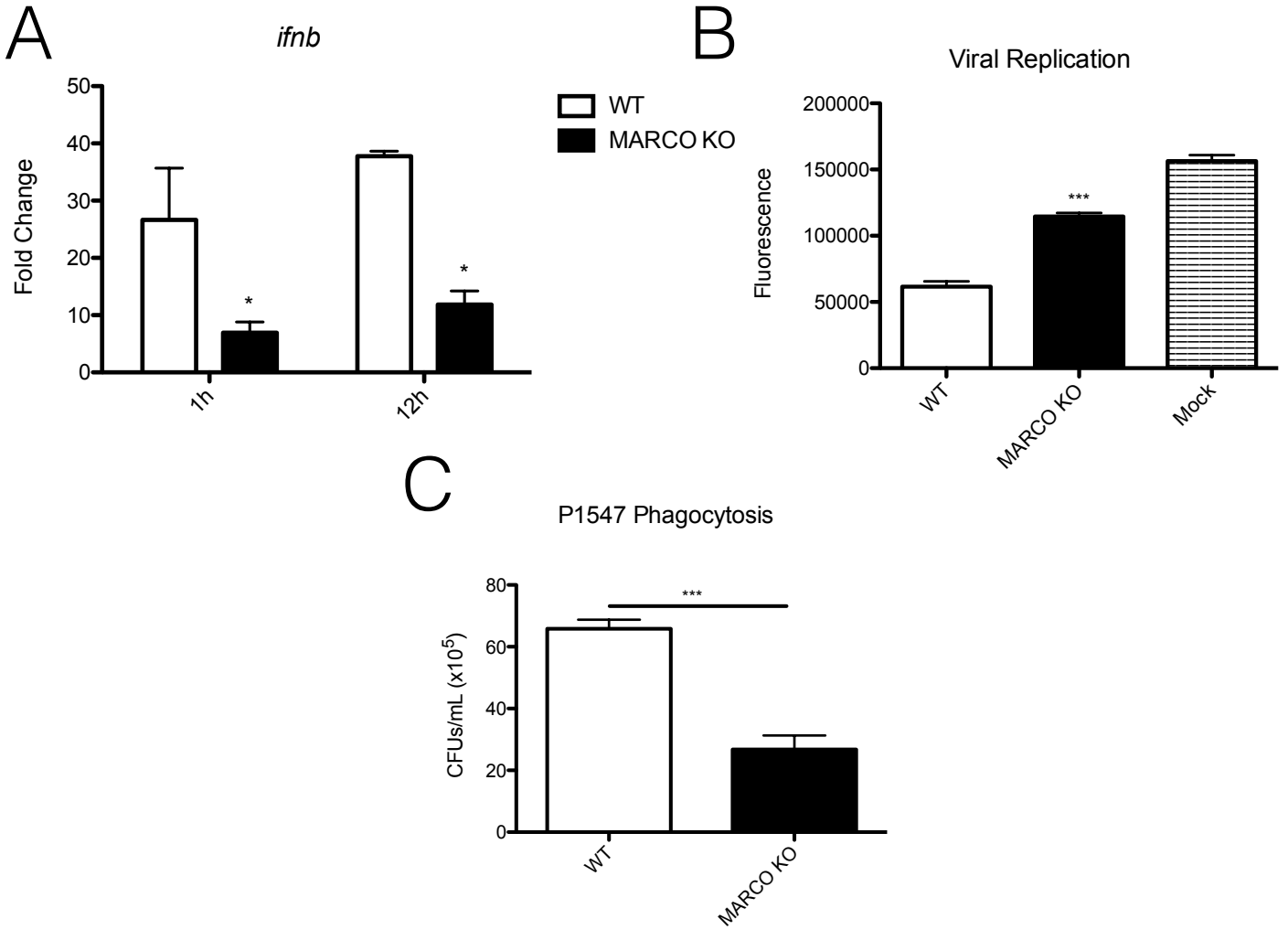

A
Detroit-562 Cells
Fold Change (IFN/Untreated)
0
1
2
3
4
5
**
**
**
tjp1
cdh1
cldn5
B
Bacterial Association
CFUs/mL
0
10000
20000
30000
40000
50000
ns
Mock
+IFN-β

Figure 4


A
ifnb
Fold Change
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40
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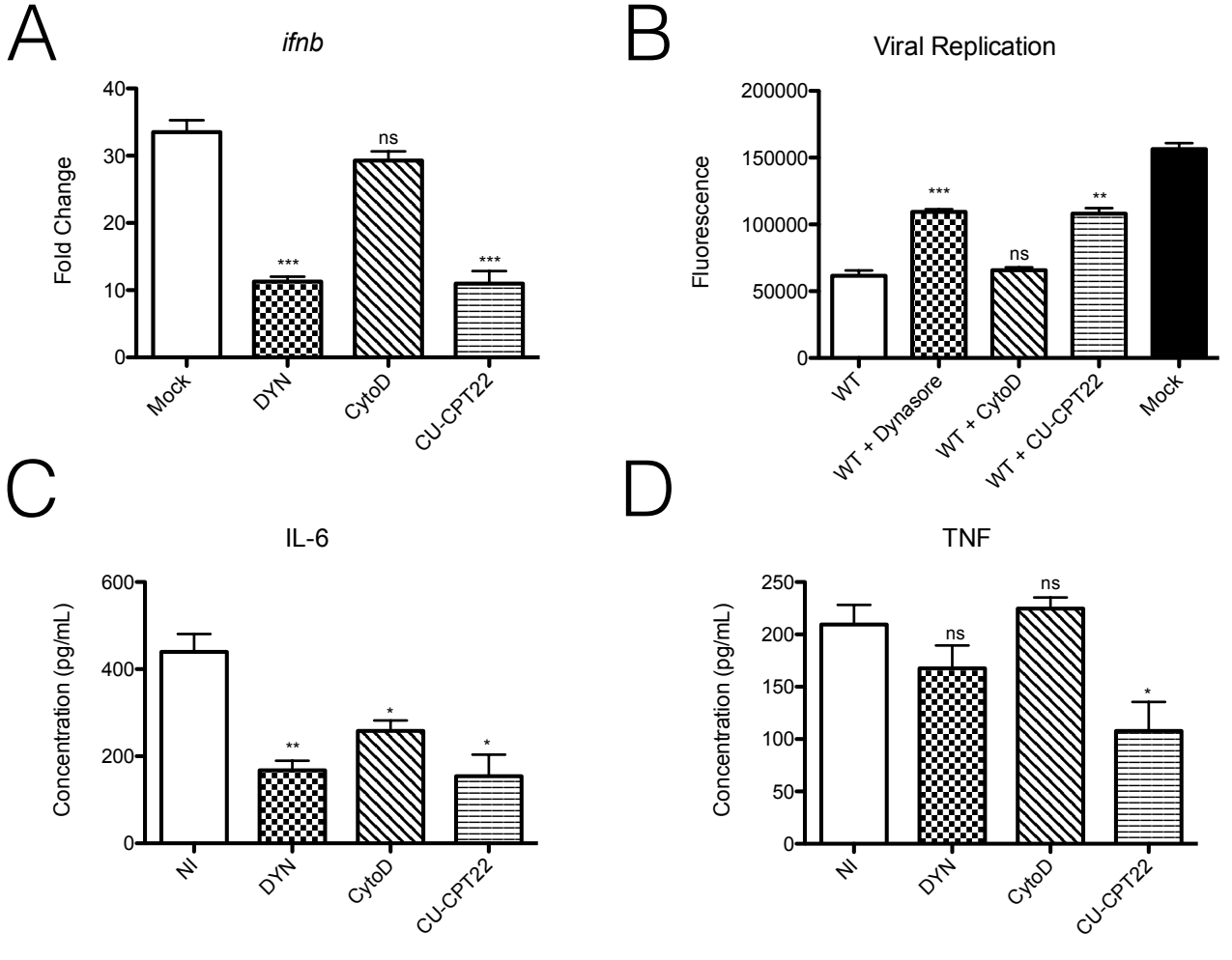
20
10
0
*
*
1h
12h
WT
MARCO KO
B
Viral Replication
Fluorescence
200000
150000
100000
50000
0
***
WT
MARCO KO
Mock
C
P1547 Phagocytosis
CFUs/mL (x10^5)
80
60
40
20
0
***
WT
MARCO KO

Figure 5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Fold Change
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30
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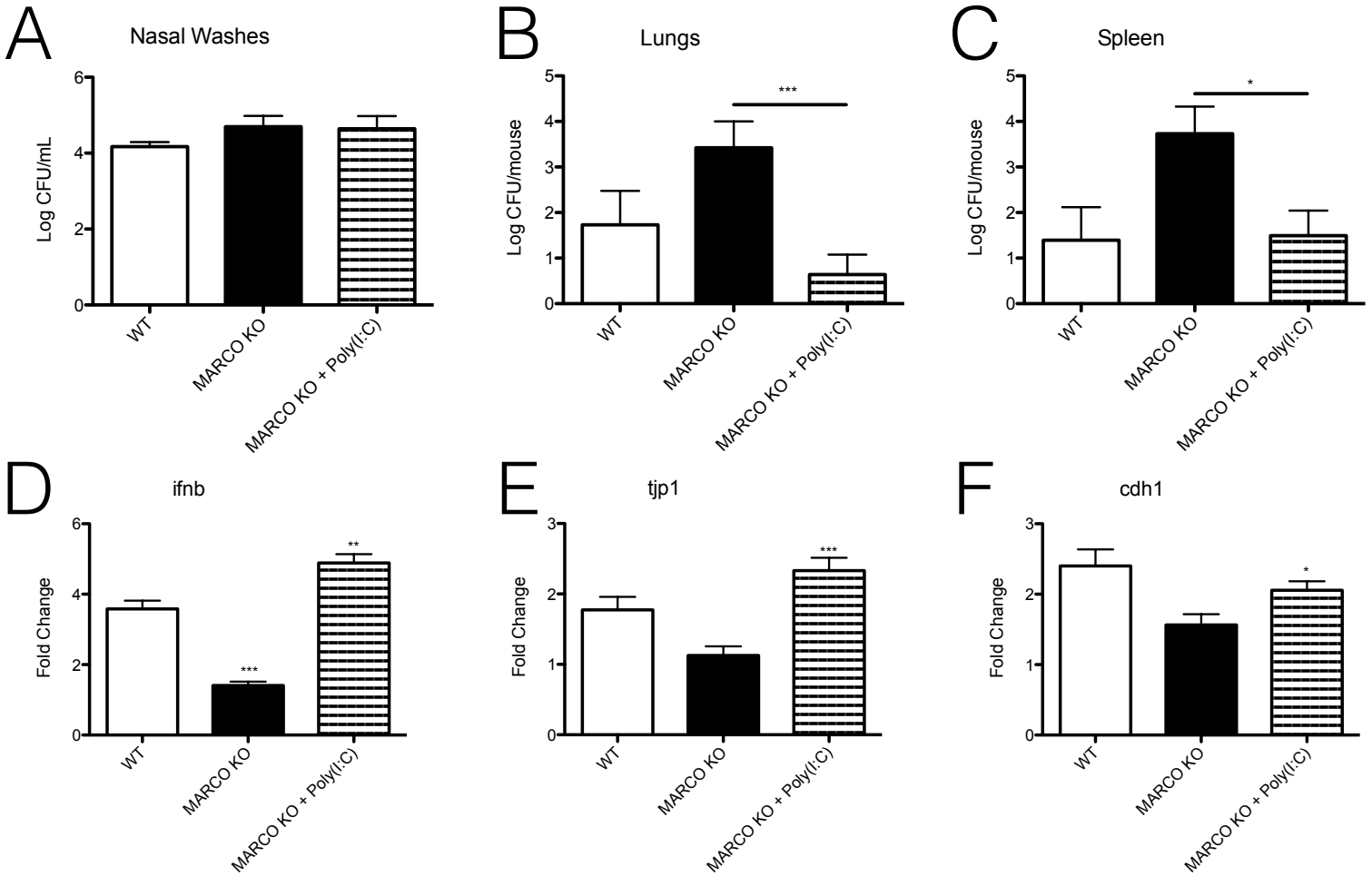
ns
***
***
Mock
DYN
CytoD
CU-CPT22
B
Viral Replication
Fluorescence
200000
150000
100000
50000
0
***
ns
**
WT
WT + Dynasore
WT + CytoD
WT + CU-CPT22
Mock
C
IL-6
Concentration (pg/mL)
600
400
200
0
**
*
*
NI
DYN
CytoD
CU-CPT22
D
TNF
Concentration (pg/mL)
250
200
150
100
50
0
ns
ns
*
NI
DYN
CytoD
CU-CPT22

Figure 6


A
Nasal Washes
Log CFU/mL
B
Lungs
Log CFU/mouse
***
C
Spleen
Log CFU/mouse
*
WT
MARCO KO
MARCO KO + Poly(I:C)
D
ifnb
Fold Change
**
***
E
tjp1
Fold Change
***
F
cdh1
Fold Change
*

Chapter 5
MicroRNA-155 Is Required for Clearance of Streptococcus pneumoniae *from the Nasopharynx*

Preface

This chapter consists of a published study from the journal *Infection & Immunity* that unveils a role for miR-155 in the clearance of pneumococcal colonization from the murine nasopharynx. When these studies were first planned, our hypothesis was that MARCO expression was regulated by miR-155, with activity from this miRNA leading to an upregulation of MARCO expression at the expense of SR-AI expression. Based on the study found in **Chapter 3**, we believed this would lead to increased bacterial clearance. While we did uncover a role for miR-155 in pneumococcal clearance, MARCO expression was not affected in miR-155-deficient mice when compared to WT mice. A change in strategy led to our discovery that miR-155 was integral to efficient activation of Th17 cells, leading to the production of IL-17, the recruitment of macrophages to the nasopharynx, followed by clearance of the bacterial colonization.

Immune regulation is a topic that has not been sufficiently studied in the context of bacterial colonization. Here we show the importance of regulatory factors like miRNAs in fine-tuning the immune response to the particular pathogen. This opens to the door to potential therapies based on short RNAs that can help 'boost' an immune response without the need for serotype-based interventions like our current vaccines.

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MicroRNA-155 Is Required for Clearance of *Streptococcus pneumoniae* from the Nasopharynx

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Pneumonia caused by *Streptococcus pneumoniae* is a major cause of death and an economic burden worldwide. *S. pneumoniae* is an intermittent colonizer of the human upper respiratory tract, and the ability to control asymptomatic colonization determines the likelihood of developing invasive disease. Recognition of *S. pneumoniae* by resident macrophages via Toll-like receptor 2 (TLR-2) and the macrophage receptor with collagenous structure (MARCO) and the presence of interleukin-17 (IL-17)-secreting CD4$^+$ T cells are required for macrophage recruitment and bacterial clearance. Despite the fact that the primary cellular effectors needed for bacterial clearance have been identified, much of the underlying regulatory mechanisms are unknown. Herein, we demonstrate that the small, noncoding RNA microRNA-155 (mir-155) is critical for the effective clearance of *S. pneumoniae*. Our studies show that mir-155-deficient mice maintain the ability to prevent acute invasive pneumococcal infection but have significantly higher bacterial burdens following colonization, independently of macrophage recognition by TLR-2, MARCO expression, or bactericidal capacity. The observed defects in bacterial clearance parallel reduced IL-17A and gamma interferon CD4$^+$ T-cell responses *in vivo*, lower IL-17A mRNA levels in the nasopharynx, and a reduced capacity to induce Th17 cell polarization. Given that knockout mice are also limited in the capacity to generate high-titer *S. pneumoniae*-specific antibodies, we conclude that mir-155 is a critical mediator of the cellular effectors needed to clear primary and secondary *S. pneumoniae* colonizations.

Pneumonia caused by *Streptococcus pneumoniae* infection is a major cause of death and an economic burden in both developed and developing nations worldwide. Children less than 2 years old and adults older than 65 years are the most likely to succumb to invasive pneumococcal disease, with mortality rate estimates reaching as high as 36% (1) and 53% (2), respectively. As with many mucosal pathogens, asymptomatic colonization precedes invasive disease; *S. pneumoniae* is no exception. This has been proven in mouse models (3) and observed in communities that have adopted the 7-valent pneumococcal conjugate vaccine, where a clear trend of reduced nasopharyngeal colonization and invasive disease is evident (4, 5). Hence, the control of nasopharyngeal colonization has been a major focus of vaccine strategies (6).

Previous studies have shown that nasopharyngeal colonization by *S. pneumoniae* (3, 7) or intranasal pneumococcal vaccination (8), induced antigen-specific antibodies and CD4$^+$ T cells, as well as interleukin-17 (IL-17). These cellular and molecular effectors were important for the protective recruitment of neutrophils upon secondary exposure. However, depending on the site of challenge, some mediators were found to be dispensable; for example, bacterial clearance following secondary nasopharyngeal colonization required CD4$^+$ T cells but not antibodies (8), whereas secondary lung instillation required antibodies but not CD4$^+$ T cells (3). In contrast, the clearance of primary colonization has been shown to be independent of both neutrophils (9) and antibodies (10) and instead relies upon macrophage-mediated immunity. Zhang and colleagues in 2009 showed that primary nasopharyngeal clearance requires CD4$^+$ T cells and IL-17 and the recruitment of macrophages and signaling via Toll-like receptor 2 (TLR-2) (9). We recently demonstrated that bacterial recognition by the phagocytic receptor MARCO (macrophage receptor with collagenous structure) is required for optimal TLR-2 and nucleotide-binding oligomerization domain-containing 2 (NOD-2) signaling. MARCO may function as a coreceptor for CD14/TLR-2 or as a phagocytic receptor that is required for the optimal delivery of *S. pneumoniae* or pneumococcal antigens to the phagolysosome and, ultimately, cytosolic NOD-2 (11).

MicroRNAs are a class of small RNA molecules (19 to 24 nucleotides) that promote the degradation or prevent the translation of mRNA transcripts to which they are complementary. Although the study of microRNAs is in its relative infancy, more than 800 of these molecules have been identified, many of which have been demonstrated to be involved in the control of infectious and noninfectious diseases (12). MicroRNA-155 (mir-155) was first described in 1997 as a noncoding RNA housed within the B-cell integration cluster gene (13) and later as being overexpressed in patients with Burkitt lymphoma (14) and B-cell lymphomas in general (15). Since then, it has been implicated in a number of biological phenomena, including inflammation and innate and adaptive immunity (12). Mir-155 expression is increased by a wide variety of stimuli, such as TLR ligands (16), and has been shown to promote both antimicrobial (17) and antiviral responses

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of macrophages (18) through the repression of factors antagonistic to the inflammatory response. For example, by repressing the transcripts encoding suppressor of cytokine signaling 1 and Src homology 2 domain-containing inositol 5-phosphatase 1, mir-155 promotes immunity to *Staphylococcus aureus* (19) and *Francisella tularensis* (20), respectively, but negatively impacts *Mycobacterium tuberculosis* infection (21). Mir-155 has also been reported to be integral in the development of IL-17A-producing $CD4^+$ T cells (Th17 cells) (22), mainly in the context of autoimmune inflammation (16, 23), but also during gastrointestinal infection with *Helicobacter pylori* (24).

Here, we demonstrate that mir-155 appears to be critical for the effective control of primary *S. pneumoniae* nasopharyngeal colonization but not acute invasive infection. Mir-155-deficient (mir155KO) mice have impaired clearance of nasopharyngeal colonization, independently of macrophage recognition by TLR-2 or MARCO or bactericidal capacity. Mir155KO mice are impaired in the ability to induce Th1 and Th17 cells during exposure to *S. pneumoniae*, which is likely a prominent factor in the observed reduction in macrophage recruitment to the nasopharynx. Furthermore, this decrease in Th1 and Th17 cell induction, as well as the absence of circulating *S. pneumoniae*-specific IgG antibodies, indicates that antipneumococcal adaptive immunity is broadly impaired in mir155KO mice.

MATERIALS AND METHODS

Mice and pneumococcal colonization model. All of the animal care and use protocols in this study were approved by the McMaster Animal Research Ethics Board (no. 13-05-4) and performed in accordance with the guidelines stipulated by the Canadian Council on Animal Care. C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and The Jackson Laboratory (Bar Harbor, ME, USA). Mir155KO mice (B6.Cg-Mir155tm1.1Rsky/J) were purchased from The Jackson Laboratory. Wild-type (WT) mice were sex matched to the knockout groups.

Two clinical isolates of *S. pneumoniae* were employed in this study, P1547 (serotype 6A), a virulent and invasive strain that has been shown to spread rapidly to the lungs of mice following nasopharyngeal colonization (25), and P1121 (serotype 23F), a strain that does not typically spread to the periphery and can be detected up to 3 weeks following nasopharyngeal colonization (9, 11, 25). Both strains were grown to mid-log phase in tryptic soy broth. For pneumococcal colonization as described previously (11), live bacteria were concentrated 10-fold in phosphate-buffered saline (PBS) and stored on ice. Unanesthetized mice were inoculated intranasally with 10 μl of a bacterial suspension containing $\sim 1 \times 10^7$ CFU. At the time of sacrifice, the trachea was cannulated and 200 μl of PBS was instilled. Lavage fluid was collected from the nares, serially diluted in PBS, and plated on tryptic soy agar plates containing 5% sheep blood and neomycin (10 μg/ml). Colonies were counted after overnight incubation at 37°C in 5% CO_2. For *in vitro* experiments, *S. pneumoniae* strain P1121 was killed by heating at 65°C for 10 min.

Human sputum processing. Induced sputum was obtained from four adult asthmatic patients attending the Firestone Institute of Respiratory Health for routine monitoring (St. Joseph's Healthcare, Hamilton, ON, Canada). All subjects provided informed written consent, and all studies met approval from the Hamilton Integrated Research Ethics Board (no. 12-3716). For cell isolation, 5 volumes of 1× Sputolysin (Calbiochem, CA, USA) was added to raw sputum and then rocked on ice and intermittently mixed by pipetting for 15 min. This suspension was diluted 1:1 with PBS and centrifuged at 300 × *g* for 10 min. Cell pellets were resuspended in XVIVO-10 culture medium (Lonza, Basel, Switzerland) supplemented with 5% pooled human AB serum (Lonza, Basel, CH). The cellular composition of sputum was determined by Shandon cytospin centrifugation, followed by hematoxylin-and-eosin staining.

Splenocyte and macrophage culture and stimulation. Harvested spleens were passed through a 40-μm cell strainer (BD Falcon; BD Biosciences, ON, Canada) into warm PBS. Cells were pelleted by centrifugation, resuspended in 1× red blood cell lysis buffer (BioLegend, CA, USA), and incubated at room temperature for 10 min. Splenocytes were pelleted by centrifugation and resuspended in R10 medium (RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10 mM L-glutamine).

Peritoneal macrophages were obtained by Bio-Gel (Bio-Rad, ON, Canada) elicitation. Briefly, 1 ml of 2% (wt/vol) Bio-Gel P100 microbeads were injected intraperitoneally; this was followed by peritoneal lavage after 4 to 5 days. Exudate was pelleted by centrifugation, resuspended in warm R10 medium, and incubated at 37°C in 5% CO_2. The next day, nonadherent cells and Bio-Gel beads were aspirated and macrophages were washed with warm PBS. Macrophages were detached by incubation in 4 mg/ml lidocaine-HCl and 10 mM EDTA for 15 to 20 min at 4°C, followed by gentle lifting. Other than macrophage killing and phagocytosis experiments, all experiments were performed in R10 medium.

For cytokine enzyme-linked immunosorbent assays (ELISAs), capture and detection antibodies for the quantification of tumor necrosis factor (TNF) and IL-6 were purchased from eBioscience. IL-17A and gamma interferon (IFN-γ) were measured with the ELISA Max Deluxe kit (BioLegend, CA, USA) according to the manufacturer's instructions.

RNA extraction and quantitative reverse transcription (RT)-PCR. Lysates from the murine nasopharynx were obtained by nasal lavage with RLT lysis buffer (Qiagen, CA, USA), followed by RNA extraction with the RNAqueous-Micro kit (Life Technologies, CA, USA). All other RNA extractions were performed with the Illustra RNAspin minikit (GE Healthcare, ON, Canada). cDNA synthesis was performed with Moloney murine leukemia virus (MMLV) reverse transcriptase (New England BioLabs, MA, USA) and 15-nucleotide-long random oligonucleotide (pentadecamer) primers (synthesized at core facilities), and gene expression was measured with the GoTaq qPCR master mix (Promega, Madison, WI, USA) and either an ABI 7900 HT sequence detection system or a StepOne Plus real-time PCR system (Life Technologies, CA, USA). The primers used are described in Table 1. Relative expression values were calculated by the $\Delta\Delta C_T$ (relative to glyceraldehyde 3-phosphate dehydrogenase) or standard-curve method, and primer efficiencies were assessed by using a standard curve of pooled, arbitrary cDNA.

For microRNA expression analysis, TaqMan MicroRNA Assays (Life Technologies, CA, USA) were used to measure the expression of murine mir-155 (no. 2571) and SNO-202 (no. 1232) and human mir-155 (no. 2623) and RNU44 (no. 1094). cDNA was synthesized with MMLV reverse transcriptase, and quantitative RT-PCR was performed with the TaqMan Gene Expression master mix (Life Technologies, CA, USA) and the instruments described above. Relative expression values were calculated by the $\Delta\Delta C_T$ method.

Flow cytometry and intracellular cytokine staining. For whole-blood immunophenotyping, 100 μl of heparinized blood was incubated with antibodies for 30 min at room temperature and then incubated in 1× Fix/Lyse buffer (eBioscience, CA, USA) for 10 min. Leukocytes were resuspended in fluorescence-activated cell sorting (FACS) wash buffer (PBS, 0.5% bovine serum albumin, 2 mM EDTA) prior to analysis. For nasal lavage fluids and peritoneal macrophages, suspensions were centrifuged, resuspended in blocking buffer (4 μg/ml anti-CD16/32; eBioscience, CA, USA), and incubated at room temperature for 15 min. Cells were subsequently incubated with antibody cocktails for 30 min at room temperature. Cells were fixed with 2% paraformaldehyde, centrifuged, and resuspended in FACS wash buffer. The conjugated antibodies used included Ly6C-fluorescein isothiocyanate (FITC) or -peridinin-chlorophyll-protein complex Cy5.5, Ly6G-Alexa Fluor 700 (BD Biosciences, ON, Canada), F480-allophycocyanin (APC), CD45-eFluor 450, TLR2-phycoerythrin (PE) Cy7, CD11b-PE Cy7, Ter119-PE, NK1.1-PE, B220-PE (eBioscience, CA, USA), and MARCO-PE (AbD Serotec, NC, USA). The lineage cells used for whole-blood immunophenotyping in-

TABLE 1 Quantitative PCR primers employed in this study

Primer	NCBI GeneID	Sequence (5′–3′)	
		Forward	Reverse
mGAPDH	14433	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA
mIFN-γ	15978	CACACTGCATCTTGGCTTTG	TTCCACATCTATGCCACTTGAG
mIL-1β	16176	GCCTCGTGCTGTCGGACCCATA	GATCCACACTCTCCAGCTGCAGG
mIL-10	16153	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGG
mIL-17A	16171	TTTAACTCCCTTGGCGCAAAA	CTTTCCCTCCGCATTGACAC
mMCP-1	20296	GTCTGTGCTGACCCCAAGAAG	TGGTTCCGATCCAGGTTTTTA
mMIP-1α	20302	TGTACCATGACACTCTGCAAC	CAACGATGAATTGGCGTGGAA
mMIP-2	20310	CCAACCACCAGGCTACAGG	GCGTCACACTCAAGCTCTG
mTNF	21926	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
hGAPDH	2597	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
hIL-12	3593	GGCCATATGGGAACTGAAGA	CAGGAGCGAATGGCTTAGA
hIL-1β	3553	CTTGGTGATGTCTGGTCCAT	GACAAATCGCTTTTCCATCTTC
hIL-6	3569	AGGAGACTTGCCTGGTGAAA	CAGGGGTGGTTATTGCATCT
hIL-8	3576	CTGCGCCAACACAGAAATTA	CATCTGGCAACCCTACAACA
hTNF	7124	CGCTCCCCAAGAAGACAG	GAGCTGCCCCTCAGCTTG

cluded those expressing Ter119, NK1.1, and B220. Absolute cell counts were quantified with CountBright Absolute Counting Beads (Life Technologies, CA, USA).

Intracellular cytokine staining of homogeneous splenocyte suspensions from uninfected or colonized mice was performed at the time points indicated in Results. Briefly, 10^6 cells (4×10^6/ml) in R10 medium were treated with either 1× Protein Transport Inhibitor (eBioscience, CA, USA) or 1× Cell Stimulation Cocktail (eBioscience, CA, USA) for 4 h at 37°C in 5% CO_2. Surface staining was performed for 30 min at room temperature with Alexa Fluor 700-conjugated CD3 (BD Biosciences, ON, Canada) and PE-conjugated CD4 (eBioscience, CA, USA) antibodies, and fixing was performed with 1× Fix/Lyse buffer (eBioscience, CA, USA) for 10 min. Cells were permeabilized for 30 min with 1× Permeabilization Buffer (eBioscience, CA, USA) at room temperature and stained with FITC-conjugated IFN-γ and APC-conjugated IL-17A antibodies (eBioscience, CA, USA) for 30 min at room temperature. Cells were fixed with 2% paraformaldehyde, centrifuged, and resuspended in FACS wash buffer prior to analysis. Th17 cells were defined as expressing CD3, CD4, and IL-17A but not IFN-γ, and Th1 cells were defined as expressing CD3, CD4, and IFN-γ but not IL-17A.

All flow cytometry was performed with a Becton Dickinson LSR II or Canto flow cytometer, and all analyses were performed with TreeStar FlowJo 7.6.3.

IgG binding assay. Heat-killed *S. pneumoniae* bacteria (10^6) were incubated with heparinized plasma from uninfected and colonized mice for 1 h at room temperature in V-bottom 96-well plates. Optimization experiments indicated that a plasma dilution of 1/75 was within the linear range of binding (data not shown). Cells were washed two times with PBS and incubated with Alexa Fluor 633-conjugated goat anti-mouse IgG (Life Technologies, CA, USA) in FACS wash buffer for 30 min at room temperature. Cells were washed two times with FACS wash buffer and fixed with 2% paraformaldehyde for 10 min before samples were run on a Becton Dickinson LSR II flow cytometer.

Macrophage killing and phagocytosis assays. Elicited peritoneal macrophages were resuspended in PBS at a concentration of 10^6/ml and infected at a multiplicity of infection (MOI) of 25 mid-log-phase *S. pneumoniae* bacteria. Cells were placed on an orbital shaker at 37°C for 30 min and washed three times in PBS. For time zero, a 20-μl aliquot of a cell suspension was incubated 1:1 with 0.3% saponin in water for 5 min at room temperature and serial dilutions were plated on tryptic soy agar plates containing 5% sheep blood and 10 μg/ml neomycin. Cells were incubated at 37°C while shaking, and aliquots were removed at the time points indicated in Results.

Elicited peritoneal macrophages were resuspended in R10 medium at a concentration of 5×10^5/ml in 96-well plates. Cells were incubated at 37°C with pHRodo Red (Life Technologies, CA, USA)-labeled *S. pneumoniae* at an MOI of 100 bacteria. For bacterial labeling, mid-log-phase *S. pneumoniae* were pelleted, washed, and resuspended in 0.1 mM pHRodo Red dye. After a 30-min incubation at room temperature with end-over-end rotation, cells were pelleted, fixed in methanol, and washed five times in PBS. At the time points indicated in Results, fluorescence was measured with excitation at 485 nm and emission at 590 nm by a BioTek Synergy plate reader (BioTek, VT, USA). The numbers of relative fluorescence units presented represent fluorescence from bacterial uptake minus autofluorescence from cells alone.

T-cell polarization assays. To induce Th17 cell differentiation, splenocytes from uninfected mice at a concentration of 2×10^6/ml were incubated for 4 days at 37°C in the presence of 1 μg/ml plate-bound anti-mouse CD3 antibody, 10×10^6 heat-killed *S. pneumoniae* bacteria, 10 ng/ml IL-2, 5 ng/ml transforming growth factor beta, 10 ng/ml IL-23, 10 ng/ml IL-6, 10 μg/ml anti-mouse IFN-γ, and 10 μg/ml anti-mouse IL-4. For Th1 polarization assays, splenocytes were incubated only in the presence of anti-CD3 antibodies, *S. pneumoniae*, and IL-2. All reagents were purchased from eBioscience (CA, USA), and intracellular cytokine staining was performed as described above.

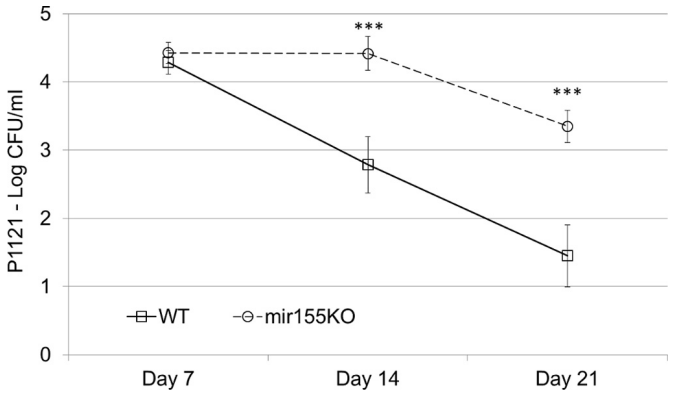

FIG 1 Loss of mir-155 impairs nasopharyngeal clearance of *S. pneumoniae* colonization. WT and mir155KO mice were inoculated intranasally with a known noninvasive colonizing strain of *S. pneumoniae* (P1121), and the bacterial loads in their nasopharynxes were enumerated. $n = 7$ to 16 per group per time point. Statistical significance was determined by the Wilcoxon rank sum test. ***, $P < 0.001$.

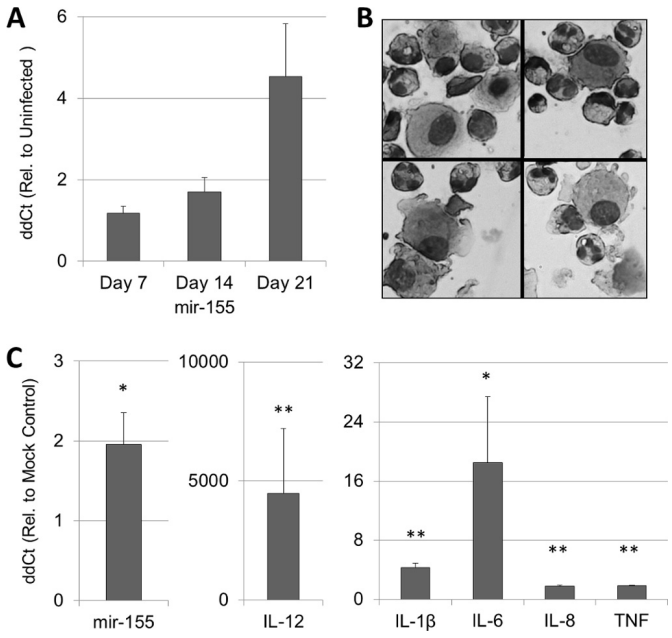

FIG 2 *S. pneumoniae* induces mir-155 in the murine nasopharynx, as well as in resident cells of the human upper respiratory tract. (A) WT mice were colonized intranasally with *S. pneumoniae* (P1121), and nasopharyngeal mir-155 expression was measured by quantitative PCR. $n = 3$ to 6 per time point. Sputum leukocytes (presented in panel B) were stimulated with *S. pneumoniae* or mock stimulated with PBS for 16 h; this was followed by the assessment of mir-155 and cytokine gene expression (C). $n = 3$ or 4 per gene. Rel., relative. Statistical significance was determined by paired *t* test on log-transformed C_T values. **, $P < 0.01$; *, $P < 0.05$.

Statistical analyses. All statistical analyses were performed in R. Where applicable, results are presented as means ± standard errors. Unpaired comparisons were performed by the Wilcoxon rank sum test, and paired comparisons were performed by Student's *t* test on log-transformed values to approximate normality.

RESULTS

Loss of mir-155 impairs the clearance of pneumococcal colonization. To assess the importance of mir-155 in the clearance of pneumococcal colonization, strain P1121 (serotype 23F) was used. This strain does not typically spread to the lungs or the periphery and can be detected in the nasopharynx up to 3 weeks following nasopharyngeal colonization (9, 11, 25). To model primary nasopharyngeal colonization, WT and mir155KO mice were inoculated intranasally with 10^7 CFU of *S. pneumoniae* and sacrificed at days 7, 14, and 21. Whereas WT mice clear nasopharyngeal bacteria in a linear fashion beginning at day 7, mir155KO mice did not begin to clear them until after day 14 (Fig. 1). Additionally, bacterial burdens in the nasopharynxes of mir155KO mice were significantly higher at days 14 ($P < 0.001$) and 21 ($P < 0.001$) and, as expected, bacterial invasion of the lungs was not detected in either group (data not shown).

During clearance of colonization with *S. pneumoniae* P1121, WT mice exhibit an increase of mir-155 in the nasopharynx as early as day 14 postinoculation, which continues to increase to day 21 (Fig. 2A). Given that nasopharyngeal lavages represent a heterogeneous mixture of local and recruited cell types, we sought to ascertain that mir-155 transcription in the nasopharynx does indeed originate from leukocytes. To test this, we purified leukocytes from human induced sputum, the majority of which were neutrophils and alveolar macrophages (approximately 60 and

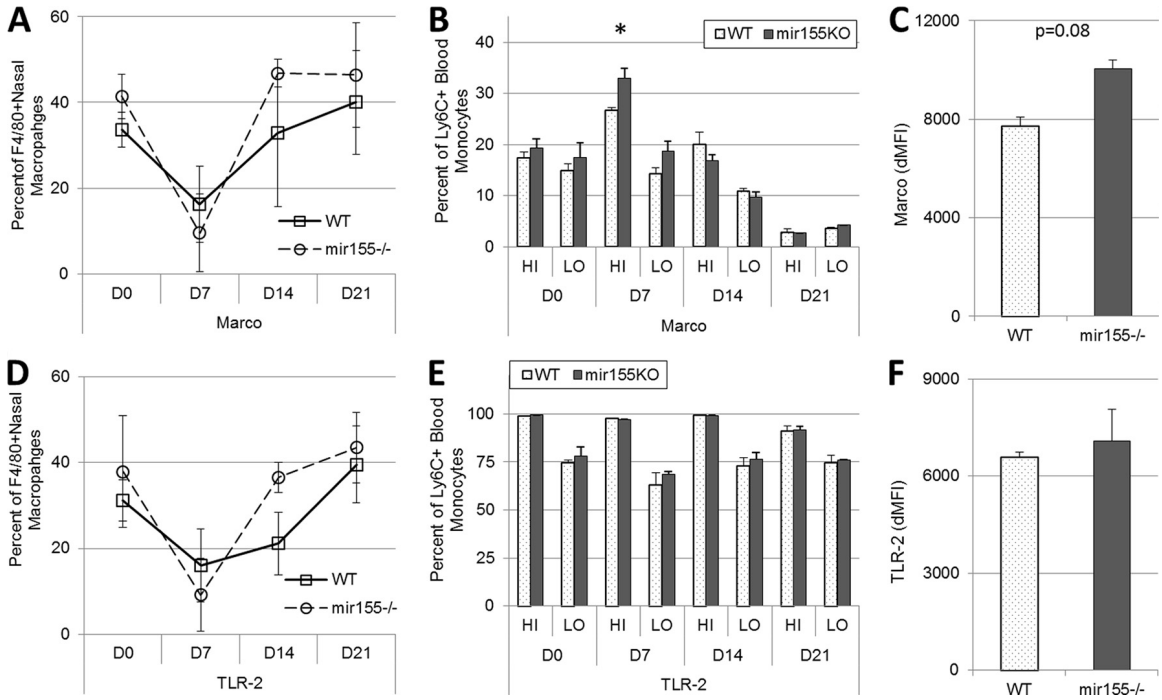

FIG 3 MARCO and TLR-2 expression is not impaired on monocytes or macrophages from mir155KO mice. The surface expression of MARCO and TLR-2 on nasal macrophages ($F4/80^+$) (A, D), and $Ly6C^+$ blood monocytes subsets ($Ly6C^{hi}$, HI; $Ly6C^{lo}$, LO) (B, E) in uninfected mice (D0) and over the course of colonization and elicited peritoneal macrophages (C, F) are shown. WT mice, squares with solid lines; mir155KO mice, circles with dashed lines. $n = 3$ to 5 per time point per group. Statistical significance was determined by Wilcoxon rank sum test. dMFI, mean fluorescent intensity minus isotype control level. *, $P < 0.05$.

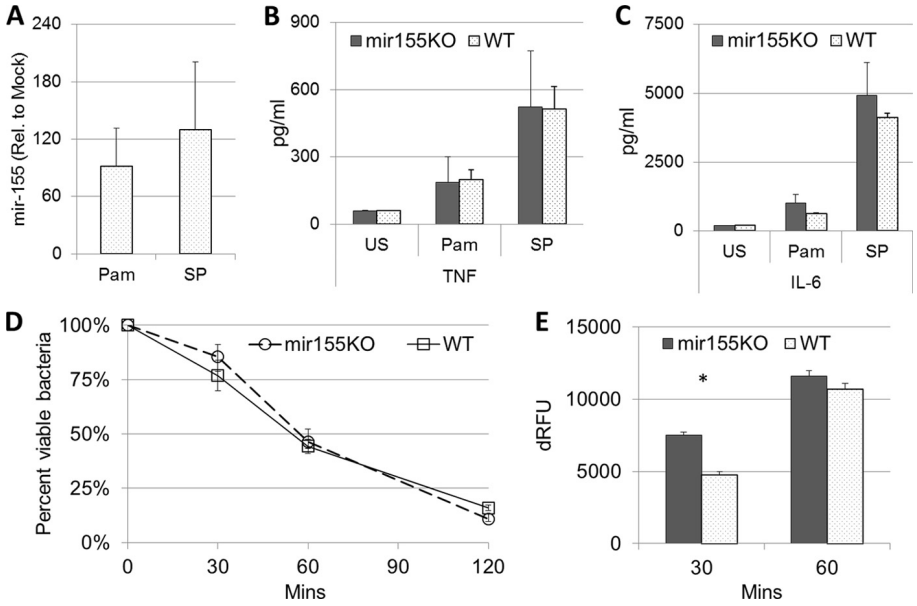

FIG 4 Loss of mir-155 does not impair innate cytokine responsiveness or bactericidal capacity of macrophages to *S. pneumoniae*. Elicited peritoneal macrophages from mir155KO (solid bars) and WT mice (dotted bars) were stimulated with Pam3CSK4 (Pam) and heat-killed *S. pneumoniae* (SP) for 24 h. This was followed by assessments of mir-155 expression (A) and TNF (B) and IL-6 (C) secretion ($n = 3$ per group per treatment). (D) Bacterial killing was measured by incubating macrophages at an MOI of 25 *S. pneumoniae* bacteria and enumerating the remaining viable bacteria at the time points indicated. (E) Bacterial uptake was measured with pHRodo-Red-labeled *S. pneumoniae* ($n = 4$ per group). Rel., relative; dRFU, relative fluorescent units minus the background level. Statistical significance was determined by Wilcoxon rank sum test. *, $P < 0.05$.

30%, respectively) (Fig. 2B), and stimulated them with heat-killed *S. pneumoniae* for 16 h. Indeed, *S. pneumoniae* induced mir-155 nearly 2-fold ($P = 0.046$, Fig. 2C), along with the cytokines IL-1β, TNF, IL-8, IL-12, and IL-6, all of which are important in the innate inflammatory response to infection (Fig. 2C).

Monocyte/macrophage MARCO and TLR-2 are unaffected by the loss of mir-155. We have previously shown that MARCO is required for the optimal clearance of *S. pneumoniae* colonization and is required for TLR-2 signaling (11). Loss of either of these innate receptors substantially diminishes the ability to clear nasopharyngeal colonization (9, 11); hence, we sought to characterize their expression in mir155KO mice. In uninfected WT and mir155KO mice and over the course of colonization generally, only subtle differences in the surface expression of MARCO or TLR-2 on nasopharyngeal macrophages ($CD11b^+$ $F4/80^+$) or blood monocyte subsets ($CD45^+$ $CD11b^+$ $Lineage^-$ and $Ly6C^{hi}$ or $Ly6C^{lo}$) were identified (Fig. 3A, B, D, and E). MARCO was found to be expressed more highly on $Ly6C^{hi}$ blood monocytes but only on day 7 postcolonization (Fig. 3B). Although there were no significant differences in the expression of TLR-2 (Fig. 3F), MARCO expression was found to be 25% greater on elicited peritoneal macrophages from mir155KO mice ($P = 0.08$, Fig. 3C).

To further characterize mir155KO macrophages, elicited peritoneal macrophages were stimulated with the synthetic TLR-2 ligand Pam3CSK4 and infected with heat-killed *S. pneumoniae*. Although mir-155 was strongly induced by both Pam3CSK4 and *S. pneumoniae* (Fig. 4A), there were no significant differences in the secretion of TNF or IL-6 at 24 h poststimulation (Fig. 4B and C). Similarly, when incubated with live *S. pneumoniae* under nonopsonic (serum-free) conditions, WT and mir155KO macrophages were equally capable of killing bacteria (Fig. 4D). However, mir155KO macrophages exhibited a subtle yet significant increase in phagocytosis ($P = 0.029$, Fig. 4E), which may be related to the aforementioned elevated expression of MARCO (Fig. 3C).

Macrophage recruitment to the nasopharynx is abolished in mir155KO mice. Myeloid cell recruitment to the nasopharynx is a critical event in the clearance of both primary and secondary *S. pneumoniae* colonizations (9). Here, we demonstrate that neutrophil ($CD11b^+$ $Ly6G^+$) (Fig. 5A) and monocyte ($CD11b^+$ $Ly6C^+$) (Fig. 5B) recruitment to the nasopharynx peaks at day 7 postinoculation, with no differences observed between WT and mir155KO mice. However, macrophage ($CD11b^+$ $F4/80^+$) recruitment, which peaked at day 14 postinoculation in WT mice, was completely abrogated in mir155KO mice ($P = 0.018$, Fig. 5C). This recruitment defect parallels a reduction in nasopharyngeal levels of the mRNA transcript encoding IL-1β (Fig. 5D), which is produced at high levels by macrophages during pneumococcal infection (26), as well as the initiation of bacterial clearance in WT mice (Fig. 1C). Also measured over the course of colonization were the mRNA transcripts encoding TNF, IL-10, MCP-1, MIP-1α, and MIP-2; however, no significant differences between WT and mir155KO mice were observed (data not shown).

Loss of mir-155 abrogates Th1- and Th17-mediated immunity. Previous studies also emphasized the importance of Th17 cells in the clearance of pneumococcal colonization and as a prerequisite for the recruitment of macrophages to the nasopharynx (6, 9). Others have indicated that IFN-γ-producing Th1 cells are also important in the protection against pneumococcal infection (27, 28). To monitor the induction of Th1 ($CD3^+$ $CD4^+$ IFN-γ^+) and Th17 ($CD3^+$ $CD4^+$ IL-$17A^+$) cells over the course of colonization with *S. pneumoniae* P1121, splenocytes from WT and mir155KO mice were examined by intracellular cytokine staining, while *S. pneumoniae*-specific splenic Th1 and Th17 cell responses were assessed by IFN-γ and IL-17A ELISAs, respectively. It ap-

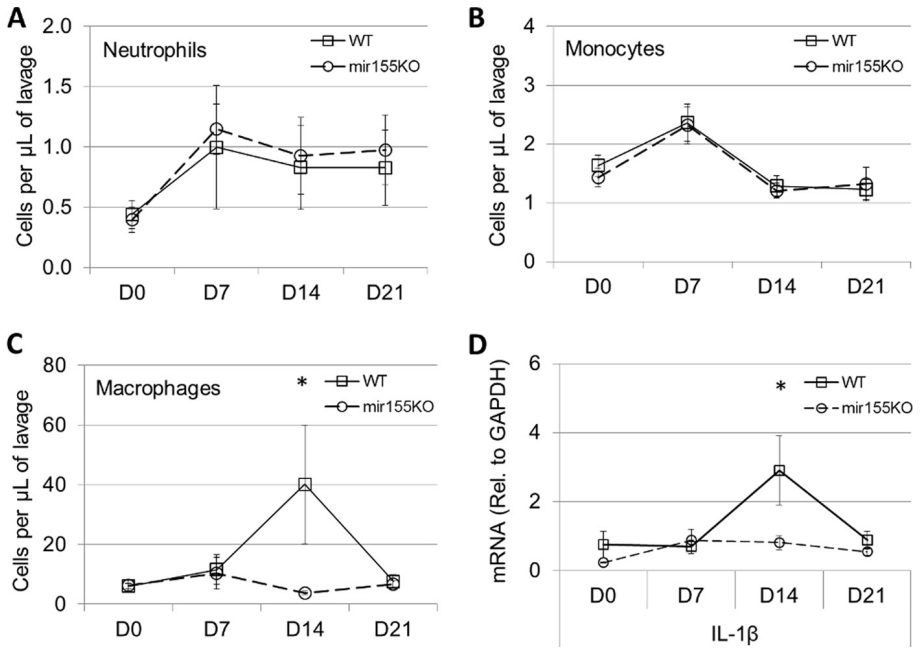

FIG 5 Macrophage recruitment is impaired in mir155KO mice over the course of colonization. In uninfected mice (D0), and at 7, 14, and 21 days postcolonization, the numbers of monocytes ($Ly6C^+$) (A), neutrophils ($Ly6G^+$) (B), and macrophages ($F4/80^+$) (C) in nasal lavage fluid were enumerated by flow cytometry. $n = 8$ to 16 per time point per group. (D) IL-1β expression in the nasopharynx was measured by quantitative PCR. $n = 4$ to 6 per time point per group. Rel., relative. Statistical significance was determined by Wilcoxon rank sum test. *, $P < 0.05$.

pears that mir155KO mice have inherently lower levels of Th1 (Fig. 6A) and Th17 (Fig. 6C) cells and a reduced capacity to induce Th1 cells over the course of colonization (Fig. 6A). Recall responses to *S. pneumoniae* indicate that antigen-specific Th1 immunity is maintained in mir155KO mice (Fig. 6B), while antigen-specific Th17 responses are reduced (Fig. 6D). In the nasopharynx, the levels of mRNA transcripts encoding IL-17A and IFN-γ were reduced in mir155KO mice at day 21 postcolonization; however, this reached significance only for IL-17A ($P = 0.043$, Fig. 6E). Finally, a significant reduction in the levels of *S. pneumoniae*-specific serum IgG antibodies was also observed in mir155KO mice ($P = 0.015$, Fig. 6F), further suggesting that these mice would be impaired in subsequent exposures to *S. pneumoniae*.

The above-described experiments indicate that mir155KO mice have a reduced capacity to generate Th1 and Th17 cells at the baseline, as well as in response to *S. pneumoniae* colonization. To ascertain whether this is an intrinsic defect of $CD4^+$ T cells, we cultured splenocytes from uninfected WT and mir155KO mice in the presence of *S. pneumoniae*, as well as under Th1 or Th17 cell-polarizing conditions. In concordance with our *in vivo* findings, there was significantly greater induction of Th17 cells from cultured WT splenocytes than from mir155KO splenocytes ($P = 0.016$, Fig. 7A and B); however, similar percentages of Th1 cells were induced in both groups (Fig. 7C and D).

The protection against pneumococcal invasion is not influenced by mir-155. To assess the importance of mir-155 in protecting against acute invasive infection, pneumococcal strain P1547 (serotype 6A), a virulent and invasive clinical isolate that has been shown to spread rapidly systemically following experimental nasopharyngeal colonization (25), was employed. Following inoculation with strain P1547, 30% of WT and 44% of mir155KO mice had detectable bacteria in their lungs on day 3 (chi-square $P = 0.86$) and 33% of WT and 50% of mir155KO mice had detectable bacteria in their lungs on day 5 (chi-square $P = 0.84$). The percentages of mice with bacteria in their lungs were not significantly different, nor were the overall bacterial loads in their lungs or nasopharynxes (data not shown). Similarly, the percentages of mice surviving to 5 days postinoculation were not significantly different (data not shown).

DISCUSSION

Our finding of mir-155 expression by leukocytes of the human upper respiratory tract led us to hypothesize that it regulates the response to *S. pneumoniae* and that changes in the kinetics or magnitude of expression may alter the ability to control pneumococcal colonization. Interestingly, while we observed no differences between WT and mir155KO mice regarding bacterial invasion of the lungs or survival following inoculation with a virulent and invasive strain of *S. pneumoniae*, mir155KO mice were significantly impaired in the ability to clear a less virulent colonizing strain. Specifically, whereas WT mice began to clear nasopharyngeal colonization at day 14 postinoculation, clearance by mir155KO mice was not apparent until day 21 and their bacterial loads were >2-fold greater than those of WT mice. The delayed induction of bacterial clearance by knockout mice correlated with significantly lower levels of mRNA for the inflammatory cytokine IL-1β in the nasopharynx. This is not surprising, as IL-1β has been previously shown to be essential in the control of nasopharyngeal pneumococcal colonization and subsequent invasive disease (26). The importance of IL-1β during primary colonization and secondary pneumococcal responses is likely due to its known role in the polarization of Th17 cells and sustained production of IL-17, as well as the indirect and direct recruitment of neutrophils to sites

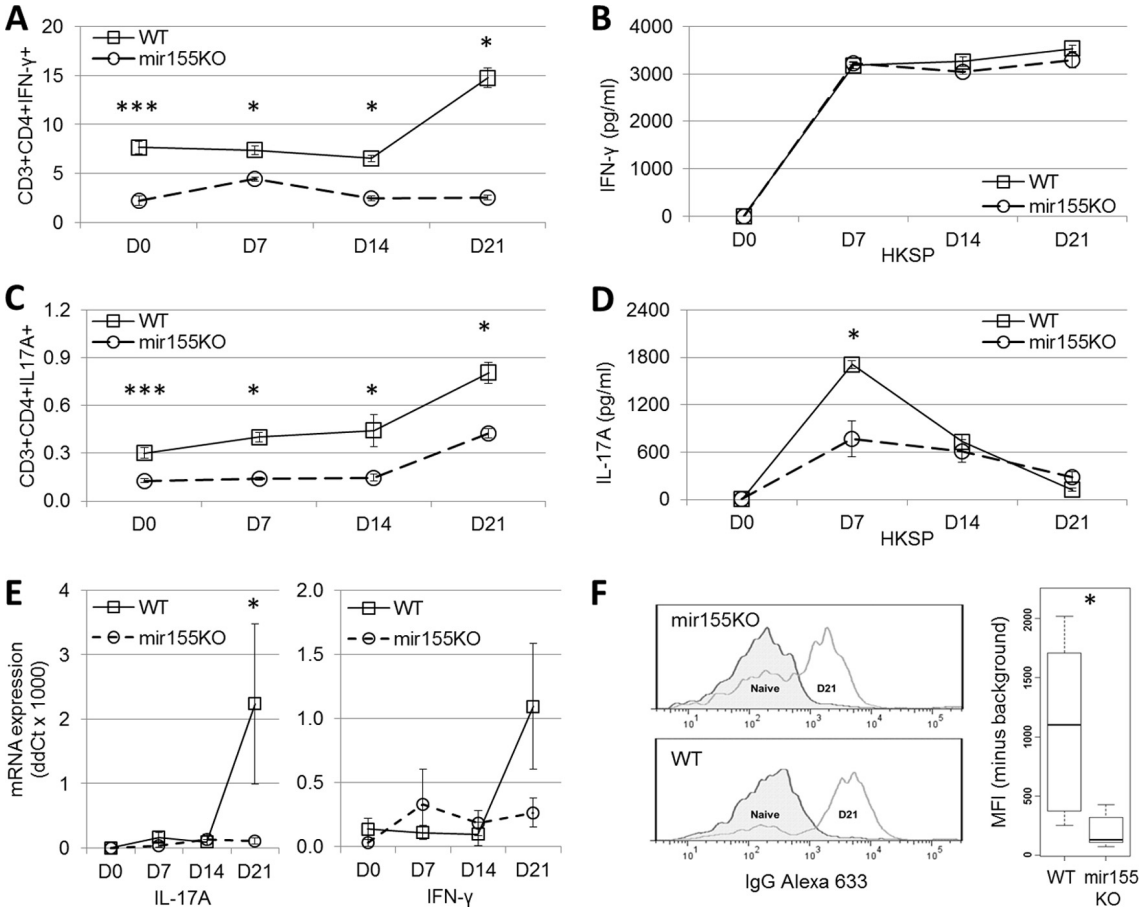

FIG 6 Adaptive immune responses to *S. pneumoniae* are impaired in mir155KO mice. Over the course of colonization, the frequencies of IFN-γ (A)- and IL-17A (C)-expressing $CD3^+$ $CD4^+$ splenocytes were enumerated by intracellular cytokine staining ($n = 4$ to 10 per group per time point) and the secretion of IFN-γ (B) and IL-17A (D) by splenocytes following 6 day of exposure to *S. pneumoniae* was measured by ELISA ($n = 4$ or 5 per group per time point). Levels of IL-17A and IFN-γ mRNAs in the nasopharynx (E) over the course of colonization ($n = 3$ or 4 per group per time point) and serum IgG levels (F) at day 21 postcolonization ($n = 4$ to 6 per group per time point) were measured. Representative flow cytometry histograms (left) and IgG mean fluorescent intensity (MFI) levels (right) are presented. Statistical significance was determined by Wilcoxon rank sum test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. D0, uninfected; D7, D14, and D21, days 7, 14, and 21 postcolonization, respectively.

of infection (29). Reduced IL-1β expression in mir155KO mice at day 14 postinoculation is likely due to the decrease in macrophage recruitment, as these cells are a major source of IL-1β during *S. pneumoniae* responses (30).

Previous studies by our lab and others (9, 11, 31) have indicated the critical importance of the innate receptors MARCO and TLR-2, as well as IL-17A-producing Th17 cells and macrophage recruitment to the nasopharynx for the clearance of primary nasopharyngeal colonization. In particular, we have shown that MARCO is required for sufficient TLR-2-mediated pneumococcal immunity, as well as being the primary phagocytic receptor for *S. pneumoniae* (11). Although MARCO is not a known target of mir-155, results from a recent study of macrophage responses to infection following bone marrow transplantation suggest that mir-155 may be at least indirectly related to MARCO-mediated phagocytosis of *Pseudomonas aeruginosa* (32). Additionally, mir-155 has been previously shown to be induced by TLR-2 signaling (16), as well as to modulate TLR-2-mediated responses (20, 33). Despite these findings, we found no evidence of impaired TLR-2 expression or signaling in mir155KO mice. Similarly, there were no differences in the expression of MARCO in the murine nasopharynx, although a subtle increase was observed on $Ly6C^{hi}$ blood monocytes at 7 days postcolonization and on elicited peritoneal macrophages from mir155KO mice.

A major finding of the present study was that mir-155 is integral for the recruitment of macrophages to the nasopharynx, a process that has been shown to be dependent on IL-17 (6, 9). During nasopharyngeal colonization, mir155KO mice exhibited lower nasopharyngeal IL-17A mRNA levels and reduced splenic Th17 numbers and IL-17A secretion upon restimulation with *S. pneumoniae*. A reduction of Th17 cells and IL-17A production in the nasopharynx would abrogate macrophage recruitment since IL-17A is a potent chemotactic factor for both monocytes (34) and macrophages (35, 36). Another important chemotactic factor for these cells, MCP-1 (CCL2), was also investigated in the nasopharynxes of WT and mir155KO mice; however, no differences were observed. As shown by our observations from Th17 polarization experiments, as well as previous studies (22), mir-155 is integral to the development of Th17 cells. The mechanism of this phenomenon is at least partly related to the repression of transcription factor Ets-1 (37), which is known to contribute to a myriad of cellular processes, including energy metabolism and innate in-

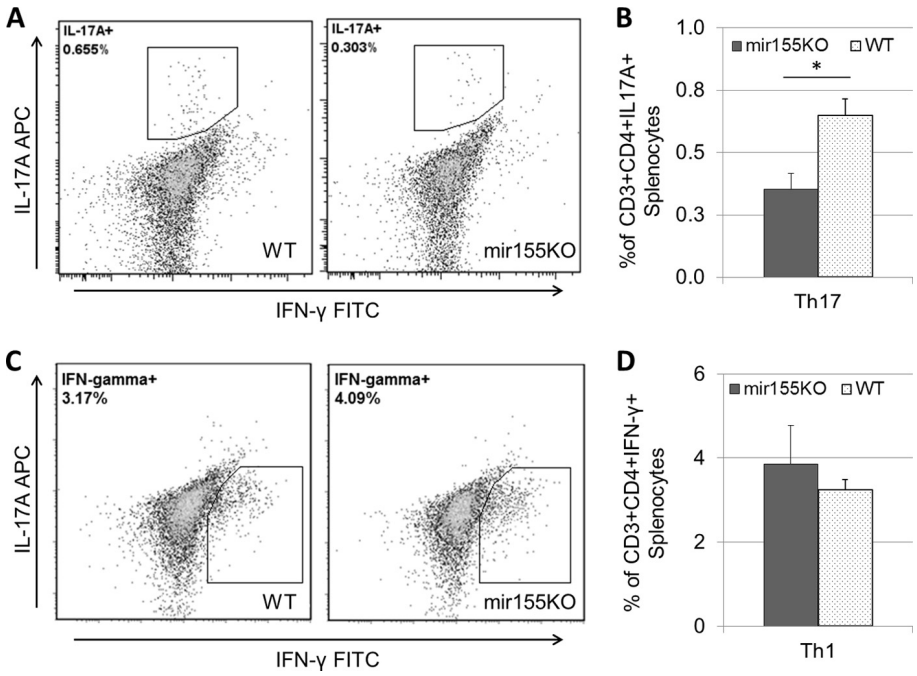

FIG 7 Th17, but not Th1, polarization is impaired in the absence of mir-155. Splenocytes from uninfected WT and mir155KO mice were stimulated with *S. pneumoniae* under Th17 or Th1 cell-polarizing conditions for 4 days. Representative dot plots and cumulative frequencies of $CD3^+$ $CD4^+$ IL-$17A^+$ splenocytes following incubation (A, B) and representative dot plots and cumulative frequencies of $CD3^+$ $CD4^+$ IFN-γ^+ splenocytes following incubation (C, D) are shown. $n = 5$ per group. Statistical significance was determined by Wilcoxon rank sum test. *, $P < 0.05$.

flammatory response-related signaling (38). We also observed reduced levels of IFN-γ-producing $CD4^+$ lymphocytes prior to and following *S. pneumoniae* colonization in mir155KO mice but no defect in the antigen-specific production of IFN-γ or the capacity to generate IFN-γ-expressing cells *in vitro*. Similar defects in the Th1 cell compartment of mir155KO mice have been previously reported (39); however, they are likely to be inconsequential during nasopharyngeal colonization, given that clearance is not effected in the absence of the IFN-γ receptor (25).

While it has been shown that IL-17A is required for the elevation of macrophage numbers in the nasopharynx during colonization (9), there is little evidence indicating that these macrophages originate from recruited precursor monocytes or via a self-renewing proliferative mechanism, which has been shown to occur for resident alveolar macrophages (40). Davis and colleagues in 2011 showed that the loss of CCR2, an essential chemotactic receptor for monocytes, abolishes the increase in cells expressing the macrophage marker F4/80 during pneumococcal colonization and that the expression of its ligand MCP-1/CCL2 correlates with bacterial clearance (41). In our studies, we observed an influx of monocytes 7 days prior to the elevation of F4/80-expressing macrophage numbers. Also, a small secondary population of macrophages expressing elevated levels of Ly6C (a prominent monocyte marker) and lower levels of F4/80 could be observed in the nasopharynx during colonization, although its frequency changed little over time (data not shown). These cells might represent monocytes that have not completely differentiated into mature nasopharyngeal macrophages, but this has yet to be determined.

Most individuals have been temporarily colonized by *S. pneumoniae* at least once in their lifetimes (42). Thus, antibody- and neutrophil-mediated defenses, in addition to Th17 memory cell subsets, are likely to be prominent effectors for the prevention of invasive disease following colonization (3, 7). Our observations suggest that mir-155 would be instrumental in conferring protective immunity to subsequent exposures, given that following primary colonization, mir155KO mice have fewer Th17 cells, as well as *S. pneumoniae*-specific circulating IgG levels. Presumably, this decrease in circulating IgG would be critical for the opsonic phagocytosis and subsequent killing of *S. pneumoniae* in the lung by resident macrophages and recruited neutrophils (3). Clearly, further studies of the importance of mir-155 following pneumococcal vaccination are warranted.

In summary, our study has provided compelling evidence that mir-155 is a necessary component of the mucosal immune response to *S. pneumoniae*. It is the first to describe a molecular mediator that is intimately involved in the recruitment of macrophages and the induction of protective Th17 immunity, which is fundamental for the clearance of primary nasopharyngeal colonization. Further exploration of the roles of mir-155 in protection against invasive pneumococcal disease and the promotion of effective vaccine responses would be most interesting.

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Chapter 6
Discussion

Summary

Streptococcus pneumoniae remains a significant medical and economic burden across the globe despite the relatively wide use of prophylactic measures, such as vaccines, and therapeutic medicines like antibiotics. Indeed, the latter is a significant source of this burden, as antibiotic resistance has risen to unmanageable levels in the past half century (1). The current focus of pneumococcal research remains producing a more comprehensive vaccination strategy that targets all pneumococcal serotypes in a safe, effective, and cost-efficient manner. This is a worthy task to be sure, but there remains a distinct lack of understanding regarding natural immunity to this bacterium. It is my opinion that better vaccination strategies will remain elusive until we are able to appreciate the complexity of the anti-pneumococcal response in healthy individuals. By gaining a more mechanistic understanding of these responses, we will be better able to replicate these responses in at-risk populations such as children, the elderly, and the immunocompromised. In this chapter, I will place the previous chapters in the context of the current pool of knowledge while highlighting their significance and aiming to limit repetition from the discussion sections held therein.

The overall goal of the work presented in this thesis was to establish a role for the class A SRs in the serotype-independent protection of mice from pneumococcal colonization and disease. We refined this somewhat vague and limited research question over time to create a rigorous inspection of the effects that MARCO expression has on pneumococcal colonization and the transition to invasive pneumococcal disease as well as the role of miR-155 on colonization dynamics. In-so-doing, we have attempted to

build on previous knowledge of macrophage-mediated anti-pneumococcal immune responses and push us further towards a universal pneumococcal vaccination strategy.

Discussion of Experimental Approach

While models of pneumococcal pneumonia are important for understanding how the immune system manages lung infection and invasive diseases like bacterial meningitis, much less is known about the response to colonization. Since all human pneumococcal disease is preceded by a colonizing event (8), we think it especially pertinent to understand how the immune system responds to these events and prevents serious infection. A reliable prophylaxis strategy will depend on preventing or limiting nasopharyngeal colonization and so anti-colonization immune activities should be the target of any universal vaccine against *S. pneumoniae*.

The experimental approach used throughout the previous chapters is based on a validated murine model of pneumococcal colonization used heavily by laboratories such as Jeff Weiser's [for example in (29, 36)]. The model involves inoculating unanaesthetized mice with a significant, but biologically relevant, number of viable bacteria in a small volume of PBS. The small inoculum volume combined with the lack of anaesthesia allows us to colonize the mice without the bacteria gaining immediate access to the lower respiratory tract. This fact is especially important for the study presented in **Chapter 4**, as we were measuring bacterial *invasiveness* from the nasopharynx and therefore did not want any other tissues being disturbed by our inoculum. By contrast, the majority of research on anti-pneumococcal responses use a significantly larger volume of PBS given intranasally to lightly anaesthetized mice whose deeper breaths force the bacteria directly into the lungs.

The time points chosen for euthanization of the infected mice were based on previous studies as well as optimization experiments performed in our laboratory. It is generally accepted that pneumococcal colonization lasts, in both humans and mice, for a two to three week period before the bacterial burden is reduced below limits of detection (9, 10). Previous studies also show that cellular recruitment of neutrophils, monocytes, and macrophages peak at three, seven, and seven days, respectively (40). Interestingly, humans and mice appear to be identical in these respects. As such, we chose to measure bacterial burden and cell densities at days one, three, seven, 14, and 21 post-inoculation for the studies shown in **Chapters 3 and 5**. These time points provided us with an accurate representation of the clearance of bacterial burden, shown by the steady decline in CFUs between days seven and 21. They also allowed for a robust analysis of the differences between bacterial burden in MARCO$^{-/-}$ mice and their WT counterparts. For the studies presented in **Chapter 4**, we chose a one day colonization method based on previous experiments performed in our laboratory (data in press). We had observed that when WT mice had significant negative reactions to bacterial colonization, they tended to succumb to pneumonia at days two or three post-inoculation with the invasive strain of *S. pneumoniae* P1547. As such, we euthanized mice at day one to determine which of the animals had bacteria escape from the nasopharynx at this time point.

Many studies regarding the role of a particular protein or gene are limited by the availability of a particular genotype of mouse. This can be due to the gene being necessary for fetal development or hardiness (i.e. mice lacking the gene cannot survive to maturity), or simply because no-one has engineered the particular mouse strain. Luckily, both MARCO- and miR-155-deficient mice are viable and reach maturity with no obvious abnormalities. miR-155 are also commercially available, making them easy to

procure. MARCO$^{-/-}$ mice are not available commercially, but a breeding colony has been established in our laboratory built on animal donations from Siamon Gordon's laboratory at Oxford University. Other knock-out mice used in the studies were either donated by other laboratories or bought from commercial suppliers.

The above-stated congruencies between murine and human colonization dynamics make the mouse an excellent model for pneumococcal colonization. This limits the need for other experimental animal models such as rats, non-human primates, or other larger animals that are more expensive and ethically unnecessary. Consequently, it is widely accepted that the mouse is the most preferable model of human pneumococcal carriage and disease. There are, however, limits to the mouse model as far as experimental approaches go. The size, shape, and orientation of the murine nasal passages make them near-impossible to collect samples from without euthanizing the animal, eliminating the chance for single-animal longitudinal studies. As a result, we are limited to studying colonization dynamics using separate groups of mice for each time-point. To reduce variation effects and properly power our statistical approaches in our experiments, we used *n* values of at least 10 for all *in vivo* experiments in our studies.

We use a number of bacterial strains throughout the previous three chapters, with P1121 and P1547 being the most used strains. These strains are serotype 23F and 6A, respectively. These serotypes were commonly found in people before the introduction of the current pneumococcal vaccines and are constituents of these vaccines (13). P1121, a clinical isolate used in **Chapters 3 and 5**, is an excellent colonizer of the nasopharynx but does not cause invasive disease regardless of the level of immunocompetence of the mouse. P1547 is a more invasive clinical isolate, with 50% of WT mice colonized with it having bacteria move to their lungs or other tissues within

two days and ~20% of mice succumbing to infection. There are significant differences between these bacterial strains and the immune response to them, which can account for discrepancies within the previous studies and between these studies and others. We have attempted, when we can, to make sure that we test a number of strains to ensure that MARCO (or miR-155) is necessary for clearance of a multitude of different pneumococcal serotypes.

The restriction of MARCO expression to monocytes, macrophages, and DCs leads us to believe that MARCO-related early cytokine and chemokine production can be attributed to resident macrophage and DC populations in the nasal mucosa and lumen. This is difficult to show directly, due to very low cell numbers in nasal lavages, hence our use of RNA lysis lavages in each of the studies presented in previous chapters. These lavages do not make it possible to determine which cell types are producing a particular cytokine and, as such, we were unable to determine which of MARCO's effects *in vivo* were directly associated with macrophages and which were indirect.

While no model is perfect, we believe that the mouse model of pneumococcal colonization has provided us with robust and highly replicable data establishing MARCO and miR-155 as important mediators of both the innate and adaptive immune responses. Our animal studies were supported by *in vitro* studies using murine and human macrophages, splenocytes, and cell lines. This variable approach has provided us with a great deal of confidence in our data.

MARCO and Pneumococcal Colonization

Insights from our mouse model

Class A SRs, including MARCO, have been shown to be important mediators of macrophage responses to a plethora of infectious bacteria (45, 52, 62). We have shown in **Chapters 3 and 4** that MARCO is central to the initiation of the inflammatory response to pneumococcal colonization. We first showed that neither the complement protein C3 nor the complement receptor MAC-1 were necessary for clearance of colonizing strains of the pneumococcus, adding to previous data from the Weiser laboratory showing that opsonizing antibodies were not necessary for this either (36). This contributed to our hypothesis that anti-pneumococcal clearance does not depend on opsonins and, thus, recognition of the bacteria must be through a non-opsonic mechanism. Previous studies showed that MARCO and SR-AI did not require opsonization to recognize their ligands and we were able to show that this applies to recognition of *S. pneumoniae* as well (45). Our initial colonization studies using both $MARCO^{-/-}$ and $SR\text{-}AI^{-/-}$ mice provided us with the key evidence that MARCO, and not SR-AI, is vital to the clearance of pneumococcal colonization, making this receptor the focus of the rest of our investigations.

Our data agree with the previously published timeframe for clearance of pneumococcal colonization in WT mice and show that this clearance is significantly limited in $MARCO^{-/-}$ mice (40). Therefore, the interaction between the pneumococcus and MARCO is vital to the expedient clearance of the bacteria from the nasopharynx. The reasons for this are multiple. Firstly, the induction of early inflammatory mediators such as TNF, IL-6, and IL-1β are all delayed in $MARCO^{-/-}$ mice. These cytokines are all necessary for establishing the inflammatory state, increasing blood flow, metabolic rate,

and cellular extravasation to the infected tissue. In this case, these cytokines provide the ideal environment for the influx of neutrophils and monocytes from the blood to the mucosal surface of the nasal passages (108). In our model, WT mice have a rapid recruitment of neutrophils to the site within one day of bacterial inoculation, followed by a comparatively delayed monocyte influx between days one and seven. These monocytes differentiate into macrophages. These data are in temporal agreement with previous studies showing that the neutrophils are unable to reduce bacterial load, which only begins to lessen as the macrophage population reaches its peak at day seven (40). MARCO-deficient mice lacking the early induction of pro-inflammatory cytokines also have delayed recruitment of these cell types, contributing to a delay in bacterial clearance.

Also contributing to the delayed cellular recruitment is a lack of chemokine production in the MARCO-deficient mice. RNA analysis of the nasal passages post-inoculation showed a paucity of *ccl2* transcription. Jeff Weiser's group has shown an unequivocal need for CCL-2 in order to coax CCR-2-expressing monocyte egress from the bone marrow early in colonization (29). The production of CCL-2 was shown to require the cytosolic PRR NOD-2, which recognizes MDP released from *S. pneumoniae* after bacterial uptake. MARCO's role in CCL-2 production likely stems from its phagocytic capacity, providing access for NOD-2 to its ligand. Indeed, we showed in **Chapter 2** that MARCO increases NOD-2-mediated NF-κB signaling in response to pneumococcal stimulation to a significant degree.

MARCO's role in NOD-2 and TLR-2 signaling [discussed below] could mean it is a central mediator of macrophage recruitment, with NOD-2 responses leading to CCL-2 production and TLR-2 signaling leading to the production of IL-17 by Th17 cells

(42). While CCL-2 is necessary for early recruitment of monocytes to the nasopharynx, IL-17 is required for sustained cellular recruitment during the clearance phase (days seven to 21) (26). Therefore, MARCO might have the dual roles of establishing early chemokine responses while supporting the transition to an IL-17-based adaptive response to colonization.

The focus of both **Chapters 3 and 4** was on MARCO's role in establishing the inflammatory response and preventing bacterial invasion early in colonization, respectively. In each of these studies we found that MARCO is important very early in the response, leading us to believe that MARCO expression on resident macrophages is a key factor in protecting mice from pneumococcal disease. Corresponding with this, we did not detect an increase in *marco* gene expression in the first seven days of colonization, despite the influx of macrophages to the nasopharynx at this time. Therefore, we believe that MARCO expression on recruited cells is not vital to the eventual clearance of the bacteria. As such, there must be other receptors on these cells that are capable of inducing uptake of the bacteria into the macrophages, which subsequently kill them. This also leads us to believe that bacterial colonization would eventually be cleared in the MARCO$^{-/-}$ mice, as we do see a significant influx of macrophages to the nasopharynges of these mice at day 21 post-inoculation. As such, further study is warranted on the mechanisms by which recruited macrophage populations recognize and destroy pneumococci.

MARCO clearly has an effect on the clearance of pneumococcal colonization from the murine nasopharynx. This stems from a lack of cytokine production and cellular recruitment in the nasopharynx itself, although MARCO's direct role in the

production of these cytokines or the recruitment of these cells remained a mystery based solely on nasal lavage data.

Innate immune signaling

Our *in vitro* work on the role of MARCO in the establishment of inflammatory responses used a combination of primary cells and cell lines. The use of HEK-293T cells for our signaling pathway analyses provided us with a platform that was easy to manipulate (through simple transfection techniques) and also provided us with a "blank slate" of sorts. HEK-293T cells do not express any of MARCO, SR-AI, TLR-2, CD-14, or NOD-2 on their own. However, they do express all of the signaling machinery necessary to activate NF-κB. This means that we were able to mix and match receptors and then stimulate the cells with pneumococci to see how each combination affected NF-κB activity.

These assays established evidence that MARCO interacts with another important mediator of the anti-pneumococcal inflammatory response - TLR-2, a PRR recognizing diacylated and triacylated lipoproteins. TLR-2's importance was also highlighted by Jeff Weiser's group in a previous paper (35, 40). Using the HEK-293T cells, we were able to show that MARCO works in conjunction with TLR-2 (along with its co-receptor CD-14) to boost NF-κB responses to pneumococcal stimulation. The boost in NF-κB activity associated with TLR-2 and NOD-2 fits in well with previous studies on MARCO's relationships with pro-inflammatory signaling. Both Siamon Gordon and Lester Kobzik's groups have shown that NF-κB responses are modulated in MARCO-deficient cells when stimulated with TLR and NLR ligands - though this has never been shown with *S. pneumoniae* before (52, 60, 62).

Explaining how MARCO's ability to recognize and take up bacteria could boost NOD-2 responses is quite simple, but this is not the case for membrane-bound receptors like TLR-2. Interestingly, though when MARCO was co-transfected with TLR-2 and CD-14, the cells responded with ~4-fold greater NF-κB activation compared to cells with just TLR-2 and CD-14, this trend was reversed when the cells were transfected with SR-AI instead of MARCO. This is an important distinction, as another laboratory has recently shown that SR-AI is able to directly diminish TLR-4 responses by binding to TRAF-6 and sequestering it away from the rest of TLR-4's signaling machinery (109). While we do not currently have the data to support it, it can be hypothesized that MARCO's interactions with TLR-2 might have more to them than simply bacterial uptake and a gain of ligand accessibility.

We showed in **Chapter 3** that the overall cellular association of *S. pneumoniae* with HEK293Ts did not require MARCO. That is, there must be more than one receptor on these cells capable of binding to the bacteria. However, MARCO-expressing cells were far superior to mock transfected cells when it came to internalizing the bacteria. These data appear to be supportive of Siamon Gordon's work showing that the class A SRs are capable of 'ligand scavenging' from the surface of cells, reducing the signaling capabilities of membrane-bound receptors like TLR-4 (62). However, these studies were performed in the context of *Neisseria meningitidis* infection, where LPS is the dominant inflammatory stimulus, so it remains unclear as to whether the same mechanisms can apply to TLR-2 and Gram-positive bacteria like *S. pneumoniae*. More in-depth analyses of the physical interactions between MARCO and TLR-2 will be necessary to parse out the mechanistic basis of how the SRs contribute to TLR-mediated NF-κB activity.

Invasive Pneumococcal Disease

Chapter 4 presents a study on how MARCO-mediated effects protect mice from IPD during a colonization event. There has been very little research performed on the transition from colonization to invasive disease, with most groups choosing to either study the nasopharynx and how bacteria are contained there, or on specific organs and how the immune system responds once an invasive disease state has been established. In this section, I will discuss the fine line between the two states of colonization and invasive disease and how some of our findings in **Chapter 3** have led to a better understanding of how immune cells and epithelial cells work together to protect the body from bacterial invasion.

Before I begin, however, it is important to establish what we mean by 'invasive disease'. There are two general ways in which one can separate colonization and disease. Many clinicians and research scientists use the crossing of the epithelial barrier as the cut-off between the two. Because bacteria can travel from the nasal passages to the lungs without crossing an epithelium, bacterial burden in the lungs is not considered invasive under this paradigm. The other way to define IPD is any acute and serious disease associated with *S. pneumoniae*, which includes pneumonia. This is the way in which we define IPD, with any bacterial burden outside of the nasopharynx being capable of instituting these diseases.

MARCO and type I IFN production

MARCO's role in protecting mice from IPD is, like its role in clearing colonization, based on modulating cytokine responses to the pneumococcus through binding and uptake of the bacteria. In this case, however, the important protein

mediators are not chemokines or interleukins but, rather, type I IFNs. Previous studies showed that pneumococcal colonization induces type I IFN production in the nasopharynx (80). The beginning of our studies on IFNs came during the production of the manuscript found in **Chapter 3**, where we found that *ifnb* gene transcripts were reduced in MARCO$^{-/-}$ nasal lavages when compared to those from WT mice early in colonization. We also found that macrophages from MARCO$^{-/-}$ mice produce less type I IFN in response to lysozyme-digested P1121. At the time, we hypothesized, based on a number of other studies, that MARCO's bacterial uptake abilities were leading to intravesicular signaling via PRRs leading to type I IFN production (87, 110).

As described in the Introduction, there is a healthy amount of literature describing type I IFN production in macrophages in response to bacteria and what receptors and signaling molecules are involved. A common thread in this literature is the necessity for PRR signaling from either the cytoplasm or intracellular vesicles. In the context of pneumococcal stimulation, it has been found that macrophages can respond to pneumococcal DNA via a STING-mediated mechanism (41, 86). STING is a cytosolic DNA sensor that activates IRF-3. This is certainly not the only way in which pneumococci induce type I IFN production in macrophages, however, since DNase-treated pneumococcal lysates were only ~35% worse at inducing *ifnb* transcription than untreated lysates in one paper in particular (41). Both of these studies have shown that pneumolysin must be present for the bacterial DNA to enter the cytoplasm, with one study showing that both phagocytosis and phagosome acidification are also necessary. Presumably, bacteria are taken up and then broken down in the phagosome, releasing pneumolysin, which can then produce pores in the phagocytic vesicle.

None of the studies mentioned in the previous paragraph studied the mechanisms by which pneumococci were engulfed by the macrophages, making it possible that MARCO is facilitating the type I IFN response through bacterial uptake. Indeed, we see in **Chapter 4** that cells pretreated with the uptake inhibitors dynasore hydrate and cytochalasin D are both less capable of producing type I IFN in response to P1547 than untreated cells. The magnitude of the type I IFN response in these cells is very similar to that of MARCO-deficient cells.

There is also evidence, in the context of other bacterial infections, that TLR-2 can contribute to type I IFN responses if it is signaling from endocytic vesicles. Purified ligand stimulation of macrophages causes TLR-2 to signal from these compartments in a MyD88-specific manner, and does so directly (i.e. not as part of an auto- or paracrine loop) (87). Again, the mechanism of uptake has not been elucidated in these studies, nor has the ability of TLR-2 to induce type I IFNs in response to pneumococci. In our studies, we showed that TLR-2 can, indeed, lead to the production of type I IFN in response to pneumococci and that these responses require uptake of the bacteria, likely through a MARCO-mediated mechanism.

Type I IFNs and pathogenesis of extracellular bacteria

MARCO-mediated uptake of pneumococci has a role in the production of type I IFN by macrophages. However, this is only one side of the story. The next question we asked was what effect this type I IFN production had on the transition from pneumococcal colonization to IPD. When we challenged mice with P1547 for one day, we saw that MARCO$^{-/-}$ mice were significantly more likely to experience bacterial invasiveness compared to WT mice. Work by Vanessa Redecke's laboratory has shown

that type I IFN can protect mice against IPD by inhibiting pneumococcal migration across epithelia, though in this study invasiveness was considered travel from the lung to the blood (81). Despite significant differences in our models, we aimed to show that MARCO-mediated type I IFN production was important for blocking bacterial transmigration across the nasal epithelium to the blood as well as transit from the nasopharynx to the lungs.

Epithelial barriers protect the host from invasive bacterial disease by forming a physical barrier to infection through the regulation of cell junction proteins. The junction proteins that we saw were regulated during colonization included ZO-1 and e-cadherin, but not claudin-5. Interestingly, based on the study mentioned above, ZO-1 and claudin-5 appear to be the most important junction proteins involved in protection of the lung epithelium from bacterial migration. This discrepancy is easily attributed to the fact that the two epithelial barriers we are dealing with are made up of different cells and that these two populations are regulated differently.

In order to show that the defect in protection seen in MARCO$^{-/-}$ mice is due to type I IFN, we chose to induce type I IFN production in these mice in a way that would bypass MARCO by giving a pre-treatment with poly(I:C). While it has been shown that MARCO can bind to and deliver double-stranded RNA molecules into a cell, deleting out one class A SR is not enough to significantly abrogate this process as SR-AI and SCARAs 3,4, and 5, are all capable of doing the same (111, 112). Poly(I:C) is able to stimulate the production of type I IFN in a wide variety of cells and has been used to do so in a plethora of mouse tissues in previous literature. When MARCO-deficient mice were given a small amount of poly(I:C), it induced *ifnb* gene transcription as well as increased levels of cell junction gene and ISG transcription. This, in turn, reversed the

MARCO$^{-/-}$ phenotype with the mice being as protected as their WT counterparts as far as bacterial invasiveness.

A recent study showed that treating influenza-infected mice with MALP-2, a TLR-2 agonist, provided them with protection against pneumococcal superinfection (113). While this study was performed using direct lung instillations of both the immunomodulator and the bacteria, and they did not measure type I IFN production, it would be interesting to see if intranasal application of TLR-2 agonists before pneumococcal colonization has a similar effect to our use of poly(I:C).

Occult bacteraemia, in humans, is the presence of bacteria in the blood without any of the expected symptoms associated with a systemic infection. These infections are almost exclusively found in children and caused by *S. pneumonia* (114). In the context of animal models of colonization, occult bacteraemia is the spread of bacteria directly from the nose to the blood, a very rare occurrence in mice. We are confident that the bacterial spread we see from the nasopharynx to the blood is occult bacteraemia and not transfer from the nose to the lungs and then to the blood because there are MARCO$^{-/-}$ mice that have bacteria in their spleens but not their lungs. To our knowledge, this is the first time that occult bacteraemia has been shown to such a degree in a mouse model. Interestingly, unpublished data of mine shows that, like humans, mice that have bacteria in their blood, but not their lungs, tend to lose less weight and have better overall survival than mice with active lung infections. We have not, however, tested what the long-term survival is for MARCO-deficient mice compared to WT mice when they are colonized with P1547. The data in **Chapter 4** present the case that type I IFN is important for protecting the host against occult bacteraemia and could be applied in the clinical setting.

As stated in the Introduction, there is an appreciable dichotomy in the literature as to whether type I IFN is beneficial or detrimental in the context of pneumococcal colonization. We show here that type I IFN is vital during the establishment of colonization to prevent the acute spread of bacteria from the nose to more vulnerable organs such as the lungs. The discrepancy in our data with those of other groups likely lies in the temporal variability with which primary colonization studies (like ours) and secondary pneumonia studies are performed. In studies on post-influenza pneumococcal pneumonia, massive amounts of type I IFN are produced either well before bacterial colonization or sustained for a longer time than in our studies. In the case of type I IFN production before pneumococcal colonization, it is known that this indirectly leads to the induction of high levels of IL-10 through the production of IL-27 (78). This IL-10 production, like that associated with many intracellular bacterial infections (see **Chapter 1**), will lead to hyporesponsiveness in macrophages once the colonization event occurs. This is likely to also occur when type I IFN or IFN-stimulating factors are given to mice throughout the first week of colonization, as is done in Nakamura *et al.*'s study (85). Interestingly, in this study the prolonged type I IFN induction leads to decreased macrophage recruitment associated with reduced *ccl2* transcription in the nose, much like our MARCO$^{-/-}$ mice. This would suggest that both too little and too much type I IFN has a detrimental effect on macrophage recruitment.

The study laid out in **Chapter 4** shows that type I IFN produced in response to extracellular bacteria can be very important for protecting the host against serious disease. While this is clearly not applicable in all cases, as seen in the studies on *Salmonella* Typhimurium infection wherein it is beneficial to lack type I IFN responses

(*77*), we believe that this work represents an important step in better understanding of the benefits of type I IFN responses to extracellular bacteria. Many potentially pathogenic bacteria colonize the nasal passages and, as such, type I IFN responses to these bacteria should be studied to see if the transition to invasiveness is occluded by the actions of these cytokines.

miR-155 and Th17 Cells

As stated in **Chapter 1**, our research on the effects of miR-155 on pneumococcal colonization began with a study published by Bethany Moore's group on the effects of prostaglandin-E_2 on SR expression and phagocytosis (*107*). This study showed that miR-155 is capable of repressing SR-AI expression in alveolar macrophages and that SR-AI upregulation can cause a concomitant downregulation in MARCO expression. While they did not test it directly, this infers that miR-155 expression is indirectly linked to MARCO expression. Indeed, the study showed that blocking miR-155 activity increased SR-AI expression and phagocytosis of *Staphylococcus aureus*, but did not look at long-term effects on MARCO expression in these cells. Preliminary data we had gathered showed that miR-155 expression was increased in the nasopharynges of pneumococcus-colonized mice as well as human sputum leukocytes stimulated with pneumococci *ex vivo*. As a result, we hypothesized that miR-155-deficient mice would have decreased MARCO expression on their macrophages leading to a phenotype similar to that which we found in **Chapter 3**.

When we colonized miR-$155^{-/-}$ mice with the colonizing strain P1121, we found that there was, indeed, a defect in the clearance of colonization in these mice. However,

there was no difference in MARCO protein expression on the cells from the nasal lavages of these mice compared to their WT counterparts. If anything, there was slightly increased MARCO expression. Compounding this, macrophages taken from the miR-155-deficient mice had no defect in the uptake or killing of pneumococci. Having found that our original hypothesis regarding MARCO regulation by miR-155 was incorrect, we expanded our search to other cell types that were important in pneumococcal clearance and had known effects for miR-155 activity.

Other phenotypic elements of the anti-pneumococcal response were shared between the MARCO- and miR-155-deficient mice, including macrophage recruitment to the nasopharynx. Interestingly, the graphs for macrophage recruitment and *il1b* gene transcription for miR-155$^{-/-}$ are remarkably similar. Since macrophages are a significant producer of IL-1β, and we had shown that IL-1β production at the cellular level was not affected by miR-155 expression, it is likely that the paucity of IL-1β we see in the nasopharynx is directly attributable to the lack of macrophage recruitment. This contrasts with what we see in **Chapter 3**, in that IL-1β production was significantly reduced in MARCO-deficient macrophages and the *il1b* gene transcription data did not match well with macrophage recruitment dynamics. These differences are further evidence that the effects of miR-155 on pneumococcal colonization are not directly macrophage-driven.

A significant portion of miR-155-related immune studies centre on its role in the activation and differentiation of inflammatory helper T cell types, namely Th1 and Th17 cells (115). Th17 cells, and IL-17 itself, have also been shown to be important for the recruitment of monocytes and macrophages to the nasopharynx later in pneumococcal colonization. Jeff Weiser and Richard Malleys's group showed that TLR-2 signaling

leads to the production of $CD4^+IL\text{-}17^+$ cells which produce high levels of the IL-17 necessary to sustain monocyte and macrophage recruitment to the nasopharynx during pneumococcal clearance (26, 42). Our preliminary data showed that clearance of pneumococci from the nasopharynx was impaired in miR-$155^{-/-}$ mice, as was macrophage recruitment at day 14 post-inoculation. As such, our hypothesis was changed to reflect miR-155's role in IL-17 production and the subsequent clearance of the bacteria.

In agreement with our data showing reduced macrophage recruitment in the nasopharynges of miR-155-deficient mice, there were also reduced levels of *il17a* gene transcription in the nasal lavages of these mice at day 21 post-inoculation. This was the only time point that the gene was significantly induced in WT mice, with miR-$155^{-/-}$ mice showing very little induction. This correlated with a significant reduction in $CD3^+CD4^+IL17^+$ cells in the spleens of these mice throughout colonization compared to their WT counterparts. Interestingly, there were less IL-17- and IFN-γ-producing T cells in the spleens of the miR-155-deficient mice even at day 0, showing an intrinsic defect in the production of both Th1 and Th17 cells. However, only Th17 responses were suppressed when splenocytes from uncolonized mice were stimulated with pneumococci under the appropriate polarizing conditions. These data suggest that miR-155-deficient mice are fully capable of inducing Th1 cells, though there may be a bias towards other helper T cell subtypes such as Th2 cells. This is in agreement with previous studies showing elevated IL-10 production in these mice (116). More importantly here, the miR-$155^{-/-}$ mice appear to be incapable of effective Th17 cell differentiation.

We did not attempt to uncover the mechanisms by which miR-155 modulates Th17 differentiation, as this topic would easily fill a thesis on its own. Stefan Muljo's

group recently published a study showing that miR-155 can influence epigenetic factors to increase cytokine gene expression (117). In their model, Th17-associated cytokines were silenced in the absence of miR-155 due to a lack of transcriptional regulation of Jarid-2, a DNA-binding protein that recruits the Polycomb Repressive Complex 2 (PRC-2) to chromatin. In the absence of Jarid-2, PRC-2-mediated gene silencing was reduced. As such, it would be interesting to see if Jarid-2-deficient mice were better equipped to clear pneumococcal colonization or if silencing of Jarid-2 could rescue the miR-155$^{-/-}$ phenotype.

Interestingly, it has been shown that miR-155 can be positively regulated by type I IFNs in macrophages as well as inducing type I IFN production itself, though this has only been shown in CD8^{+} T cells (97, 118). While we did not look into type I IFN production in our miR-155$^{-/-}$ mice, we did not see any difference in invasiveness between these mice and their WT counterparts when they were colonized with P1547 instead of P1121. This suggests, based on the study in **Chapter 4**, that type I IFN production is not significantly affected in the nasopharynges of miR-155$^{-/-}$ mice.

To conclude, while our original hypothesis regarding miR-155-dependent MARCO regulation was not supported by our studies, we uncovered an important facet of the anti-pneumococcal response. miR-155's regulation of Th17 polarization and the subsequent IL-17-mediated macrophage recruitment to the nasopharynx is indispensable for the clearance of pneumococcal colonization. Also, while MARCO was not directly involved in this process, its interactions with TLR-2 signaling pathways may very well play into the transition to Th17-mediated immunity.

Applications to Human Health

Streptococcus pneumoniae is one of the most prevalent and lethal pathogens in the world. As such, any research being performed on the interactions between this bacterium and the immune system should maintain an eye on improving human health through prevention of colonization or treatment of IPD. We believe that the best strategies for eliminating pneumococcus-related morbidity and mortality are prophylactic ones that target the colonization of the nasopharynges. Unfortunately, we still do not know enough about the development of protective immunity at mucosal surfaces or the natural protective immune response against the pneumococcus.

The use of innate immune modulating agents, and especially TLR agonists, as adjuvants has become common in vaccine design in recent decades (119). Since MARCO is so important in establishing the inflammatory response and the transition from innate to adaptive immunity in the nose, it could be postulated that any agent boosting MARCO expression would help vaccine efficacy in this anatomical niche. Intranasal vaccines, such as the FluMist influenza vaccine, are safe, effective, and, most importantly, circumvent the need for injections. An intranasal pneumococcal vaccine would have these benefits while also inducing protective immunity at the site of colonization. If an agent that increases the expression of MARCO were added, this could potentially increase vaccine constituent uptake by local macrophages and DCs, facilitating antigen presentation.

Type I IFN-related prophylaxis against IPD would be a more difficult proposition. Since it has been shown that prolonged type I IFN expression (as occurs during influenza infection) is detrimental to anti-pneumococcal responses, it would be unwise to establish a pro-IFN milieu in the nasopharynx for long periods of time. It is

also impossible to know when an individual will be colonized with *S. pneumoniae* due to its ubiquity in the environment. However, increasing MARCO expression in the nasopharynges of at-risk individuals could provide for a boost in type I IFN production at the outset of colonization, therefore protecting them from IPD. Of course, more research will need to be done regarding MARCO's role in this type I IFN production and the longer-term effects of this cytokine production in animal models.

The miRNAs have become a very promising avenue for treatment and prevention of diseases due to their small size and specificity (120). If clinicians were able to harness the powers of gene regulation presented by miRNAs, the potential for therapy would be unrivalled. Current studies on miRNA-based therapeutics are mostly targeted to oncolytic therapies and cardiovascular disease as opposed to infectious diseases. These therapies all suffer from an inability to properly target particular cells as well as the speed at which the body eliminates the miRNAs. However, there have been advances in these areas and multiple miRNAs are being used in clinical trials today. As such, it is easy to believe that the widespread use of miRNA-based therapeutics could be on the horizon.

We, and others, have shown that miR-155 is important for regulating Th17 responses (102, 116, 121). Others have also shown that Th17 cells are vital to clearing pneumococcal clearance (26, 42). Clinical studies have also seen that children with persistent pneumococcal colonization tend to have lower levels of Th17 cells with higher $Foxp3^+$ regulatory T cell frequencies in their adenoidal tissues (122). It is possible that increasing miR-155 activity in these children (and other at-risk populations) would lead to more robust anti-pneumococcal responses and less chance of recurrent nasopharyngeal colonization. The potential outside of pneumococcal colonization is

significant as well, with a lack of Th17 cell differentiation being associated with many other diseases as well. A recent publication in *Mucosal Immunology* shows that a novel nasal vaccine against *S. pneumoniae* induces miRNA-associated Th17 cells, conferring protection in macaques (123). This is certainly a step in the right direction as far as a universal pneumococcal vaccine.

The basic research performed in this thesis could have clinical ramifications in the near future due to the rapid maturation of gene editing, immunomodulating, and miRNA-based therapeutic tools occurring today. Tuning the expression of MARCO, miR-155, or any of the other proteins and receptors described herein could have monumental effects of pneumococcal clearance, especially in at-risk populations.

Comments and Future Directions

The studies held within this thesis represent a collection of steps towards a full understanding of how the immune system reacts to, and disposes of, colonizing pneumococci in the nose. Like all immune responses, this is an intricate process involving a multitude of cells, proteins, and other molecules that are all tightly regulated. As such, we do not yet fully understand the role of MARCO in co-ordinating these reactions. However, the following points have now been established and will aid in future research into the anti-pneumococcal immune response:

1. MARCO is an uptake receptor for *S. pneumoniae* and helps establish the inflammatory response to this bacterium in the nasopharynx.
2. MARCO expression modulates the activity of PRRs, including TLRs and NLRs, in response to the pneumococcus.

3. MARCO is vital for protection against IPD through the production of type I IFNs post-pneumococcal colonization.
4. Type I IFN in the nasopharynx leads to increased epithelial barrier integrity and decreased bacterial invasiveness.
5. miR-155 aids in the differentiation of Th17 cells in colonized mice, allowing for the production of IL-17 and the sustained recruitment of macrophages to the nasopharynx.

Given unlimited time and resources, we could delve deeper into many of these statements. The most intriguing, in my opinion, is the modulation of TLR-2 and NOD-2 signaling by MARCO. While work in our laboratory has established how MARCO binds to its ligands via the SRCR domain (data in review), the signaling mechanisms associated with MARCO-mediated uptake and cytokine responses remain a mystery. We have evidence that MARCO's role in this regard is based around bacterial uptake, however we do not know whether this simply leads to increased ligand availability to the PRRs or if MARCO is able to alter signaling dynamics in a more direct manner. Evidence that SR-AI can alter signaling by sequestering scaffold proteins give us reason to believe MARCO may have a similar role, but with opposite effect (109). Regardless, a better understanding of how MARCO affects pro-inflammatory signaling would increase the therapeutic potential of MARCO modulation.

There are many questions left to be asked regarding MARCO's role in colonization dynamics as a whole. For example, what is MARCO's role in editing the nasal microbiota? The nasal mucosa is home to a plethora of bacterial species making up the natural flora. While most of these bacteria are harmless, or even beneficial, to the host, there are constantly potential pathogens colonizing these surfaces as well. The

immune system requires a way in which to distinguish these two populations, which may be through the expression of ligands for putative bacterial recognition receptors like the class A SRs. In fact, unpublished data from our laboratory points to significant alterations in the microbiota of MARCO$^{-/-}$ and SR-AI$^{-/-}$ mice compared to WT. Changes in microbiota have been shown to be both indicative of, as well as predictive of vulnerability to, infectious disease. As such, it would be pertinent to research how these receptors affect the nasal microbiota and how these effects alter pneumococcal colonization.

The effects of MARCO expression on DCs has been understudied compared to that on macrophages. Antigen uptake by DCs is vital to the transition from innate to adaptive immune responses. Since MARCO is a major uptake receptor of bacteria, it stands to reason that MARCO aids in this process in DCs. While there has been some study of this (124), a more thorough look at how MARCO affects DC responses to bacteria will be beneficial for understanding how colonizing bacteria elicit adaptive immune responses and could aid in vaccine development.

Occult bacteraemia, in general, remains poorly understood. While patients presenting with occult bacteraemia normally clear the infection without the need for intervention, a small percentage of patients may have secondary complications that can be fatal (114). Uncontrolled pneumococcal colonization may contribute to bacteraemia in these children, and as such it is important that we are able to study the causes of this infection in an animal model. MARCO$^{-/-}$ mice may represent such a model. These mice could be used to research the cause and effects of pneumococcal bacteraemia as well as generate a better understanding of how the body clears these infections without the need for outside intervention.

To conclude, the studies presented in this thesis provide a wealth of novel knowledge in how the body protects itself from pneumococcal colonization and IPD. These studies also provide background for a number of potential hypothesis-driven inquiries into the role of MARCO in innate immune responses as well as the importance of type I IFNs and microRNAs in response to extracellular bacterial pathogens. I hope that these studies, and those that they inspire, will lead to more effective prophylactics or interventions for those that suffer from pneumococcal disease.

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