Regulatory Role of Monocytes and Macrophages in Infection and Immunity

Adam G. Peres

Department of Microbiology & Immunology

McGill University, Montreal

June 2018

A thesis submitted to McGill in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

Abstract

The immune system uses a network of defense mechanisms that protects the host from infections. Although essential for host survival, this system can also be detrimental due to excessive inflammation and tissue damage. Therefore, hosts have evolved anti-inflammatory mechanisms aimed at controlling pro-inflammatory responses and contributing to host-commensal or -pathogen interactions and disease tolerance. Monocytes and macrophages are cells that are often the initiators and targets of both pro- and anti-inflammatory responses, and play major roles in the pathogenesis and regulation of many diseases. This thesis explored the role of two such immune-regulatory molecules, interleukin 10 (IL-10) and the aryl hydrocarbon receptor (AHR) in the regulation of monocyte responses.

The first manuscript investigated the capacity of nasal *Staphylococcus aureus* isolates to induce the production of the anti-inflammatory cytokine IL-10 by human primary monocytes. Using laboratory strains of *S. aureus*, our lab had previously reported the importance of IL-10 in regulating T cell activation by staphylococcal superantigens (SAg). Here, we demonstrated that the induction of IL-10 by 16 nasal isolates of *S. aureus* differed by up to 3-fold. Mechanistically, the magnitude of IL-10 induction was due to differential degree of activation of the PI3K-Akt-mTOR pathway by *S. aureus*. The IL-10 response to *S. aureus* could also be uncoupled from the pro-inflammatory TNF-α response by both the signaling pathways used and the requirement for internalization and phagosome maturation. Lastly, the IL-10-inducing capacity of a nasal *S. aureus* isolate correlated with the suppression of SAg-induced T cell activation and lower Th1 cytokine production.

The other manuscripts included in this thesis focused on the regulation of AHR in the context of monocyte and macrophage responses. We first examined the expression of the AHR

gene program in monocytes during inflammation. To do this, we concomitantly stimulated human monocytes with either the toll-like receptor 4 (TLR4) ligand LPS or *S. aureus*, and an AHR ligand FICZ and found that LPS or *S. aureus* specifically blocked the induction of *Cyp1a1* and *Cyp1b1* mRNA. These enzymes metabolize AHR ligands to negatively regulate its activation. Interestingly, FICZ-induced CYP1 protein activity was restricted to monocyte-derived macrophages differentiated by GM-CSF (GM-MDMs) and was also regulated by LPS. Lastly, AHR ligands regulated cytokine production in GM-MDMs.

Next, we explored the CYP1-AHR axis in the host response to endotoxin and endotoxin tolerance. Mice lacking AHR were more susceptible to endotoxemia than wild-type mice due to excessive cytokine production, but did not present an impairment in the establishment of endotoxin tolerance. We corroborated these findings *in vitro* using primary human monocytes. We also observed that, during endotoxemia, liver *Cyp1a2* expression and activity was significantly reduced. We therefore hypothesized that *Cyp1a2*-deficient mice would be protected from LPS-driven immunopathology because of an increased bioavailability of AHR ligands that would dampen the inflammatory response by macrophages. However, we observed higher susceptibility of these mice to a primary LPS challenge. The mechanistic explanation for this finding is unclear and is still currently being investigated.

Collectively, the work in this thesis furthered our understanding of the involvement of IL-10 and the AHR gene program in the immune regulatory role that monocytes and monocyte-derived macrophages play during commensalism and infectious diseases.

Résumé

Le système immunitaire possède un vaste réseau de mécanismes de défense essentiel à la survie de l'hôte, mais qui peut aussi lui être néfaste en causant une inflammation excessive et des lésions tissulaires. Les hôtes ont ainsi développé des mécanismes anti-inflammatoires visant à contrôler les réponses pro-inflammatoires contre un pathogène et à contribuer aux interactions hôte-microbe, ainsi qu'à leur tolérance aux maladies. Les monocytes peuvent être à la fois les initiateurs et les cibles des réponses pro- et anti-inflammatoires. Ils jouent par conséquent un rôle majeur dans la pathogénicité d'un grand nombre de maladies et leurs régulations. Ce travail de thèse a étudié le rôle de deux molécules anti-inflammatoires, IL-10 et le AHR, dans la régulation des réponses monocytaires.

Dans le premier article, nous avons analysé la capacité d'isolats nasaux de *S. aureus* à induire la production d'IL-10 par les monocytes. Grâce à l'utilisation de souches *S. aureus* de laboratoire, notre équipe a déjà mis en évidence l'importance de l'IL-10 dans la régulation de l'activation des cellules T par les SAg de *S. aureus*. Nous avons démontré que l'induction d'IL-10 par 16 souches nasales de *S. aureus* pouvait différer jusqu'à 3 fois en intensité. D'un point de vue mécanistique, la variation d'amplitude de l'induction d'IL-10 était causée par une activation différentielle de la voie de signalisation PI3K-Akt-mTor par *S. aureus*. Aussi, la production d'IL-10 en réponse aux différents isolats de *S. aureus* se différenciait de la réponse du TNF-α, au niveau des voies de signalisation utilisées, mais aussi au niveau de l'internalisation et de la maturation des phagosomes. Enfin, la capacité de souches nasales de *S. aureus* à induire l'IL-10 corrélait avec la suppression de l'activation des cellules T par leurs SAg et une plus faible production de cytokines Th1.

Les autres publications étaient plus particulièrement focalisées sur la régulation du facteur de transcription AHR dans le contexte des réponses monocytaires et macrophagiques. Nous avons tout d'abord étudié l'expression des gènes régulés par l'AHR dans les monocytes au cours du processus d'inflammation. Pour cela, nous avons stimulé des monocytes humains soit avec du LPS, ligand du TLR4, soit avec *S. aureus*, simultanément avec FICZ, un ligand de l'AHR. Les résultats ont montré que le LPS et *S. aureus* bloquaient spécifiquement l'induction des gènes *Cyp1a1* et *Cyp1b1*. Ces deux enzymes métabolisent les ligands de l'AHR afin d'inhiber son activation. L'activité de la protéine CYP1 induite par FICZ était restreinte aux macrophages dérivés de monocytes différenciés par GM-CSF (GM-MDMs), mais était aussi régulée par le LPS. Enfin, les ligands activateurs de l'AHR régulaient aussi la production de cytokines dans les GM-MDMs.

Nous avons ensuite exploré l'axe CYP1-AHR dans la réponse de l'hôte aux LPS et la tolérance aux endotoxines. Les souris dépourvues d'AHR étaient plus susceptibles que les souris de type sauvage au LPS suite à une surproduction de cytokines, mais ne présentaient pas de défaut dans l'établissement de la tolérance aux endotoxines. Ces résultats ont ensuite été confirmés *in vitro* dans des monocytes primaires humains. Nous avons également observé que lors de l'endotoxémie induite par LPS, l'expression et l'activité hépatique de *Cyp1a2* étaient significativement réduites. Nous avons ainsi émis l'hypothèse que les souris déficientes pour *Cyp1a2* seraient protégées de l'immunopathologie causée par le LPS grâce à une meilleure biodisponibilité des ligands de l'AHR, ce qui permettrait ainsi de modérer la réponse inflammatoire des macrophages. Nous avons cependant observé une susceptibilité accrue de ces souris à l'endotoxémie primaire.

Dans l'ensemble, cette thèse a permis d'approfondir notre compréhension du rôle de l'IL-10 et des gènes régulés par l'AHR dans le rôle immuno-régulateur que jouent les monocytes et les macrophages, lors de commensalisme et de maladies infectieuses.

Acknowledgements

My PhD could not have been completed without the tremendous support from my supervisor Dr. Joaquín Madrenas. Quim, I can not over-state how much your mentorship has meant to me. Your knowledge and enthusiasm for science made every day in the lab enjoyable. I thank you for your dedication to my project and for always taking the time to discuss science. I never felt neglected. I would also like to thank the members of the Madrenas lab: Camille, Andrey, Ben, Benoit, Cynthia, Jelani, Joao, Pete, and Zhigang, for your collegiately and discussions over the years. I was also fortunate to work with numerous motivated undergraduates, most importantly An Qi.

I would also like to thank Dr. Irah King for adopting me over the final year of my PhD. Irah, you opened my eyes up to a new way of approaching science and eased my transition to field mouse immunology. I am grateful for the time you devoted to understanding my project. I thank the members of the King lab: Ghislaine, Alex, Amicha, Barbara, Cindy, Hualin, Marilena, and Yue, for accommodating me over the past year.

The work in this PhD could also have not occurred without our collaborators, including Drs. Benoit Cousineau, Martin Desrosiers, Michael Surette, and Robert Zamboni. I need to thank Dr. Benoit Cousineau a second time for his guidance while sitting on my PhD advisory committee. I would also like to thank the Department of Microbiology & Immunology at McGill University for their continuous support during my PhD, and the CIHR Neuroinflammation Training Program and Fonds de la Recherche Santé de Québec for salary support.

I also need to thank those in my personal life. To my Sault and Montreal friends: I thank you for providing the essential balance to my lab life. To my parents, Gregory and Louisanna, and my sisters, Erica and Nadia: thank you for the unconditional love and support, and endless supply of care packages. I love you all. And finally, to Amandine: I thank you for your patience and for your understanding. You are the light during my darkest days. Je t'aime.

Table of Contents

List of Tables

Chapter 1

Chapter 2

Appendices

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Contribution to Original Knowledge

In Chapter 2, published in *Infection & Immunity* in 2015 [1], we documented the uncoupling of pro- and anti-inflammatory responses to *S. aureus* by human PBMCs. Specifically, we showed that:

1. The capacity of nasal *S. aureus* isolates to induce pro- and anti-inflammatory responses in human peripheral blood mononuclear cells is heterogeneous.
2. Diverse *S. aureus* isolates differentially activated the PI3K-Akt-mTOR, which correlated with their IL-10-inducing capacity.
3. The pro- and anti-inflammatory responses to *S. aureus* used different pathways.
4. The anti-inflammatory response to *S. aureus* did not require phagocytosis and phago-lysosome maturation, unlike the pro-inflammatory response.
5. The IL-10-inducing capacity of a *S. aureus* isolate correlated with its capacity to regulate adaptive immunity.

In Chapter 3, published in the *Journal of Leukocyte Biology* in 2017 [2], we demonstrated that PAMPs suppressed *Cyp1* family expression in human monocytes and macrophages. Specifically, we showed that:

1. PAMPs inhibited the AHR-dependent induction of *Cyp1a1* and *Cyp1b1* in primary human monocytes.
2. Inhibition of *Cyp1a1* did not require *de novo* protein production, nor p38, ERK, or PI3K-Akt-mTOR signaling.
3. GM-CSF-derived human macrophages upregulated CYP1 activity following AHR activation.

4. AHR activation regulated the cytokine production by GM-CSF-derived human macrophages.

In Chapter 4, which is currently unpublished, we described the importance of the CYP1-AHR pathway during endotoxemia and endotoxin tolerance. Specifically, we showed that:

1. AHR regulated the inflammatory response to a primary LPS challenge but was not required for endotoxin tolerance in mice.
2. AHR was functional in refractory monocytes generated *in vitro* and regulated their cytokine production
3. Liver *Cyp1a2* expression and activity was decreased during endotoxemia.
4. AHR was not required for decreased *Cyp1a2* expression and activity during endotoxemia.
5. CYP1A2-deficient mice were susceptible to endotoxemia.

Contribution of Authors

Chapter 1:

A.G.P. wrote the literature review. The section "*The broad landscape of immune interactions with Staphylococcus aureus: From commensalism to lethal infections"* was adapted from a review written by A.G.P. and J.M. [3].

Chapter 2:

A.G.P. and J.M. designed the experiments, analyzed the data, and wrote the manuscript. A.G.P., C.S., J.L., A.Q., and B.L. performed experiments and analyzed data. B.C., M.G.S., and M.D. provided scientific input and reagents.

Chapter 3:

A.G.P. and J.M. designed the experiments, analyzed the data and wrote the manuscript. A.G.P. performed experiments and analyzed data. R.Z., and I.L.K. provided reagents and scientific input.

Chapter 4:

A.G.P. and J.M. wrote the manuscript. A.G.P and V.A.G.R. performed experiments. A.G.P. analyzed the data. I.L.K. and J.M. supervised experiments.

Chapter 5:

A.G.P. wrote the discussion.

List of Abbreviations

3MC – 3-methylcholanthrene

AHR – Aryl hydrocarbon receptor

AHH – Aryl hydrocarbon hydralase

AHRR – Aryl hydrocarbon receptor repressor

AIP – AHR-interacting protein

AP – Activator protein

ARNT – AHR nuclear translocator

BP – Benzo-[α]-pyrene

CCR – Chemokine receptor

CD – Cluster for differentiation

CHX – Cycloheximide

CRS – Chronic rhinosinusitis

CYP1 – Cytochrome P450 family 1

DC – Dendritic cells

DMBA – 7,12-dimethylbenz[α]anthracene

EAE – Experimental autoimmune encephalitis

ERK – Extracellular signal-regulated kinase

EROD – 7-ethoxyresorufin-*O*-deethylase

FICZ – 6-formylindolo(3,2-b)carbazole

FMO3 – Flavin-containing monooxygenase 3

GPCR – G-protein-coupled receptor

GM-CSF – Granulocyte macrophage colony-stimulating factor

HIF – Hypoxia-inducible factor

HLA – Human leukocyte antigen

HSP – Heat-shock protein

IC3 – Indole-3-carbinol

IFN – Interferon

IL – Interleukin

ILC – Innate lymphoid cell

IMQ – Imiquimod

IRAK – Interleukin-1 receptor-associated kinase

IRF – Interferon response factor

Kyn – Kynurenine

LAMA – Laminin, alpha

LBD – Ligand-binding domain

LBP – LPS binding protein

LPS - Lipopolysaccharide

MAMPs – Microbial-associated molecular patterns

MAPK – Mitogen-activated protein kinase

M-CSF – Macrophage colony-stimulating factor

MDM – Monocyte-derived macrophage

MDP – Muramyl dipeptide

MHC – Major histocompatibility complex

MLST – Multi-locus sequence typing

moDCs – Monocyte-derived DCs

MOI – Multiplicity of infection

MRSA- Methicillin-resistant *Staphylococcus aureus*

mTOR – Mammalian target of rapamycin

MyD88 – Myeloid differentiation primary response gene 88

NES – Nuclear export sequence

NF-κB – Nuclear Factor kappa-light-chain-enhanced of activated B cells

NLS – Nuclear localization sequence

NOD – Nucleotide-binding oligomerization domain-like receptor

NPTX1 – Neuronal pentraxin 1

NQO1 – NADPH quinone dehydrogenase 1

PAH – Polycyclic aromatic hydrocarbon

PAS – PER-ARNT-SIM

PAMP – Pathogen-associated molecular pattern

PBMC – Peripheral blood mononuclear cells

PCR – Polymerase chain reaction

PFT – Pore-forming toxin

PGN – Peptidoglycan

PI3K – Phosphoinositide 3-kinase

Poly I:C – Polyinosinic:polycytidylic acid

PRR – Pattern recognition receptor

RPMI – Roswell park Memorial Institute medium

RT-qPCR – Reverse-transcriptase quantitative polymerase chain reaction

SAg - Superantigen

SEE – Staphylococcal enterotoxin E

SIRS – Systemic inflammatory response syndrome

SOFA – Sequential organ failure assessment

STAT – Signal Transducers and Activators of Transcript

TAB – TAK1-binding protein

TAK1 – TGF-β-activated kinase 1

TANK – TRAF family member-associated NF-κB activator

TBK1 – TANK-binding kinase 1

TCDD – 2,3,7,8-tetrachlorodibenzodioxin

TGF-β – Transforming growth factor β

TiPARP – TCDD-induced poly(ADP)-ribose polymerase

TIR – Toll/IL-1R domain

TIRAP – Toll-interleukin 1 receptor domain containing adaptor protein

TLR – Toll-like receptor

TNF-α – Tumor necrosis factor-α

TRAF – TNF receptor-associated factor

TSA – Tryptic soy broth

TSB – Tryptic soy agar

TSS – Toxic shock syndrome

TSST – Toxic shock syndrome toxin

UGT1A6 – UDP glucuronosyltransferase family 1 member A6

VLA – Very late antigen

XAP-2 – hepatitis B virus X-associated protein 2

Chapter 1 : Literature Review

1.1 Regulation of Inflammatory Mechanisms of the Immune System

The immune system is a complex network of cells and molecules that has been selected to protect the host from invading pathogens and cancerous cells. It can be broken into two separate types of mechanisms, the innate and adaptive mechanisms, that have distinct roles but that are in constant communication with one another and function in concert. The effector mechanisms of these systems are responsible for the defense against hazardous agents. These mechanisms sense and initiate a tailored response, depending on the nature of the risk, clear the pathogen or damaged cells, and provide memory in case of re-exposure [4]. However, this response is not perfect and can be associated with damage to the host. Many of the mediators of the inflammatory response are cytotoxic and cause severe tissue damage and organ failure, known as immunopathology. Moreover, uncontrolled effector mechanisms in the context of the microbiome may cause unnecessary harm and eradicate beneficial microbes that limit the out-growth of pathogenic ones [5]. Therefore, a set of "immune regulatory" mechanisms has evolved to control and/or reset the system once the danger has been removed. They are activated in parallel to the effector mechanisms and antagonize them through extracellular degradation of inflammatory mediators, blocking receptor signaling, preventing target gene transcription, or inducing apoptosis of inflammatory cells. Some of these mechanisms may also partake in disease tolerance strategies that aim at limiting tissue damage without affecting microbial burden [6].

However, mechanisms of immune regulation may also be detrimental to the host. Over-reach by these responses can cause immunosuppression and expose the host to infection by opportunistic bacteria or latent viruses, or the growth and spread of cancerous cells. For example, immunosuppression is a common problem associated with death from sepsis and bacteremia [7,

8]. The tumor microenvironment is also highly suppressive towards infiltrating leukocytes and promotes tumor growth and metastasis [9]. Therefore, a fine equilibrium of effector and anti-inflammatory responses must be established and a breakdown in this balance can lead to disease [10].

The work in this thesis explores the role of two immune-regulatory mediators, interleukin-10 (IL-10) and the aryl hydrocarbon receptor (AHR), in the host response to *S. aureus* and the pathogenesis of sepsis. Chapter 1 will provide a detailed overview of our current understanding of the immunomodulatory properties of these proteins.

1.2 Innate Immune Activation by Toll-like Receptors

1.2.1 Overview of Innate Immune Mechanisms

The innate immune system is the first line of defense against invading pathogens. Its fundamental principle of operation is to detect molecular patterns commonly found on invading microbes, whether it be a bacteria, virus, parasite, or cancerous cell, that are not present on healthy host cells. It is mostly mediated by myeloid cells, such as monocytes, macrophages, dendritic cells (DCs), neutrophils, basophils, eosinophils, and mast cells, but also includes cells of lymphoid lineage (e.g. innate lymphoid cells) and non-hematopoietic cells (e.g. epithelial and endothelial cells). The key functions of these cells are microbial sensing, phagocytosis and pathogen elimination.

1.2.2 Ontogeny and Function of Monocytes, Monocyte-derived Cells, and Macrophages

Monocytes and macrophages are mononuclear phagocytes of the innate immune system that sense and clear microbes. Monocytes are typically found in the blood, while macrophages are localized to tissues. The original paradigm was that tissue-resident macrophages were terminally

differentiated monocytes that had egressed from the blood [11]. These macrophages were unable to divide, thus requiring constant replenishment from circulating monocytes to be maintained. However, using bone marrow chimeras and murine Cre-loxP fate mapping tools, we now know that tissue-resident macrophages are seeded during embryonic development, are long-lived and self-renewing, and are not generally maintained by monocytes in the steady state [12]. However, during an infection or tissue injury, monocytes will infiltrate into the inflamed tissue and differentiate into macrophages or DCs [12]. These infiltrating monocyte-derived cells will perform many of the same functions as the tissue-resident cells, but may also perform additional functions depending on the infection and tissue microenvironment [12]. Following the resolution of the inflammation, the monocyte-derived macrophages will be retained in the tissue and obtain a genetic signature similar to embryonic-derived macrophages [12]. This section will outline the ontogeny of monocytes, monocyte-derived cells and macrophages.

Monocytes are predominantly found in the blood and spleen, are short-lived, and are continuously generated in the bone marrow from precursors [13]. Blood monocytes can be broken into two subsets based on their expression of CD14 in humans and Ly6C in mice [14]. $CD14^{hi}$ monocytes ($Ly6C^{hi}$ in mice) account for ~90% of the blood monocyte population and are classical "M1" inflammatory monocytes. They express the chemokine receptor CCR2, are very phagocytic and produce large amounts of pro-inflammatory cytokines, chemokines, and reactive oxygen species in response to microbial stimulation [15]. On the other hand, $CD14^{low}$ monocytes ($Ly6C^{low}$ in mice) are 5-10% of the total blood monocytes and are non-classical "M2" macrophages. They produce very small amounts of pro-inflammatory cytokines, but large amounts of anti-inflammatory cytokines and tissue repair factors when stimulated [15]. These monocytes also express high levels of CX_3CR1 that allows them to "patrol" the vascular endothelium and resolve

inflammation [16]. Ly6C^{low} monocytes may not be a distinct subset but rather a mature form of Ly6C^{high} monocytes [17].

Monocyte migration into the inflamed tissue requires CCR2 and the VLA4 (α4β1 integrin) [18]. Thus, most transmigrating monocytes are classical inflammatory monocytes [19]. Once in the tissue, the monocytes obtain cues from the tissue microenvironment to begin differentiating into macrophages and DCs [12]. These environmental signatures will also dictate the function and responses of the monocyte-derived cells. Genetic analysis of the monocyte-derived macrophages indicates that these cells sequentially converge towards a tissue-resident macrophages phenotype until the point that they are nearly indistinguishable [12]. Monocytes can also cross the vascular endothelium during steady state and replace tissue resident macrophages. Most notably, intestinal macrophages are constantly being replenished by circulating Ly6C^{high} monocytes [20]. In conclusion, monocyte-derived cells and the tissue-specific factors that control their differentiation and function are critical for understanding the immunological network during infection and immunity.

Macrophage development begins in the embryo [21]. The first site of macrophage generation is the yolk sac at murine embryonic day 8 (E8) and does not use a monocyte intermediate, with a second wave occurring at murine E11.5 in the fetal liver from aorta-gonad-mesonephros-derived hematopoietic stem cells. Both waves contribute to the initial seeding of macrophages, with the exception of microglia, the brain-resident macrophage, that are exclusively derived from the yolk sac [22, 23]. However, the relative contribution of each wave is dependent on the seeding tissue. For example, intestinal macrophages are mostly derived from the yolk sac, with little contribution from the fetal liver, while Langerhans cells – macrophages residing in the epidermal layer of the skin – are derived almost exclusively from the fetal liver [21]. Yolk sac and

fetal liver derived-macrophages also require different transcription factors. Yolk sac macrophages depend on the transcription PU.1 for their development [24] and begin seeding tissues immediately when the circulatory system is formed at murine E8.5-9. Fetal liver-derived macrophages, on the other hand, likely require the transcription factor Myb [12] and migrate to the tissues at murine E12.5-13 [25]. In either case, following seeding, the tissue microenvironment completes embryonic-derived macrophages maturation and the acquisition of tissue-specific functions [26-28].

1.2.3 Signaling from Pattern Recognition Receptors Initiate the Inflammatory Response

Whether an infection is caused by a bacteria, virus, fungus or parasite, the immune response is triggered by the detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) on the surface of the epithelium, endothelium and leukocytes. Activation of PRRs leads to the up-regulation of expression of cell surface molecules as well as production of soluble molecules that mediate both the mechanisms critical for pathogen clearance and the detrimental processes that cause pathology. Moreover, all ensuing inflammation will be imprinted by the original response initiated by the activated PRR. As such, a fundamental understanding of these innate immune mechanisms is critical for understanding and treating inflammatory diseases.

PRRs can be broadly separated into four families: C-type lectin receptors (CLRs), NOD-like-receptors (NLRs), RIG-like receptors (RLRs) and Toll-like receptors (TLRs) [29]. These receptors have evolved to recognize a diverse set of microbial products including nucleic acids (both DNA and RNA), polysaccharides, lipoproteins and liposaccharides. CLRs are located exclusively on the cell surface and recognize polysaccharides on the membranes of bacteria and

fungus [30]. RLRs are cytosolic receptors that recognize RNA produced by viruses. They use the adaptor protein MAVS to drive IRF3- and IRF7-dependent type I interferon (IFN) production [31]. NLRs are cytosolic receptors that recognize a diverse range of microbial molecules and are best known for initiating the formation of the inflammasome [32]. TLRs are membrane-bound receptors that recognize structurally different microbial components (**Table 1.1**) and primarily cause cytokine production through either NF-κB or IRFs [29]. Most of the work covered in this thesis involves TLR ligands and will be described below.

1.2.4 Lipopolysaccharide: The Prototypical TLR Ligand

Lipopolysaccharide (LPS; also known as endotoxin) is a major component of the cell membrane of Gram-negative bacteria. It is composed of three structural regions: 1) a phosphorylated lipid A portion containing up to six fatty acids that anchor the molecule to the outer membrane; 2) a core oligosaccharide that contains a 3-deoxy-D-manno-oct-ulosonic acid (Kdo) sugar that attaches it to the lipid A portion; and 3) an outer O-saccharide (or O-antigen) that is highly variable [33]. LPS that contains all three structural components is classified as smooth (i.e. S-form), while the absence of the O-saccharide is a rough (R)-form LPS [34]. Functionally, LPS is critical for the integrity of the Gram-negative bacterial membrane and generates an important barrier that protects Gram-negative bacteria from environmental stresses [33]. Therefore, the unique structure and essential function of LPS makes it an excellent "molecular pattern" to be recognized by PRRs.

The lipid A component of LPS is recognized by the PRR TLR4 [35]. Conventional hexaacetylated lipid A, such as the one found on *Escherichia coli*, is the strongest TLR4 ligand known [36]. Initial binding to LPS is by the TLR4 accessory molecules LPS-binding protein (LBP) and CD14 that shuttles LPS to the TLR4 receptor complex [37]. In the absence of ligand, TLR4

forms a weak heterodimer with its co-receptor MD2, which is required for optimal LPS recognition [38]. TLR4 binds lipid A through five of its lipid chains, leaving the sixth lipid chain accessible for binding to MD2 [39]. The deep embedding of the six lipid chains into the TLR4 & MD2 binding pockets, in combination with secondary interactions through the phosphorylated lipid A sugars, forms a tight TLR4-LPS-MD2 complex that induces robust TLR4 signaling [40]. As such, the removal of one lipid chain from Lipid A can convert LPS from a very strong agonist to a weak agonist. This lipid A modification is a commonly used by both commensal and pathogenic Gram-negative bacteria as an immune evasion strategy [34]. Moreover, changing the length of the lipid chain or the phosphorylation of the lipid A sugars also reduces TLR4's affinity to LPS and these modifications are commonly found in pathogenic bacteria [36].

1.2.5 The TLR Signaling Pathway

TLRs are probably the best studied class of PRRs. They are characterized by their common structure of extracellular leucine-rich repeats (LRRs) that serve as the basis of the PAMP-binding domain. TLRs also have a transmembrane region and a short intracellular tail containing a Toll/IL-1R (TIR) domain that is essential for signaling [41, 42]. The TLR family is composed of 10 members in humans (TLRs 1-10) and twelve members in mice (TLRs 1-9, 11, 12, and 13). In mice, TLR10 is a pseudogene and it is not expressed as a result of a retroviral insert [29]. TLRs 1, 2, 4, 5, and 6 are found in the plasma membrane, while TLRs 3, 7, 8, and 9 are found in endolysosome membranes [29]. TLR10 is found both on the plasma and endolysosome membranes. The subcellular location of TLRs has been selected for optimal detection of their ligands (**Table 1.1**). Most TLRs function as homodimers to signal [42], with the exception of TLR2 that heterodimerizes with either TLR1 [43], TLR6 [44] or TLR10 [45].

TLR signaling is a well-defined pathway that requires either the adaptor proteins MyD88 (all TLRs except TLR3) or TRIF (only for TLRs 3 and 4) [29]. TLR4 is the model receptor for this family because it is the only member that uses both of these signaling pathways (**Figure 1.1**). TLR4 binding to its ligand, LPS [46, 47], causes receptor dimerization and recruitment of MyD88 through a TIR-TIR interaction [48, 49]. TLR2 and TLR4 require an additional adaptor protein, TIRAP/Mal, to recruit MyD88 [50-53]. The serine/threonine kinases IRAK-4, IRAK-1, and IRAK-2 then bind to the signaling complex through a death domain homodimer interaction between MyD88 and IRAK-4 [48, 54-56]. Auto- and trans-phosphorylation of the IRAK proteins leads to their dissociation of the MyD88-bound receptor and interaction with TRAF6, an E3 ubiquitin protein ligase [48, 57]. TRAF6 K63 ubiquitinates itself and generates a free K63 polyubiquitin chain that binds to TAB2/3 to activate TAK1 [58]. Downstream TLR signaling begins with TAK1-mediated IKK phosphorylation and multiple MAPK kinases. Phosphorylated IKK then phosphorylates IκB and tags it for ubiquitin-dependent proteasome degradation [59]. IκB degradation frees the NF-κB family for nuclear translocation and transcriptional induction of pro-inflammatory cytokines, such as *Il1b*, *Il6*, *Il8*, *Il12p35/p40*, and *Tnfa*, and the antigen-presentation machinery (e.g. *MHC class II, CD80*, *CD86*, and *CD40*) required to activate the adaptive immune system. Moreover, TAK1 also phosphorylates multiple MAPK kinases that initiate p38, ERK, and JNK signaling that also drives cytokine and chemokine production.

Ligand binding by TLR4 also leads to internalization and endosomal signaling through the TRIF-dependent pathway, which is also used by TLR3 [60, 61] (**Figure 1.1**). Endosomal translocation of TLR4 is dependent on the accessory protein CD14 and signaling through Syk-PLCγ2 [62]. MyD88-independent signaling begins by bridging ligand-bound TLR3 and TLR4 to TRAF-6 and TRAF-3 through the TIR-containing adaptor proteins TRIF and, for TLR4, TRAM

[63]. TRAF-6 then feeds into the NF-κB and MAPK signaling pathways described above, while TRAF-3 [64] leads to the activation of the transcription factor IRF-3 and IRF-7 [65-67] through the IKK-like kinases TBK-1 and IKK-*i* [68-70]. Phosphorylated IRF-3 and IRF-7 homo- or heterodimerize, enter the nucleus, and induce the production of type I IFNs, which are critical for mounting an effective antiviral defense.

1.2.6 Defects in TLR and Human Disease

The importance of TLR in fighting infections is highlighted by the susceptibility of humans that are deficient in components of TLR signaling. Individuals with loss-of-function mutations in either MyD88 or IRAK4 have recurrent invasive infections caused by *S. aureus*, *Streptococcus pyogenes*, and *Pseudomonas aeruginosa* and require continuous antibiotic treatment until adolescence [71, 72]. Interestingly, once these patients reach adulthood they no longer require antibiotics, suggesting the development of redundant mechanisms to detect these pathogens. Moreover, human deficiencies in UNC93b [73], a protein required for the trafficking of TLR3, TLR7, and TLR9, TBK1 [74], or other components of TLR3 signaling [75] are highly susceptible to herpes simplex virus (HSV) encephalitis. The unique susceptibility of these individuals to HSV encephalitis is because of the lack of redundancy of TLR3 functions in neurons, astrocytes, and oligodendrocytes [76, 77]. In other tissues, TLR3 responses can be performed by other nucleic acid sensing PRRs. Collectively, these studies of human deficiencies in TLR signaling demonstrate the importance of PRRs in immunity to infections.

1.2.7 Introduction to Immune Regulatory Responses Triggered by TLR Signaling

In addition to the NF-κB-dependent pro-inflammatory responses described above, certain TLR ligands can also activate immune-regulatory responses. Activation of both TLR2 and TLR4 induces the production of the anti-inflammatory cytokine IL-10 and expression of PD-L1 that suppress conventional T cell activation [78-81]. Similar to their pro-inflammatory counter-parts, these immune regulatory mechanisms have important consequences on host-pathogen interactions. For example, IL-10 induction in response to *S. aureus* has major implications in the pathogenicity and commensalism of this microbe [82], and this response will be discussed further in the next section.

1.3 The Broad Landscape of Immune Interactions with *Staphylococcus aureus*: From Commensalism to Lethal Infection

S. aureus is a gram-positive facultative anaerobe of the *Firmicutes* phylum. Its name is derived from the Greek *staphylē* (meaning grape) and *kókkos* (meaning granule), and Latin *aureus* (meaning golden) that describe its golden, round, clustered appearance. *S. aureus* lives a dual lifestyle as both the cause of life-threatening infections and as part of the normal human microbiota. A remarkable feature of this lifestyle is that a single strain of *S. aureus* can be both a pathogen and a commensal, as evident by the routine isolation of pathogenic strains of *S. aureus* (e.g. USA300) from the noses of healthy adults [83, 84]. Therefore, understanding the cellular and molecular determinants that dictate its ability to cause severe disease or asymptomatically colonize its host is an important field of study.

1.3.1 The dual relationship between *S. aureus* and human beings: commensalism vs. pathogenicity

S. aureus is the most common microbe isolated from inpatient cultures and the second most common microbe isolated from outpatient samples [85]. *S. aureus* can infect almost any tissue in the body and cause localized infections (i.e. pneumonia, abscess) or systemic conditions such as sepsis and toxic shock syndrome (TSS). In particular, burn patients are at a high risk of *S. aureus* infections because of the damaged skin barrier [86-89]. The number of hospitalizations and deaths due to *S. aureus* infections has risen over the past decade and in 2005 were estimated at 500,000 and 20,000, respectively, in the United States alone [90, 91]. This high morbidity and mortality and the rise in frequency of antibiotic-resistance strains, including methicillin-resistant *S. aureus* (MRSA), have made *S. aureus* a very serious health care problem with a high associated financial burden [91].

In contrast to its high frequency as a pathogenic microbe, *S. aureus* also resides chronically in the upper respiratory tract of up to 25% of the population without causing any apparent complications [92, 93]. A person is given the 'persistent carrier' status if two nasal swabs taken more than 1 week apart are both positive for *S. aureus* ($> 10^3$ cfu / swab), whereas non-carriers are negative in both swabs [94, 95]. Carriage is more common in men than women [96, 97] and during childhood [98, 99]. Historically, a third classification 'intermittent carrier' was given to people when only one of the two cultures was positive [95]. However, because the risk of infection between non-carriers and intermittent carriers is not significantly different [100], and the two groups develop similar profiles of anti-staphylococcal antibodies and staphylococcal elimination kinetics during artificial nose colonization [101], it is now thought that non-carriers may have carried *S. aureus* transiently.

Both host genetics and microbial factors contribute to staphylococcal nasal carriage, but their relative contributions are uncertain [102]. A study of 617 pairs of Danish twins found that the concordance rate of *S. aureus* colonization of monozygotic twins was only slightly higher than dizygotic twins [97], suggesting that the contribution of host genetics is minor. Only two other twin concordance studies on *S. aureus* nasal carriage have been done [103, 104]. The studies had conflicting results and limited statistical power (less than 100 twin pairs), indicating the need for more host genetics studies. Moreover, the associations between *S. aureus* nasal colonization and host and bacterial adhesion proteins [105, 106], composition of the nostril microbiota [107, 108], and gene-environment interactions [109] reflect the complexity of the determinants of *S. aureus* nasal carriage.

The nasal carriage status of a patient is of clinical importance. Persistent carriers are at a higher risk of developing staphylococcal bacteremia but are also 3-fold less likely to die from it, and thus may be partially protected [92]. However, eradication of *S. aureus* from the nose can effectively reduce the risk of post-operative *S. aureus* infections [110]. Therefore, the precise clinical implications of *S. aureus* nasal carriage are still debatable, especially at the mechanistic level, and may differ depending on the clinical setting.

Given its ability to interact with the human immune system as both a commensal and pathogenic organism, *S. aureus* can be labeled as a ‘pathobiont’, a microbe that is normally commensal but can turn pathogenic under certain conditions. These conditions are unknown but identifying the mechanisms that *S. aureus* uses to balance its commensal and pathogenic states is important to understand the origin of invasive staphylococcal infections and to develop more effective therapies for these infections.

1.3.2 *S. aureus* Can Modulate the Immune Response to its Toxins

TSS is an acute, toxin-mediated illness caused by *S. aureus* or group A streptococci, such as *Streptococcus pyogenes*. Staphylococcal TSS presents itself abruptly causing fever, hypotension and multi-system failure (**Table 1.2**). It was first described in 1978 when seven patients developed systemic complications secondary to infections with these microbes [111]. These patients were *S. aureus* positive in their mucosa but not in their blood, urine or cerebrospinal fluid, suggesting a toxin-mediated illness. Soon thereafter, a link between staphylococcal TSS and the use of highly absorbent tampons was established [112]. The initial incidence of staphylococcal TSS was estimated at 6.2 cases per 100 000 women 12 to 49 years of age [113], but has declined and stabilized at the current incidence of 0.5 to 1 case per 100 000 women 18 to 44 years of age [114, 115]. The mortality rate ranges from 5 to 30% and is more common in non-menstrual TSS than menstrual TSS [116]. The incidence of TSS-like symptoms in burn patients has been reported as high as 13% [117, 118].

The pathogenesis of TSS is caused solely by the expression of bacterial pyrogenic exotoxins called superantigens (SAg). The majority of these toxins are encoded on mobile genetic elements (i.e. phages, plasmids) [119] and close to 100% of clinical isolates of *S. aureus* harbor at least one SAg [120-123]. In 1996, 55% of *S. aureus* isolated from burn wounds expressed at least one SAg [124]. Thus far, 20 members of the SAg family have been identified and are separated into five phylogenetic groups, with staphylococcal SAgs falling into four of these groups (I, II, III, V) [119]. In addition, the staphylococcal SAg family is loosely classified into staphylococcal enterotoxins (SE followed by a letter, e.g. SEA, SEB, SEC, etc.), toxic shock syndrome toxin-1 (TSST-1) and staphylococcal superantigen-like proteins (SSL). Although structurally similar, the SSL family members do not function as SAgs and only some have a known function (reviewed in

[125]). All SAgs fold into a conserved N-terminal β–barrel domain, similar to an oligonucleotide/oligosaccharide-binding (OB) fold, and a C-terminal β–grasp domain [126].

The capacity of SAgs to cause TSS is linked to their ability to activate large numbers of T cells. SAgs bind to the V region of the selective β chains of the T cell receptor (TCR) and to the α or β chains of human leukocyte antigen (HLA) class II molecules outside of the peptide binding groove on antigen presenting cells (APCs). As a result of this unique interaction, SAgs can bypass conventional antigen processing and activate up to 25% of all T cells, contrary to the activation of less than 10^{-5} to 10^{-6} T cells in the response to conventional antigens [127]. In both cases, T cells are activated by the canonical TCR-signaling pathway (reviewed in [128]). Briefly, binding of the TCR to the MHC brings it within proximity to the CD4/CD8 co-receptor. Co-receptor-bound kinase Lck phosphorylates immune tyrosine-based activation motifs (ITAMs) on the CD3 and ζ-chains of the TCR. This provides a docking site for the kinase ZAP-70 (ζ-chain-associating protein of 70 kDa), which is activated by phosphorylation by Lck. Downstream signaling cascades are initiated by ZAP-70 phosphorylation of LAT and SLP-76. The Lck-ZAP-70-LAT pathway can also be activated in a co-receptor-independent manner when activated by SAgs, although the molecular basis is unknown [129]. In addition to this canonical pathway, a secondary pathway independent of CD4/CD8 ligation and Lck activation further enhances T cell activation by signaling through a Gα11/PLCβ-dependent cascade [129]. Moreover, it has been reported that SAgs can also activate the corresponding APC, although the specific molecular mechanism is still unclear [130-132].

The over-activated T cell response causes massive cytokine production and the development of a ‘cytokine storm’, dominated by a T helper type 1 (Th1) profile characterized by high levels of interferon-γ (IFN-γ) and lymphotoxin. Moreover, other pro-inflammatory cytokines

(i.e. tumor necrosis factor-α (TNF-α), IL-1, and IL-6, and the chemokine IL-8) are produced by T cells, APCs, and parenchymal cells.

The clinical manifestations of TSS are determined by the massive cytokine release, which induces severe hemodynamic alterations. Tissue ischemia soon follows and translates to multi-organ failure, cardiovascular collapse, and ultimately, death. The hypotension also prevents bacterial clearance by polymorphonuclear cells and slows the delivery of antibiotics. Moreover, the hyperactive state of T cells causes many clones to apoptose and others to enter a transient state of anergy. Importantly, the induction of TSS requires only picomolar concentrations of SAgs, because of the potent immunostimulatory capacity of these toxins [133].

Despite the unique stimulatory potency of SAgs and the high frequency of *S. aureus* nasal carriage, the incidence of staphylococcal TSS remains relatively low. Considering almost all isolates of *S. aureus* express at least one SAg [120-123], one would expect carriers to be constantly bombarded by these toxins, yet disease rarely develops. The inhibitory properties of the α- and β-chains of hemoglobin on SAg production may contribute, but are probably not the only answer because the presence of staphylococcal bacteremia is detected in less than 5% and 25% of all TSS cases [134, 135], and *S. aureus* hospitalizations [90, 91], respectively. The production of neutralizing antibodies by carriers against SAgs may also provide protection [136], as low anti-SAg antibody titers are a risk factor for invasive staphylococcal infections [123, 137]. However, these antibodies have poor cross-reactivity to SAg produced by other *S. aureus* isolates and, it remains unclear if nasal carriage is enough to enhance the production of anti-staphylococcal antibodies [138-141]. Indeed, 85% of women have detectable antibodies against TSST-1 by age 40 [123]. If carriers had protective antibody titers to their endogenous strain, one would expect that if they would be more susceptible to invasive infections by exogenous *S. aureus* strains rather

than their own endogenous strain. However, 80% of bacteremia cases in carriers, and almost all resulting deaths, are caused by an endogenous *S. aureus* isolate [92]. Therefore, anti-SAg antibodies may provide some general protection against staphylococcal TSS, but this protection is not specific for carriers.

The paradox of high frequency of staphylococcal infections with low incidence of TSS led us and other groups to explore the potential evolutionary pressure for *S. aureus* to regulate its own pathogenicity. It is currently unknown what evolutionary advantage SAg expression provides to *S. aureus*. Unlike the advantageous effects observed in *S. pyogenes* colonization [142], SAg production by *S. aureus* did not promote nasal colonization in a mouse model [143], and more work is required to determine if these toxins favor colonization or spreading in other tissues. However, one can argue that from the host's perspective, tolerance to SAg may provide an advantage in limiting immunopathology. Host-pathogen interactions could serve as a selective platform for mechanisms that regulate microbial toxins and minimize their toxicity. Recent evidence of a mechanism of modulation of the host immune response by the cell wall of *S. aureus* supports this idea, and can partially explain why certain individuals are carriers, as well as the different outcomes of *S. aureus* infections [80].

1.3.3 Cellular Basis of Immunomodulation by *S. aureus*

The initial recognition of *S. aureus* is primarily dependent on epithelial cells or cells of the innate immune system. *S. aureus* contains pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors (PRRs) on these cells. The thick peptidoglycan (PGN) cell wall of gram-positive bacteria contains lipoproteins, covalently and non-covalently attached proteins, teichoic acid and membrane-anchored lipoteichoic acid (LTA) [144]. These molecules

are ligands for the TLR2 PRR. TLR2 signals in conjunction with other receptors to recognize this diverse group of molecules and to appropriately tailor the immune response. For example, TLR1 and TLR6 heterodimerize with TLR2 to detect triacyl and diacyl lipoproteins respectively, and the accessory molecules CD14 and CD36 act as co-receptors to further help these TLR2 heterodimers respond to certain ligands, such as LTA [145]. Once inside the cell, digestion of *S. aureus* generates additional ligands for intracellular PRRs, including peptidoglycan fragments (i.e. muramyl dipeptide; MDP) and unmethylated DNA that are recognized by NOD2 and TLR9, respectively [146]. This degradation can also release staphylococcal-specific epitopes that can be loaded onto MHC molecules and presented to cells of the adaptive immune system by APCs to activate cellular and humoral responses. The TLR2- and TLR9-dependent responses require the recruitment of MyD88 by MAL and activation of the MAPK and canonical NF-kB pathways, while the NODs activate NF-kB mostly through RIP2 [147]. Recognition of PAMPs by PRRs elicits a robust pro-inflammatory immune response that is coordinated by the production of cytokines (e.g. TNF-α, IL-1β, IL-6) and chemokines (e.g. MCP-1 and IL-8) by macrophages (MΦ), DCs, neutrophils, and epithelial cells.

Recently, a regulatory role for TLR2 in response to *S. aureus* stimulation has emerged. Embedded in the staphylococcal cell wall are molecules that can downregulate the initial T cell response to SAgs (as measured by IL-2 production) [80]. This mechanism is mediated by TLR2 signaling, likely in combination with TLR6, on APCs and induces IL-10 production and apoptosis of these cells. These staphylococcal-derived anti-inflammatory TLR2 ligands may also regulate inflammation of the skin by blocking TLR3 signaling and induction of pro-inflammatory cytokines by keratinocytes in an IL-10-independent mechanism [148]. To differentiate these responses from the pro-inflammatory reaction, we have labeled this response the ‘immunomodulatory’ response.

IL-10 is an anti-inflammatory cytokine that suppresses the production of Th1 cytokines and downregulates the expression of the co-stimulatory molecules CD80 and CD86 on APCs [149, 150]. Combined, these suppressive factors can down-regulate the 'cytokine storm' induced by SAgs and lower the risk of TSS. *In vivo* studies in mice have supported this conclusion: mice administered live *S. aureus* or SAg in conjunction with staphylococcal PGN have reduced inflammation and survive longer than those given live *S. aureus* or SAg alone [80, 151]. During early stages of infection, up-regulation of PD-L1 on the surface of APCs also contributes to immunomodulation of the host adaptive T cell responses [81].

This mechanism can accommodate different scenarios. Other microbes, such as *Yersinia enterocolitica, Mycobacterium tuberculosis,* and *Candida albicans*, can modulate the immune response through activation of TLR2 on the surface of APCs [152, 153], non-immune cells [148, 154], or murine $CD25^{+}Foxp3^{+}$ regulatory T cells (Treg) [155, 156]. We have only observed immunomodulation by *S. aureus* in APCs, and it is unlikely that staphylococcal PGN-embedded molecules can also modulate through Tregs because human resting T cells do not express TLR2 [157]. However, SAgs may directly regulate their own toxicity by inducing proliferation of Tregs in a Vβ-specific manner [158]. The generation of IL-10-producing Th17 cells in response to *S. aureus* has also been reported [159]. The generation of this Th17 subset was attributed to a lack of IL-1β during T cell differentiation. We have observed IL-1β production by human peripheral blood mononuclear cells in response to *S. aureus* at 18 h post-infection (Peres *et al.*, 2012, unpublished data), but whether the IL-1β response is maintained during later stages of infection (i.e. days) is unknown. Therefore, *S. aureus* may have strategies of modulating the immune system during many stages of infection.

The IL-10 response to *S. aureus* is 4-20 times stronger when human monocytes/macrophages are the APCs rather than DCs [160]. DCs, instead, activate a Th1/Th17 response by producing IL-12 and IL-23. This difference may have an impact on the clinical outcome of *S. aureus* depending on the site of infection because DCs and monocytes/macrophages are not equally represented in all tissues. For example, the primary APC in the nasal sub-mucosa is the MΦ, while in the skin it is the Langerhans cell, an epidermal-resident DC. Their differential capacities to produce IL-10 in response to staphylococcal PGN-embedded molecules can partially explain why the nose is a site of carriage and the skin is a site for a strong pro-inflammatory response (i.e. TSS) (**Figure 1.2**).

The underlying mechanism of immunomodulation by *S. aureus* can also account for immune evasion of *S. aureus* in the blood. In the blood, where monocytes are the dominant APCs, the high IL-10 levels allow *S. aureus* to evade immune responses and cause septicemia. It is important to note that the immunomodulatory response triggered by *S. aureus* has only been studied using MΦ and DCs derived from human peripheral blood monocytes [160]. How the heterogeneity of APCs subsets within tissues reflects their functional capacity needs to be addressed, in particular the response by tissue-specific APCs found at the sites of *S. aureus* infections (i.e. dermal DCs (DDC), alveolar MΦs). Of interest would be to investigate how newly characterized subsets, such as $CD141^+$ DDCs, behave in response to *S. aureus*, because these cells have an enhanced IL-10 response compared to classical DCs and are thought to play a role in the maintenance of skin homeostasis [161].

1.3.4 Molecular Basis of the Immune Modulatory Properties of *S. aureus*

A precise molecular basis of the immunomodulatory properties of *S. aureus* is starting to emerge. A *S. aureus* strain deficient in lipoprotein synthesis (*lgt*:: Spec *S. aureus*) was still able to modulate the T cell response to SAgs [80], suggesting that molecules other than lipoproteins, such as LTA, or covalently or non-covalently attached proteins, are the inducers of the immunomodulatory response. Preliminary evidence suggested that a TLR2/6 ligand rather than their TLR2/1 counterparts are involved because zymosan (a TLR2/6 ligand) but not a Pam3CSK4 (a TLR2/1 ligand) was able to downregulate the T cell response to staphylococcal SAgs [80]. However, this may be an oversimplification, since Pam2CSK4 (also a TLR2/6 ligand) was unable to induce detectable levels of IL-10 [160]. Moreover, the TLR2 ligand LTA was also able to suppress host adaptive and innate immune responses [81, 148], indicating a role for glycopolymers in immunomodulation. In our hands, we detected higher induction of IL-10 by PGN than LTA, indicating that a ligand other than LTA is responsible for immunomodulation [160]. Alternatively, the oligomeric structure of TLR2 ligands in the PGN layer may cause extensive receptor cross-linking that enhances downstream signaling [162, 163]. Together, these findings suggest that additional ligands and/or signaling conformations contribute to immunomodulation through TLR2.

One way to explain differential pro- and anti-inflammatory signaling by TLR2 is to claim differential use of accessory molecules in recognizing certain TLR2 ligands. Indeed, the immunomodulatory response to staphylococcal PGN is independent of CD14 and CD36, while the pro-inflammatory response is dependent on CD14 [160]. The presence of these accessory molecules does not appear to negatively regulate IL-10 production.

The different requirements for co-receptors by the pro-inflammatory and immunomodulatory responses may also be associated with qualitative and quantitative differences in the activation of signaling pathways. The TLR2-dependent pro-inflammatory response requires MyD88-dependent activation of the canonical NF-kB and MAPK pathways [164]. On the other hand, immunomodulation by the staphylococcal cell wall is dependent on activation of the PI3K/AKT pathway [160]. In this response, activation of the PI3K/AKT pathway decreases Th1 profile cytokines production because inhibition of PI3K by wortmannin reduced the production of IL-10 and enhanced production of Th1 cytokines. The inhibition of Th1 cytokine production could be a direct result of PI3K/AKT pathway activation or the result of IL-10 dependent suppressive activity.

How the PI3K/AKT pathway is activated downstream of TLR2 signaling is still uncertain. TLR2, TLR1, and TLR6 all contain PI3K binding domains but why PI3K is recruited in response to some ligands but not others is unclear. The TLR adaptor protein MAL has been shown to connect TLR2/6-dependent signaling and PI3K activity in a MyD88-independent pathway [165] and mediate IL-10 expression through CREB in the response to the TLR2/6 ligand Pam2CSK4 [166]. We have found MAL is similarly expressed in monocyte-derived MΦ and DCs (moDCs) [160], but differential involvement of downstream molecules in different APC subsets needs to be addressed. Another candidate to consider is DC-SCRIPT. This transcriptional regulator is expressed exclusively in DCs and negatively regulates IL-10 production by TLR signaling. When DC-SCRIPT was knocked down by siRNA in moDCs, these cells displayed enhanced IL-10 production and decreased IL-12 in response to TLR4 and TLR7/8, but not TLR3, ligands [167]. Interestingly, TLR2, 4, 7 and 8 can all signal through MyD88, while TLR3 signals exclusively through TRIF. It is possible that TLR2 signaling in DCs suppresses IL-10 production by activating

DC-SCRIPT, and thus maintains the production of Th1 cytokines. In contrast, TLR2 signaling in monocytes/macrophages activates PI3K/AKT, enhances IL-10 production and inhibits Th1 cytokine production in an autocrine/paracrine-manner.

The different co-receptor requirements and divergent signaling pathways suggest that the pro-inflammatory and immunomodulatory response to *S. aureus* can be uncoupled. We predict that the cell wall of *S. aureus* contains two sets of PAMPs that can bind TLR2. One includes ligands that induce a predominantly pro-inflammatory response with a mild immunomodulatory response (i.e. LTA). The other includes ligands that induce a more immunomodulatory response, with less of a pro-inflammation response. The relative abundance of these two sets of ligands may vary from strain to strain, as does the response from person to person, and this may contribute to the clinical outcomes seen. As an extension, one can argue that there may be a selective pressure for strains producing large quantities (or multiple types) of superantigens to also express more immunomodulatory IL-10-inducing ligands on their cell wall.

1.3.5 Clinical Implications of Immunomodulation by *S. aureus*

An understanding of the relationship between *S. aureus* and humans has implications on many different clinical scenarios. Although the biological effects of immune modulation by *S. aureus* can emerge as important contributors to the outcome of any systemic infections by this microbe, it is not difficult to foresee that they may also play a role in the context of cutaneous injuries, such as burns. Direct disruptions of the cutaneous barrier, or qualitative or quantitative changes in the microbiota, associated with cutaneous injuries, may tilt the balance towards *S. aureus* pathogenicity, instead of commensalism. Factors such as the site of infection, the primary responding APC, host genetics and specific *S. aureus* strain all may contribute in parallel to the

outcome of the *S. aureus* encounter with humans. For example, we would predict that nasal carriers of *S. aureus* are genetically hardwired to produce more IL-10 than non-carriers. Alternatively, the strain that these individuals carry may have higher amounts of IL-10 inducing ligand(s). In either condition, chronic carriage may be facilitated. However, this same strain-donor interaction may lead to more severe staphylococcal bacteremia because the robust IL-10 response by monocytes in the blood would allow for *S. aureus* to evade the host adaptive immune response and cause a more severe systemic infection. The complexity of these interactions may require a systems biology analysis before accurate predictions of specific scenarios can be made.

A high IL-10 response may also be a risk factor for patients with bacteremia, regardless of their carrier status. Patients who had serum IL-10 levels above normal (> 7.8 pg mL^{-1}) at the time of hospitalization with staphylococcal bacteremia died in 25% of the cases compared to no mortality observed by patients with IL-10 within normal range [8, 168, 169]. If our hypothesis that carrier strains are more effective at inducing IL-10 production is correct, then it could explain why carriers die more often from their endogenous strain [92], as exogenous strains may not be as effective in evading the immune system. Unfortunately, the carrier status of these patients was not investigated. On the contrary, immunomodulation in the blood will protect the patient from developing TSS by suppressing the SAg-induced immune response and could explain why bacteremia is rarely found in TSS patients [134].

Current therapies for TSS are centered on early detection and symptomatic treatment (resuscitation, controlling the spread of the infection with antibiotics, etc.). Of interest, bacteriostatic antibiotics (i.e. clindamycin) have been reported to be more effective in treating TSS than bactericidal antibiotics and lead to better clinical outcomes [170-172]. This benefit has been attributed to the ability of this antibiotic to inhibit the production of exotoxins by *S. aureus* [173].

Immunomodulation by the staphylococcal cell wall offers a second mechanism for the suitability of action for clindamycin. By maintaining the integrity of the cell wall, bacteriostatic antibiotics will preserve the immunomodulatory potential of *S. aureus* and protect the host against an 'over-driven' immune response. In contrast, bactericidal antibiotics would rupture the cell and expose the patient to more exotoxins, while simultaneously decreasing the amount of immunomodulatory ligands. The latter outcome makes a TSS patient more susceptible to further damage, and possibly death.

Identifying the PGN-embedded TLR2 ligand, and the host co-receptors, could provide a template for developing novel anti-inflammatory molecules and pro-inflammatory adjuvants. This approach has been used previously: a peptide derivative of the *Limulus* anti-LPS factor (LALF) has been shown to reduce organ damage and improve survival to bacterial sepsis in mice [174]. LALF increases IL-2, IL-12 and IL-13 mRNA but does not affect IL-4 and IL-10 levels, creating a profile opposite to what is seen by the staphylococcal PGN-embedded molecules.

1.4 Sepsis

1.4.1 History, Definition, and Epidemiology of Sepsis

Sepsis is characterized as a dysregulated systemic immune response to a severe infection [175]. It derives from the Greek word "σηψιζ" (pronounced *sêpsis*), which means the "decomposition of animal, or vegetable or organic matter in the presence of bacteria" [176]. The earliest known references to sepsis are from poems by Homer dated to 2,700 years ago. The belief at the time was that the biological decay of the colon released "dangerous principles" that could cause "auto-intoxication" [176, 177]. In his collection *Corpus Hippocraticum*, Hippocrates (460-370 BCE) described sepsis as the dangerous, foul-smelling putrefaction of the body, and

documented his attempts at treating sepsis with alcohols derived from wine and vinegar [176]. The Romans furthered these theories and believed that sepsis was caused by invisible creatures. However, it was not until Joseph Lister applied Louis Pasteur's "Germ Theory" to the development of septic surgery and wound care in the late 19th century that our understanding of sepsis truly began. However, despite early recognition of the dangers and importance of sepsis, the first consensus definition was not developed until 1991 [178].

The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) defines sepsis as "life-threating organ dysfunction caused by a dysregulated host response to infection" [179-181]. Sepsis is now clinically determined using the sequential organ failure assessment (SOFA) score, replacing the systemic inflammatory response syndrome (SIRS) criteria [178, 182]. Since SIRS can also be seen during sterile inflammation, such as in trauma or ischemia, SOFA provides a statistically supreme diagnosis of sepsis [180]. The third-generation definitions also removed the condition "severe sepsis" and defined septic shock as a "subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality" [179, 181]. This can be assessed clinically by a mean arterial pressure of 66 mm Hg or greater and serum lactate levels above 2 mmol/L (>18 mg/dL) [179, 181].

Sepsis is one of the leading causes of mortality worldwide. A meta-analysis of 1,553 reports from 1979-2015 estimated that sepsis accounted for over 30 million episodes and 5 million deaths globally each year [183]. In the U.S.A. alone, it accounts for approximately 1.7 million hospitalizations and 270,000 deaths annually [184]. In 2013, sepsis was the most costly disease to U.S.A. taxpayers at a price tag of $23.6 billion: over 6% of the national health care budget [185]. In Canada, sepsis causes over 30,000 hospitalizations and is a contributing factor in almost 10,000

deaths each year [186]. Unfortunately, the continuous rise in the incidence of sepsis over the past two decades [187-190] indicates it will be a serious health concern for the foreseeable future.

Sepsis can be caused by an infection from bacteria, fungi or viruses. Bacterial infections account for the majority of sepsis cases, and gram-negative bacteria are slightly more common than gram-positive infections (62% vs. 47%) [191]. Gram-negative bacterial infections are also associated with a higher rate of mortality [192]. *E. coli* and *S. aureus* are the most frequent bacteria isolated from patients with sepsis, collectively accounting for over a third of sepsis cases. Although fungi account for a minority of sepsis cases [191], fungal sepsis has a mortality rate that is almost double that of bacterial sepsis [192]. *Candida* is the most common fungus isolated from patients with sepsis. Sepsis arises from infections that typically originate in the respiratory tract, abdomen, blood or urinary/genital tract. Men are more susceptible to respiratory tract sepsis, while women are more likely to develop urinary sepsis [193]. However, the mortality rate of sepsis at any infection site does not differ between men and women [193].

1.4.2 Pathogenesis of Sepsis

Sepsis can be broken into two distinct phases: an early hyperinflammation phase followed by a late immunosuppression phase (**Figure 1.3**). Microbes that breach the physical anatomical barriers are recognized by PRRs and a rapid immune response is initiated. In sepsis, the activation of PRRs is immense, causing myeloid and epithelial cells to produce excessive amounts of pro-inflammatory cytokines and the development of a "cytokine storm". These pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-12 and IFNγ, are critical for the pathogenesis and immunopathology associated with sepsis [194]. Recently, innate B cells have been shown to contribute to sepsis by producing IL-3 [195]. This cytokine promotes leukocyte survival and

proliferation and amplifies the pro-inflammatory response in the cecal ligation and puncture model of sepsis. In humans, serum IL-3 levels correlated with blood monocyte numbers and were an independent predictor of day 28 survival in sepsis patients [195]. Collectively, these pro-inflammatory responses result in complement activation, thrombosis and endothelial cell dysfunction, and can cause irreversible tissue ischemia and damage [194]. Ultimately, patients with sepsis succumb to cardiovascular failure and multi-organ dysfunction.

Soon after the initiation of the pro-inflammatory response, immune regulatory counter-mechanisms begin to act. These two responses occur in parallel during early sepsis and are observed simultaneously in sepsis patients [196, 197]. The immune regulatory mechanisms are mounted to try to control the on-going cytokine storm, but the result is often a crash of the immune response and the development of sepsis-induced immunosuppression that affects both myeloid cells and lymphocytes (**Figure 1.4**). Most notably, peripheral blood monocytes have impaired pro-inflammatory cytokine production upon *ex vivo* re-stimulation and exhibit signs of exhaustion, such as high PD-L1 levels and low HLA-DR, CD80 and CD86 expression [7, 198]. Monocytes are thought to be essential orchestrators in the establishment of sepsis-induced immunosuppression [199] and low expression of HLA-DR on monocytes is one of the best predictors of mortality from sepsis [200]. As such, cytokines that target the re-activation of refractory monocytes, such as IFNγ and GM-CSF, may provide effective treatments for sepsis [201].

Patients that die from sepsis also display severe apoptosis of DCs, B cells and T cells that results in a significant loss of these populations in lymphoid tissues [202-206]. Moreover, the remaining cells display signs of immunosuppression: T cells from deceased sepsis patients have increased expression of PD-1 [7], a sign of exhaustion, and the inability to differentiate towards Th1 and Th17 cells [207, 208]. There is also a higher frequency of regulatory T cells in sepsis

patients [209, 210], and these cells likely exacerbate the refractory state of monocytes during sepsis [211]. It currently remains unclear why patients die during the immunosuppressive phase. One possibility is the re-activation of latent viruses or infection by opportunistic pathogens [212-215], but these account for less than 10% of all deaths from sepsis [216]. Most likely, these deaths are, in part, due to the lasting effects of the tissue damage caused by the early hyper-inflammatory phase.

1.4.3. The Liver in the Pathogenesis of Sepsis

The liver is one of the most commonly injured organ during sepsis [217]. Liver dysfunction and failure occurs in almost 50% and 10% of patients with sepsis, respectively, and liver damage is found in over 50% of patients who died from sepsis, second highest to only respiratory failure [217-219]. Liver dysfunction is caused by permanent damaging of hepatocytes that impairs their ability to synthesize molecules and clear toxins from the blood [217]. Damage to the liver can be caused by pro-inflammatory cytokines or toxins produced by the host during the progression of the disease, or by virulence factors secreted by the pathogen.

The liver also plays an active role in the pathogenesis of sepsis [217]. Both Kupffer cells, liver resident macrophages, and hepatocytes produce pro-inflammatory cytokines, and contribute to the pathology caused by the early hyper-inflammatory phase. Hepatocytes also produce components of the acute phase response, such as C-reactive protein and serum albumin A. These molecules are an important component of innate immune mechanisms, such as leukocyte chemotaxis and phagocytosis of the pathogen [220]. Lastly, dysregulated hepatic expression of cytochrome P450 enzymes, which are critical for toxin clearance in the blood, during sepsis [221] may lead to the accumulation of toxins in the blood that exacerbates tissue damage. The importance

of the liver during sepsis has been demonstrated in mice using hepatocyte-specific deletions that increase or decrease pathology in these models [222-226]. Therefore, studying the function of the liver during sepsis is critical to our understanding of this disease.

1.4.4. Endotoxin Tolerance in Monocytes as a Model of Sepsis

Endotoxin tolerance is a temporary state of "immune paralysis" in APCs following an exposure to LPS [199], wherein these refractory cells are unable to respond to a subsequent stimulation with LPS or another PAMP. The initial LPS stimulation induces epigenetic [227] and metabolic [228] re-programming of monocytes (termed "refractory monocytes") that prevents up-regulation of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-12 and TNF-α upon re-challenge. Blocking IL-10 and TGFβ during the primary challenge reverses the impaired cytokine production [229], indicating these anti-inflammatory cytokines are at least partially responsible for the induction of this state. Up-regulation of negative regulators of TLR signaling, such as IRAK-M [230], also contribute to endotoxin tolerance. This refractory state has been confirmed in mice and humans. Mice treated with a sub-lethal dose of LPS are protected from a subsequent lethal dose of LPS through decreased pro-inflammatory cytokine production [231, 232], and peripheral blood mononuclear cells (PBMCs) from healthy humans injected with LPS produce less TNF-α and IL-6 upon *ex vivo* re-challenge [233]. In both *in vitro* [234] and *in vivo* [231] models, endotoxin tolerance can last upwards of 7 days.

Monocytes that are tolerized to LPS recapitulate many of the features of monocytes obtained from patients with sepsis. In addition to impaired cytokine production, refractory monocytes have decreased expression of HLA-DR and the co-stimulatory molecule CD86 [235]. Low HLA-DR expression is commonly seen in patients with sepsis and is highly correlated with negative outcomes [200]. Down-regulation of HLA-DR and CD86 on refractory monocytes caused

a decrease in T cell priming and antigen-specific IFNγ production [235]. Similar to pro-inflammatory cytokine production, the impaired antigen presentation could be reversed by the addition of anti-IL-10 antibodies. However, not all monocyte functions are lost. Both refractory monocytes and monocytes from patients with sepsis exhibit enhanced phagocytosis capacity [234], increased microbicidal activity, and upregulation of genes involved in tissue remodeling [198]. Thus, endotoxin tolerance of monocytes is a useful model to study the effects of sepsis-induced immunosuppression of monocytes in the laboratory.

Not all PAMPs induce paralysis of APCs, though. Monocytes stimulated with β-glucan, a component of the fungal cell wall, have enhanced cytokine production upon a subsequent LPS challenge [236]. Similar to endotoxin tolerized monocytes, β-glucan-treated monocytes undergo a metabolic and epigenetic shift that boosts their recall response to PAMPs [237-239]. This "trained immunity" of myeloid cells acts as a form of innate immune memory and has many interesting clinical applications, including the potential to reverse immune paralysis in monocytes during sepsis [240].

1.5 Biology of the Aryl Hydrocarbon Receptor

1.5.1 History of the Aryl Hydrocarbon Receptor: from Toxicology to Immunology

AHR was first discovered as the receptor responsible for initiating the detoxification response to toxic dioxins and polycyclic aromatic hydrocarbons (PAHs) [241, 242]. Since the 1950s, it was known that benzo-[α]-pyrene (BP) and other PAHs were able to induce a "BP hydroxylase" (later called the aryl hydrocarbon hydroxylase (AHH) before being identified as the cytochrome P450 family 1 enzyme) in the rat liver that converted many PAHs into non-carcinogenic metabolites [243, 244]. However, it took almost three decades before AHR was identified as the receptor for

PAHs. In an unique approach, the laboratory of Dr. Olivier Hankinson used the fact that the cytotoxic effects of BP required the BP hydroxylase to select for "BP hydroxylase-deficient" clones that lacked the ability to express this enzyme following BP exposure [245]. Using this approach, two clones that could not bind TCDD, another prototypical AHR ligand, were generated [246] and the identity of receptor of the *Ah locus* was finally unmasked.

Since this discovery, the field of AHR has rapidly expanded and we now know much about the fundamental biology of this receptor [241, 247]. We know where AHR resides in the cytoplasm, how it recognizes its ligands, who its binding partners are, how it binds DNA and how it is turned off. The field, however, made a significant development in the mid 2000s when AHR was identified as a regulator of the immune system. The relationship between AHR and the immune system is complex: it can function as a pro- or anti-inflammatory mediator, and can be detrimental or beneficial to disease, depending on the cell type, pathogen, and environmental trigger. This section will discuss the fundamental biology of AHR and our current understanding of its role in the immune system.

Structure of the AHR gene and protein

AHR is a member of the Per-ARNT-Sim (PAS) transcription factor family named after the protein structural domain common to all family members [248]. It is widely expressed in mammalian cells and tissues [249] and is evolutionarily conserved from invertebrates to primates [250]. The AHR gene is comprised of 11 exons that are found on chromosome 7 in humans [251, 252] and chromosome 12 in mice [253]. Its promoter is unusual in that it contains neither a TATA or CCAAT box, but it does contain a GC-rich region within its multiple transcription binding sites [254, 255]. Within this region are four consensus binding sites for zinc-finger transcription factors,

such as Sp1 and Sp3, that are required for basal AHR expression [254, 256, 257]. In addition, the AHR promoter contains six activator protein-1 (AP-1) and two AP-2 binding sites [257], suggesting *Ahr* can be induced under certain environmental stresses.

The *Ahr* gene codes for an 848-amino acid protein in humans (805 amino acids in mice) that is composed of three essential functional domains (**Figure 1.5**). Its N-terminus bears a beta helix-loop-helix (bHLH) domain that is involved in DNA binding and protein-protein interactions. In this region are two arginine residues, located at positions 14 and 39, that are indispensable for DNA binding [258-260]. Site-directed mutagenesis has also identified tyrosine 9 as an essential residue for AHR to bind DNA [258]. How this residue contributes to DNA binding is unclear, but likely involves regulating the phosphorylation of an adjacent residue [261]. Next to the bHLH domain is the PAS domain, located from amino acids 111-342 in humans, that can be broken into two PAS subdomains, PAS-A and PAS-B, of approximately 50 amino acids in length each. Both PAS domains are essential for AHR protein-protein interactions with ARNT, its transcriptional activation partner, and its co-chaperones [247]. The PAS-B domain also contains the ligand-binding domain (LBD) of AHR [262]. The C-terminus of AHR contains the transactivation domain with a glutamine-rich region that interacts with multiple transcriptional co-activators [263, 264]. In addition to these functional domains, AHR also contains a nuclear localization sequence (NLS) within residues 13-39 and a nuclear export sequence (NES) at residues 63-73 [265].

1.5.2 The Ligands of AHR

Ligand binding by AHR is a pre-requisite for all of its functions. The classic AHR ligand is a planar molecule composed of multiple 5- or 6-member rings in tandem. However, outside of these constraints, the structure of AHR ligands is quite diverse (**Figure 1.6**). Moreover, some reported

AHR ligands possess only a single ring (e.g. kynurenine) or multiple rings connected by short, linear chains (e.g. dibenzoylmethane). This ligand promiscuity of AHR is due to its flexible ligand-binding pocket that can interact with molecules through multiple residues [266, 267]. In this section, the current knowledge of exogenous and endogenous ligands, and our understanding of the molecular basis of AHR ligand binding will be discussed.

Exogenous AHR ligands

The best characterized AHR ligands are dioxins that are produced as byproducts of the industrialized world. The prototypic AHR ligand of this class of molecules is 2,3,7,8-tetrachlorodibenzodioxin (TCDD), a contaminant of the inorganic herbicide Agent Orange [268]. Other exogenous AHR ligands are PAHs, such as BP and 7,12-dimethylbenz[α]anthracene (DMBA), are produced during the incomplete combustion of organic matter and are major constituents of exhaust particles from coal and diesel engines. These PAHs are typically highly potent ligands and can bind AHR at picomolar concentrations [269].

AHR ligands can also be produced naturally by organisms. Indole-3-carbinol (I3C) found in cruciferous vegetables consumed by mammals, such as broccoli and cabbage, is converted by stomach acids into two high-affinity AHR ligands, ICZ and DIM [270]. Bacterial species commonly found in the human and murine gut microbiota are also a rich source of AHR ligands by metabolizing tryptophan into indole derivatives [271, 272]. The uptake of these microbial- or dietary-derived AHR ligands has important implications for gut homeostasis and protection from gastrointestinal infections [273, 274]. Moreover, it was recently shown that pathogenic bacteria also produce molecules that act as AHR ligands. *P. aeruginosa* and *Mycobacterium tuberculosis*, two of the most common causes of respiratory tract infections, synthesized pigments that could

bind AHR and induce *Cyp1a1* expression as effectively as TCDD [275]. Similar to what happens in the gut, these pigments enhanced the protective immune responses to pulmonary infections through AHR activation of macrophages.

Endogenous AHR ligands

The search of an endogenous ligand is perhaps the biggest quest remaining in the field of AHR research [276]. Although they were hypothesized since the discovery of AHR, the idea of endogenous ligands was originally regarded as controversial [277]. It was not until the developmental defects observed in AHR-deficient mice [278-281] that the notion was strongly considered. Even today, there is much debate as to whether a true endogenous ligand of AHR has been identified.

Kynurenine (Kyn) is one of the current leading candidate for an endogenous AHR ligand. Like many AHR ligands, it is a tryptophan metabolite synthesized by the oxidizing enzymes tryptophan 2,3-dioxygenase (TDO2), indoleamine 2,3-dioxygenase 1 (IDO1), or IDO2 through an N-formyl-L-Kyn intermediate [282]. These enzymes catalyze the same reaction but have different tissue expression. TDO2 is constitutively expressed at high levels in the liver, whereas IDO1 and IDO2 are expressed outside the liver at low basal levels, but are highly inducible, including by many PAMPs (e.g. LPS, CpG) and cytokines (e.g. IFNγ, TNF-α). Kyn has been shown to bind AHR and induce *Cyp1a1* expression [232], and drive the development of tolerogenic DCs [283] and regulatory T cells (Treg) [284]. However, despite having profound AHR-dependent effects *in vitro*, the high concentration of Kyn required to activate AHR (> 50 μM, compared to the pM levels required for most AHR ligands) questions its *in vivo* significance.

Another candidate is the tryptophan UV-lysis product 6-formylindolo(3,2-b)carbazole (FICZ). Thought to be important in AHR-dependent processes in the skin, FICZ has been observed in tryptophan [285, 286], cell culture media [287], and keratinocytes [288, 289] exposed to UV. It can also be synthesized by tryptophan treatment with H_2O_2 or by skin yeast commensals of the *Malassezia* genus [290]. Importantly, FICZ binds AHR at picomolar concentrations comparable to TCDD [285], and has been reported to drive AHR-dependent mechanisms both *in vitro* and *in vivo* [291-293]. However, until FICZ is detected *in vivo* in the absence of exogenous administration, evidence that it is a true endogenous AHR ligand remains uncertain.

1.5.3 The Canonical AHR Pathway

The classical function of AHR is its ability to act as a transcription factor following ligand binding (**Figure 1.7**). In this pathway, the activation of AHR first depends on its release from the cytoskeleton. In the absence of its ligand, AHR is retained in the cytoplasm in an inactive state through an inactivation complex. This complex is composed of the chaperone proteins heat-shock protein (HSP) 90 [294, 295], p23 [296], and the AHR-interacting protein (AIP; also known as XAP2 or ARA9) [297, 298]. HSP90 interacts with the bHLH and PAS-B domains of AHR and is important in maintaining AHR in a high-affinity ligand binding conformation [299, 300]. It also masks AHR's DNA binding residues and NLS sequence located in the bHLH. AIP binds to both AHR and HSP90 to stabilize the complex [301] and helps maintain high cytosolic levels of AHR by preventing its ubiquitination and subsequent proteasome degradation [302]. p23 interacts with HSP90, and perhaps AHR, and enhances the ligand responsiveness of the complex [296]. In the nucleus, p23 also prevents AHR binding to ARNT in the absence of a ligand [296].

Ligand binding to AHR causes a confirmation change that seems to expose its NLS, while leaving most of the AHR inactivation complex intact (**Figure 1.7**). The exposed NLS allows AHR to enter the nucleus through a β-importin-dependent mechanism [303]. In the nucleus, ligand-bound AHR interacts with its DNA binding partner ARNT and is released by HSP90 and p23 [304, 305]. The AHR-ARNT dimer binds to DNA at xenobiotic response elements (DREs) located upstream of gene promoters and initiates the transcription of these genes [306]. DREs contain the core consensus sequence 5'-CACGCA-3'. The collective set of genes AHR transcriptionally activates are known as the 'AHR gene battery' [307]. The core members of this battery are the cytochrome P450 family 1 (*Cyp1a1*, *Cyp1a2*, and *Cyp1b1*) and *Ahrr*, but may include others such as *Nqo1*, *Nptx1*, *Fmo3*, *Serpine1*, and *Ugt1a6* [307] depending on the cell type.

1.5.4 Termination and Negative Regulation of AHR signaling

The detrimental effects of excessive AHR activation has led to the evolution of multiple regulatory mechanisms. The first level of regulation involves ubiquitin-targeting of AHR for proteasome degradation [308-310]. Following DNA binding and transcriptional induction of its target genes, CRM-1 recognizes the NES of AHR and exports it to the cytoplasm [308]. The export rate of AHR is partially controlled by masking the NES by phosphorylation [311, 312]. In the cytoplasm, AHR is ubiquitinated, likely on one of its 12 C-terminal lysine residues [313], by an unidentified E3 ligase and sent to the 29S proteasome for degradation. This mechanism therefore acts as a natural end to signaling through the AHR pathway and ensures the response can only be resumed by *de novo* AHR production. Cytoplasmic ubiquitination also prevents AHR from functioning when it spontaneously dissociates from its inactivation complex in the absence of ligand [314].

The second mechanism of AHR regulation is by the AHR repressor (AHRR). AHRR is a core member of the AHR gene battery and is rapidly transcribed in most cells following AHR activation. It has a higher binding affinity for the AHR PAS domain than ARNT and disrupts AHR-ARNT dimerization. Since AHRR does not have a DNA binding domain, this effectively precludes AHR binding to XRE and stops transcriptional activation of its target genes [315]. However, AHRR can only prevent AHR from acting as a transcription factor and likely does not disrupt other AHR functions, such as its E3 ubiquitin ligase activity [316].

Finally, AHR activation is negatively regulated through CYP1-dependent hydroxylation of its ligands. Similar to AHRR, the CYP1 family is induced following AHR activation. The family is composed of *Cyp1a1*, *Cyp1a2* and *Cyp1b1* and has tissue-specific expression patterns. *Cyp1a1* and *Cyp1b1* are basally expressed at low levels, but highly inducible by AHR ligands. They are expressed in almost all cells, including leukocytes [249], but the highest induction occurs in barrier epithelial cells and the liver [278]. *Cyp1a2* expression is restricted to the liver [317], and is both constitutively expressed at high levels and inducible [278, 318]. All three CYP1 proteins are found in the endoplasmic reticulum and can metabolize many of the molecules that AHR recognizes. Clearance of these ligands limits their biological effects and prevents continuous AHR activation. The importance of the CYP1 family in regulating AHR is exemplified by the enhanced and prolonged AHR activation observed in cell lines treated with CYP1A1 inhibitors [319]. More recently, it was shown that systemic over-expression of *Cyp1a1* depleted the gut AHR ligand levels and caused impaired immunity and pathogen clearance during *C. rodentium* infection [274]. Therefore, the CYP1 family acts as rheostats for AHR that link its activation to the concentrations of PAH's currently present. Moreover, unlike AHRR, the CYP1 can theoretically inhibit all ligand-dependent AHR functions [320].

1.5.5 AHR Regulation of Immune Function

As mentioned previously, in addition to controlling the detoxification response to polycyclic aromatic molecules, AHR has emerged as a critical regulator of the immune response [321, 322]. The first indication that AHR could influence immunity was from the immunosuppressive state observed in mice following repeated injections of AHR ligands [323-325]. It is now known that AHR is expressed in both the myeloid and lymphoid lineages [249], and plays an important role in the development and function of these cells (**Figure 1.8**). This section will outline how AHR expression in leukocytes impacts the function of the immune system.

AHR in the lymphoid lineage

AHR expression in lymphoid cells is thought to act as a link between the environment and the immune system [326]. As such, many of the known immunological roles of AHR are in the development of a Th17-like RORγT-dependent type 3 immune response that is critical for maintaining mucosal tissue integrity during infection. For example, activation of AHR during αβ T cell differentiation promotes the generation of Th17 cells and enhances their function [292, 293, 327]. By directly interacting with RORγT, the master transcription factor of Th17 cells, AHR reinforces the Th17 program and enhances the production of IL-17 and IL-22. It also prevents IL-2 inhibition of Th17 differentiation by blocking STAT5 phosphorylation [327]. The effects of AHR on Th17 differentiation are not only critical for protection against pathogens at barrier sites [274, 328, 329], but are also implicated in the development of autoimmune disorders. Treating mice with the AHR agonist FICZ during the experimental autoimmune encephalitis (EAE) model

of multiple sclerosis increases the number of Th17 in the draining lymph nodes of these mice and worsened their pathology [292, 293].

AHR also drives the differentiation of type 1 regulatory T cells (Tr1) [330, 331]. These cells are defined as IL-10-producing $CD4^+$ T cells that do not express Foxp3 [332] and are critical for limiting pathogenic T cell responses during murine EAE [333]. AHR is induced in T cells by IL-27, a growth factor for Tr1 cell development [334, 335], and cooperates with the transcription factor c-Maf to transactivate the expression of IL-10 and IL-21 [330]. The functional importance of this mechanism was demonstrated by the protection against EAE elicited by MOG-specific T cells cultured in the presence of IL-27. This protection was not obtained from the adoptive transfer of cultured T cells defective in AHR [330]. However, it remains unclear how AHR signaling can be protective or detrimental to EAE based on whether it drives Th17 or Tr1 differentiation. One proposed mechanism is this balance is regulated by temporal conditions of AHR activation. Ligands that cause prolonged AHR activation, such as TCDD, drive the differentiation of Tr1 cells and would be protective, whereas more transient activating ligands such as FICZ may favor the differentiation of Th17 cells and be pathogenic [292]. A second intriguing mechanism is based on the finding that AHR, along with TGF-β, promoted the trans-differentiation of Th17 cells to Tr1 cells during the resolution of inflammation [336]. In this context, the AHR may contribute to a step-wise Th0 to Th17 to Tr1 maturation process, or additional signals are required for complete differentiation to Th17 cells [322].

In addition to conventional αβ T cells, AHR is also required for the development of $ROR\gamma T^+$ group 3 innate lymphoid cells (ILC3s) and γδ T cells. AHR maintains the survival of $ROR\gamma T^+$ ILC3s in the small intestine lamina propria and Peyer's patches by upregulating Notch surface expression and stabilizing the expression of c-Kit [337, 338]. The loss of this population

in AHR-deficient mice results in impaired formation of gut-associated lymphoid tissues and susceptibility to *C. rodentium* infection [337]. AHR is also required for optimal functioning of ILC3s, as it augments IL-22 production through enhanced recruitment of RORγT to the *Il22* locus [339]. Diet and the microbiota are the major sources of AHR ligands required for the maintenance and function of intestinal ILC3s and Th17 cells [272, 274, 337, 340].

Although AHR is expressed in all γδ T cells, its importance in the survival and function of these cells is subset-specific. AHR-deficient mice have an almost complete loss in skin epidermal Vγ3$^+$ γδ T cells (Garman nomenclature [341]) and intestinal Vγ5$^+$ γδ T cells, but normal numbers of other skin γδ T cell subsets, lung Vγ4$^+$ γδ T cells, and total γδ T cells in the spleen and lymph nodes [340]. Moreover, AHR activation enhances IL-22 production and proliferation of γδ T cells *in vitro* [342]. Unfortunately, the biological ramifications of intrinsic AHR expression in γδ T cells during a disease or infection has yet to be shown.

AHR in the myeloid lineage

AHR is constrictively expressed by monocytes, macrophages and DCs, and its activation limits the pro-inflammatory responses induced by PAMPs. This was first evident by the profound susceptibility of AHR-deficient mice to endotoxemia [232, 343, 344]. These mice have elevated serum levels of pro-inflammatory cytokines and decreased serum IL-10 levels, and succumb to this hyper-inflammatory response because of greater lung and liver tissue pathology. Macrophages-specific deletion of AHR, using the LysM-cre mice, recapitulated this phenotype and demonstrated a macrophage-intrinsic role for AHR [343]. Moreover, peritoneal macrophages from AHR-deficient mice produce more TNF-α and IL-6 in response to LPS [344]. Mechanistically, AHR limits the inflammatory response by complexing with NF-κB and STAT1

and blocking NF-κB-dependent transcription without affecting its DNA binding [344]. It also modulates the production of type I IFNs to viruses by inducing the expression of the ADP-ribosylase TIPARP that blocks TBK1 activation through a post-translational modification [345]. However, not all inflammatory cytokines are reduced by AHR activation. For example, IL-8 production is augmented by the AHR ligand FICZ in human DCs [346], although the molecular details of this outlier are unknown.

AHR also regulates the antigen presentation and T cell differentiation-inducing properties of DCs. FICZ decreased the LPS-induced expression of HLA-DR, CD80, and CD86 in human moDCs, while not affecting the expression of CD83 and CD40 [346, 347]. The downregulation of the antigen presentation machinery on DCs by FICZ resulted in reduced differentiation of Th1 and Th17 cells *in vitro*. Instead, DCs stimulated in the presence of AHR ligands become tolerogenic and promote the differentiation of Treg that are protective during EAE [283, 348]. AHR can also impact the differentiation of DCs. AHR activation during human monocyte differentiation skews the culture towards a moDC-phenotype by inducing the expression of BLIMP-1 [349]. These DCs had a very similar hyper-inflammatory profile observed in conventional moDCs generated with GM-CSF and IL-4, and their gene signature was enriched in leprosy lesions with a strong immune response to *Mycobacterium leprae.* Therefore, similar to T cells [322], the conditions and timing of AHR exposure to DCs may dictate whether AHR promotes tolerogenic or inflammatory DCs *in vivo*.

The past decade has elucidated multiple mechanisms of AHR in the immune response. We know that AHR operates in many different cell types and can promote both pro-inflammatory and immune-regulatory responses depending on timing and magnitude of its activation. However, to fully understand the complex and diverse functions of AHR in leukocytes requires more work.

1.6 Rationale and Specific Aims

As outlined in Chapter 1, immune regulatory mechanisms are essential for limiting the pathogenic responses of the immune system, but can also lead to the development of disease. Two examples were these mechanisms contribute are the pathobiotic behavior of *S. aureus* and the pathogenesis of sepsis. In both scenarios, monocytes and macrophages play a central role in governing the ongoing immune response and are being targeted therapeutically [350]. Thus, a better understanding of the contribution of monocytes and macrophages to the pathophysiology of these diseases will lead to improved therapies. The objective of this thesis was to investigate monocyte and macrophage immune regulatory mechanisms involved in *S. aureus* pathobiosis and sepsis development.

The first part of my thesis focused on the anti-inflammatory cytokine IL-10 in the context of *S. aureus* pathobiosis. Previous work from our lab reported that IL-10 production to the cell wall of *S. aureus* down-regulated SAg activation of T cells and prevented staphylococcal TSS [80], and may also dictate asymptomatic nasal colonization and bacteremia by *S. aureus* [3]. However, this work was done using laboratory strains of *S. aureus* and my first specific aim was to test the operation of this mechanism in natural-occurring isolates of *S. aureus*. **We hypothesized that naturally-occurring *S. aureus* isolates would differ in their capacity to induce IL-10**. To test this hypothesis, we used 16 nasal isolates of *S. aureus* obtained from the nose of patients with chronic rhinosinusitis and characterized the pro- and anti-inflammatory responses to these isolates by human peripheral blood mononuclear cells.

The second part of my thesis investigated the role of AHR in the function of monocytes and macrophages during laboratory models of sepsis. AHR is emerging as a potent regulator of the inflammatory response to PAMPs, such as LPS [232, 344]. Moreover, AHR signaling is

regulated by three mechanisms [320], one of which is the metabolism of AHR ligands by the CYP1 family to suppresses its activation [319]. However, how this family controls AHR activity and functions in monocytes is unknown. **We hypothesized that changes in the expression of CYP1 during inflammation would alter the availability of AHR ligands to monocytes and thereby control the activation of these cells by PAMPs.** To test this hypothesis, we used *in vitro* and *in vivo* models of monocytes during both the hyper-inflammatory and immunosuppressive phases of sepsis to study the intrinsic and extrinsic effects of CYP1 expression on AHR activation in these cells.

1.7 Tables

Table 1.1. Location and ligands of TLR family.

Member	Location [29]	Ligand	References
TLR1	Plasma membrane	Triacyl lipoproteins, with TLR2	[351]
TLR2	Plasma membrane	Lipoproteins, with TLR1, TLR6, and TLR10	[351-355]
TLR3	Endolysosome	dsRNA	[356]
TLR4	Plasma membrane	LPS	[46, 47]
TLR5	Plasma membrane	Flagellin	[357]
TLR6	Plasma membrane	Diacyl lipoproteins, with TLR2	[353, 354]
TLR7	Endolysosome	ssRNA	[358]
TLR8	Endolysosome	ssRNA	[358, 359]
TLR9	Endolysosome	DNA	[360]
TLR10	Plasma membrane [361] and endolysosome	Lipoproteins, with TLR2	[355]

Table 1.2. Diagnostic criteria for toxic shock syndrome. [Adapted from [362]]

Fever ≥ 38.9°C
Rash – diffuse mascular erythroderma
Desquamation – 1-2 weeks after onset of illness, especially of palms and soles
Hypotension – systolic blood pressure ≤ 90 mm Hg for adults
Multi-system involvement – 3 or more of the following:
Gastrointestinal – vomiting or diarrhea at the onset of illness
Muscular – severe myalgia or elevated creatine phosphokinase
Mucous membranes – vaginal, oropharyngeal, conjunctive hyperaemia
Renal – blood urea nitrogen or creatinine twice-upper limit of normal
Hepatic – total bilirubin twice-upper limit of normal
Haematological – platelets ≤ 100 x 10^9/L
Negative results on the following tests:
Blood, throat, or cerebrospinal fluid culture (blood culture may be positive for *S. aureus*)
Rise in titre to Rocky Mountain spotted fever, leptospirosis, or measles

1.8 Figures

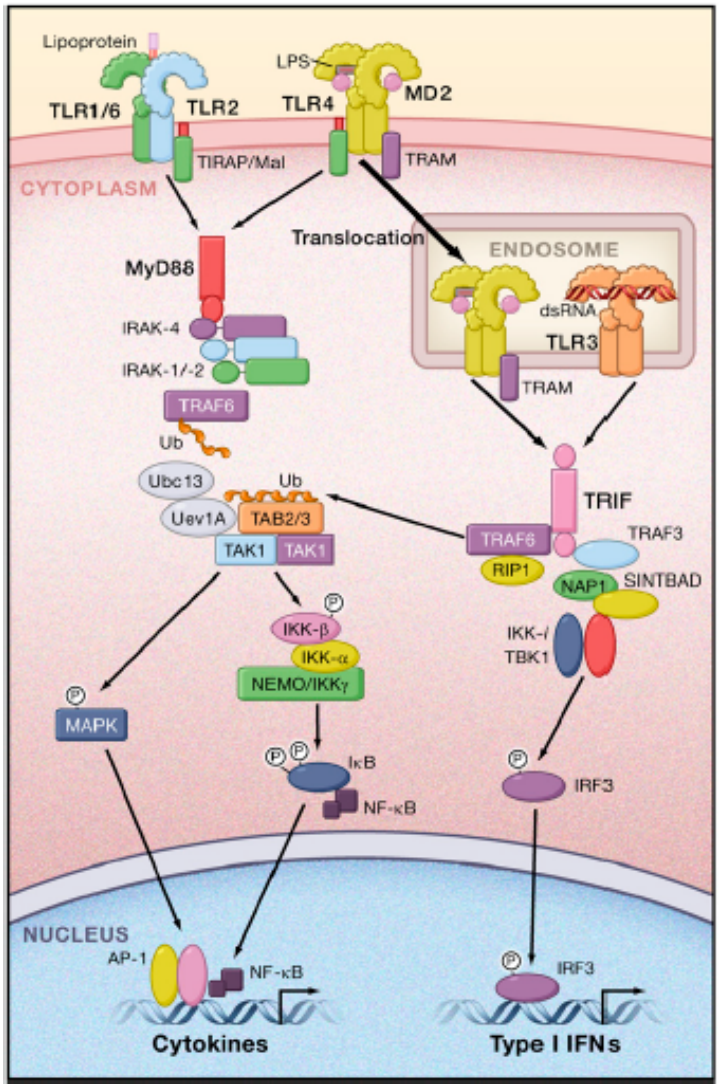


Figure 1.1. TLR signaling pathways. TLR2 heterodimers and TLR4 homodimers drive MyD88-dependent signaling to induce the production of cytokines (left cascade). TRIF-dependent signaling (right cascade) is activated following TLR3 or TLR4 activation and induces the production of Type I IFNs and cytokines. (Source: Adapted from [29])

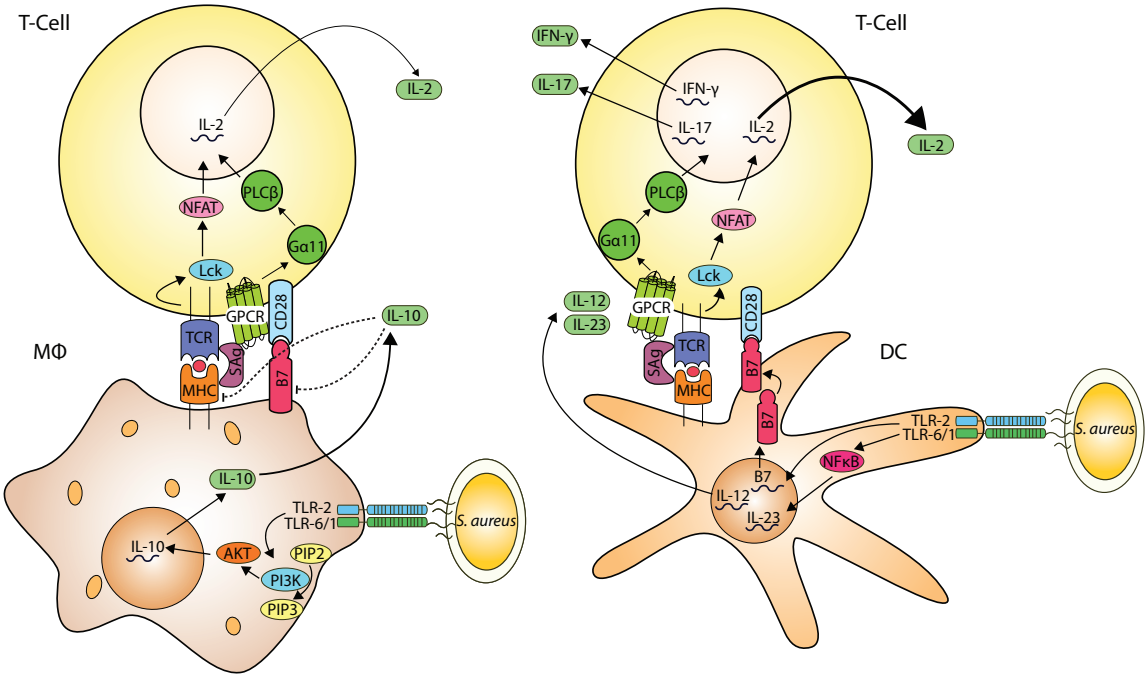


Figure 1.2. A framework for pathobiosis by *S. aureus*. When monocytes/macrophages (MΦ) are the primary APC responding to *S. aureus* (left panel), TLR2 ligands embedded in the cell wall predominantly induce IL-10 production through the PI3K/AKT pathway, block SAg-induced T cell activation. When dendritic cells (DC) are the primary APC (right panel), TLR2 ligands trigger a Th1/Th17 response, which characterizes *S. aureus* infections, and the development of T cell immunity. (Source: Adapted from [3])

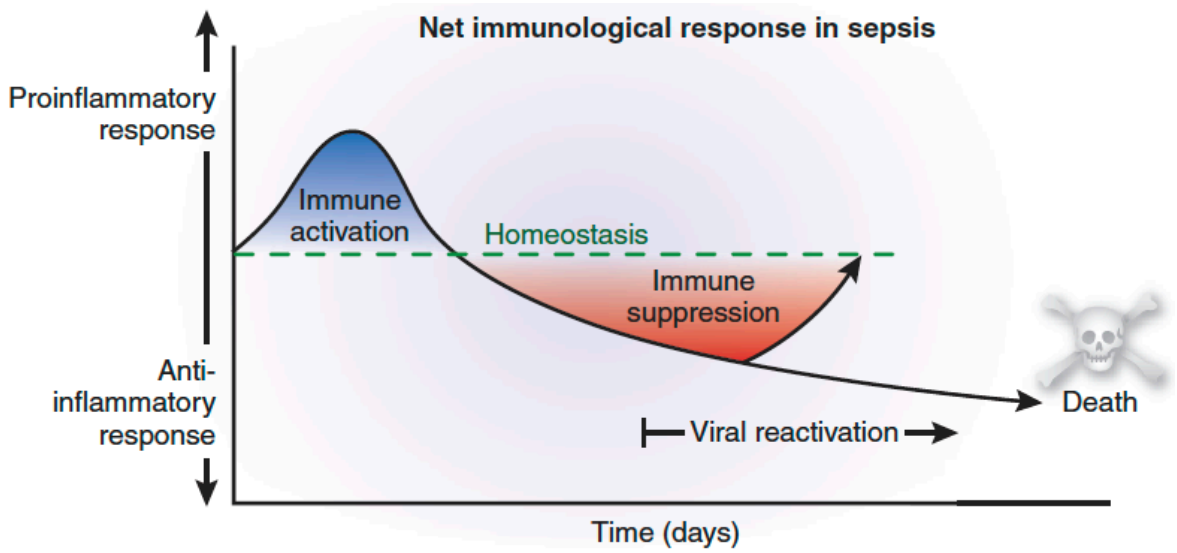


Figure 1.3. The pathogenesis of sepsis. Patients with sepsis initially present with a hyper-activated immune system and a "cytokine storm" of pro- and anti-inflammatory mediators. This phase subsides within a few days and the patient develops sepsis-induced immunosuppression. Recovery from sepsis occurs when the pathogen is cleared and the immune system returns to homeostasis. Death from sepsis may result from the tissue damage initiated by the hyper-inflammatory phase or latent viral reactivation in the immunosuppressive phase. (Source: Adapted from [175]).

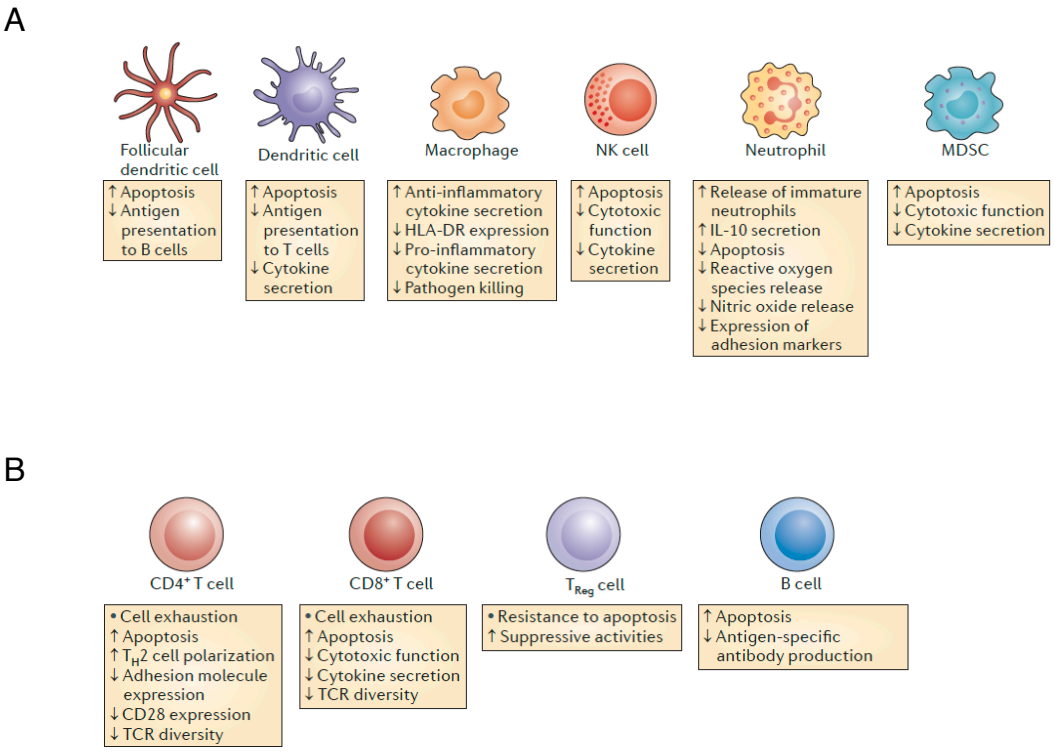


Figure 1.4. Immunological changes seen in the immunosuppressive phase of sepsis. Cellular characteristics of sepsis-induced immunosuppression in cells of the (A) innate and (B) adaptive immune systems. (Source: Adapted from [197])

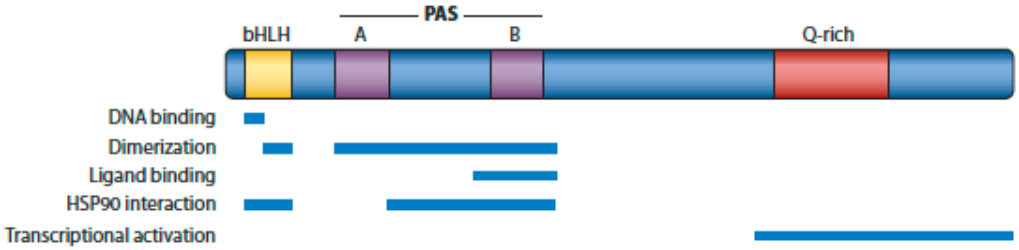


Figure 1.5. The major protein domains and key functional regions of AHR. (Source: Adapted from [321])

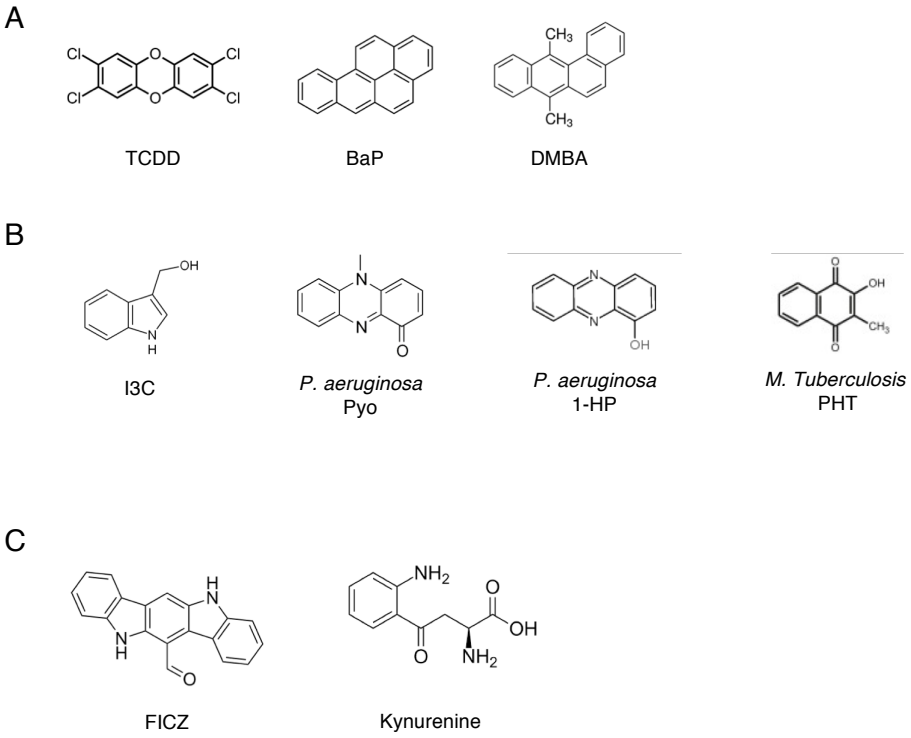


Figure 1.6. Structures of known AHR ligands. (A) Exogenous artificially-synthesized AHR ligands. (B) Exogenous natural-occurring AHR ligands. (C) Endogenous AHR ligands.

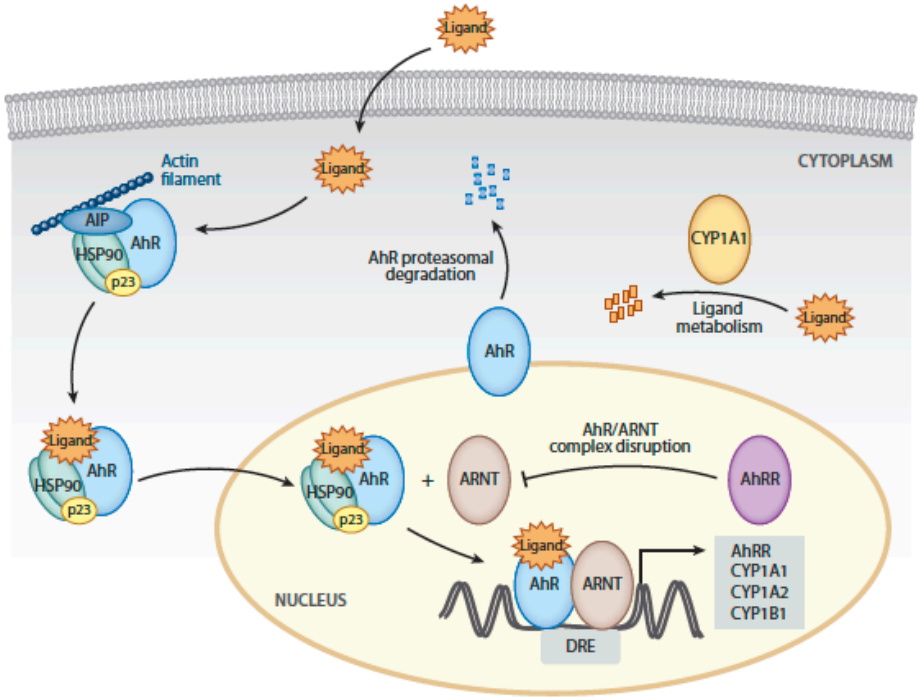


Figure 1.7. Canonical AHR signaling pathway. Ligand-induced activation of AHR leads to its nuclear translocation and dimerization with ARNT. The AHR:ARNT dimer binds to XRE in the genome and transcriptional activates the AHR gene battery (Grey Box). AHR signaling is turned off by three mechanisms: (1) AHR nuclear export and ubiquitin-dependent proteasome degradation; (2) Disruption of the AHR:ARNT dimer by AHRR; and (3) Ligand metabolism by CYP1 family. (Source: Adapted from [321]).

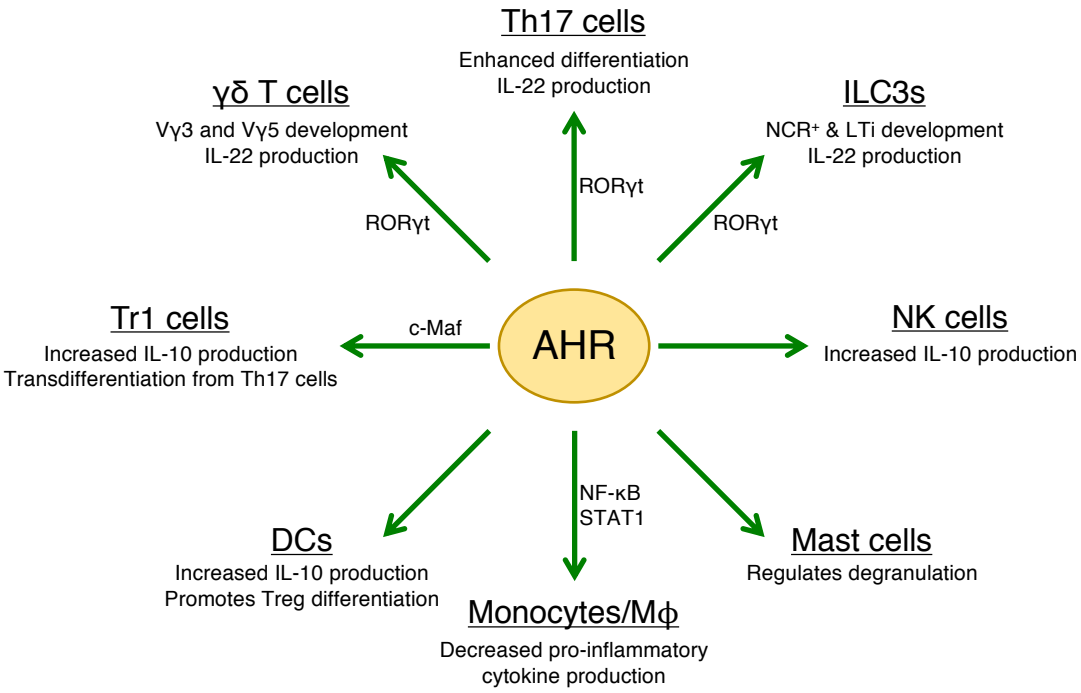


Figure 1.8. Immunological functions of AHR. Development and functions of lymphocytes and myeloid cells that are modulated by AHR. Proteins beside green arrows are transcription factors AHR directly interacts to execute its function.

Preface to Chapter 2

As outlined in Chapter 1, the IL-10 response triggered by *S. aureus* has important effects on the development of host tolerance and disease pathogenesis of this microbe. Moreover, our laboratory has previously demonstrated that this IL-10 response is induced by cell wall components of *S. aureus* through a TLR2-dependent, PI3K-Akt-mediated pathway [80, 160]. However, the majority of these results were obtained using a single laboratory strain of *S. aureus*. We therefore wanted to investigate the capacity of nasal isolates of *S. aureus* to activate this pathway and compare it to the pro-inflammatory response induced by *S. aureus*. To do this, we screened a panel of 16 isolates of *S. aureus* obtained from the noses of patients with chronic rhinosinusitis for their capacity to induce pro- and anti-inflammatory cytokines in human peripheral blood mononuclear cells. We hypothesized that these *S. aureus* isolates would differ in their ability to induce IL-10 production and occur independently of the pro-inflammatory response.

Chapter 2 : Uncoupling of Pro- and Anti-Inflammatory Properties of *Staphylococcus aureus*

Adam G. Peres[*,†], Camille Stegen[*,†], Junbin Li[*,†], An Qi Xu[*,†], Benoit Levast[*,†], Michael G. Surette[†,‡], Benoit Cousineau[*,†], Martin Desrosiers[†,§], and Joaquín Madrenas[*,†,¶].

the [*]Department of Microbiology and Immunology, and the [†]Microbiome and Disease Tolerance Centre, McGill University, Montréal, QC, Canada; [‡]Department of Medicine, McMaster University, Hamilton, ON, Canada; and [§]Centre hospitalier de l'Université de Montréal, Montréal, QC, Canada

This chapter contains the following published manuscript:

Peres AG, Stegen C, Li J, Xu AQ, Levast B, Surette MG, Cousineau B, Desrosiers M, and Madrenas J. 2015. Uncoupling of pro- and anti-inflammatory responses to nasal *Staphylococcus aureus* isolates. *Infect Immun* 83: 1587-1597. *doi: 10.1128/IAI.02832-14*

This manuscript was highlighted in the *Spotlight* section by the editors as an article of significant interest.

2.1. Abstract

Staphylococcus aureus is a gram-positive bacterium carried by a quarter of the healthy human population that can cause severe infections. This pathobiosis has been linked to a balance between TLR2-dependent pro- and anti-inflammatory responses. The relationship between these two types of responses is unknown. Analysis of 16 nasal isolates of *S. aureus* showed heterogeneity in their capacity to induce pro- and anti-inflammatory responses, suggesting that these two responses are independent of each other. Uncoupling of these responses was corroborated by selective signaling through PI3K-Akt-mTOR and ERK for the anti-inflammatory response and through p38 for the pro-inflammatory response. Uncoupling was also observed at the level of phagocytosis and phagosomal processing of *S. aureus*, which were required solely for the pro-inflammatory response. Importantly, the anti-inflammatory properties of a *S. aureus* isolate correlated with its ability to modulate T cell immunity. Our results suggest the presence of anti-inflammatory TLR2 ligands in the staphylococcal cell wall, whose identification may provide templates for novel immunomodulatory drugs.

Keywords: *Staphylococcus aureus*, Pathobiosis, Commensalism, Microbiome, Disease Tolerance, Infection, Toll-like Receptor-2, Human, Cytokines, PI3K-Akt-mTOR

2.2. Introduction

S. aureus is a gram-positive bacterium that is frequently associated with localized soft-tissue infections (e.g. impetigo, dermatitis) and also systemic complications (e.g. bacteremia, sepsis and toxic shock syndrome (TSS) [90, 91, 363]. It is the most common microbe isolated from intra-hospital microbiological samples, and the second most common microbe isolated from outpatient samples [85]. However, *S. aureus* is also part of the healthy human microbiome of the upper respiratory tract, being chronically carried by more than 25% of the general population with no long-term ill effects [92, 101, 364]. As such, *S. aureus* can be classified as a pathobiont: an organism that is typically safe to its host, but can become pathogenic under certain circumstances other than immunosuppression.

One of the remarkable features of this state of pathobiosis is that 'commensal' *S. aureus* isolates contain many, if not all, of the known virulence factors and microbial associated molecular patterns (MAMPs) linked to disease [122, 365, 366]. The pathogenic potential of these isolates is exemplified by the risk of staphylococcal nasal carriers to develop systemic infections caused by the endogenous *S. aureus* strain they carry [92, 367]. How these highly pathogenic microbes can behave as commensals and only rarely cause disease remains unknown [3, 368].

Early recognition of *S. aureus* is initiated by pattern recognition receptors (PRRs) on epithelial cells and innate phagocytic cells. Toll-like receptor 2 (TLR2) has emerged as the most important of these PRRs in detecting extracellular *S. aureus* [369]. It heterodimerizes with TLR1 or TLR6 to recognize lipopeptides and glycopolymers embedded in the staphylococcal cell envelope triggering pro-inflammatory responses. Conventional pro-inflammatory TLR2 signaling begins with the recruitment of the adaptor proteins TIRAP and MyD88 and the Ser/Thr kinases IRAK-1 and -4. Distal TLR2 signaling activates the NF-κB and MAPK pathways to upregulate

pro-inflammatory cytokine (i.e. IL-1β, IL-6, TNF-α, IL-12p70) and chemokine (i.e. IL-8, CCL2, CCL3, CCL4, RANTES) production that will then coordinate microbial clearance [29]. The importance of this pathway is highlighted by the susceptibility of MyD88/IRAK4-deficient patients to staphylococcal infections [71, 72].

TLR2 also cross-talks with other PRRs, including NOD1/2 and TLR9 that recognize fragments of the peptidoglycan (PGN) backbone and CpG-DNA, respectively [370]. TLR9 activates a similar signaling pathway as TLR2 but without the need for TIRAP bridging, whereas NOD1/2 activate the NF-κB pathway through RIP-2. Signaling from these receptors requires phagocytosis and subsequent endosomal processing of *S. aureus* to liberate typically hidden ligands on the staphylococcal cell wall or in the DNA [146, 371]. Digestion of *S. aureus* also releases additional TLR2 ligands that amplify the inflammatory response. Ultimately, cross-talk between signaling from these receptors enhances the host's ability to clear infection and avoid disease.

It has been recently shown that, in addition to the pro-inflammatory response described above, *S. aureus* is capable of inducing a robust anti-inflammatory response as measured by production of IL-10 [80, 81, 160]. We and others have shown that this anti-inflammatory response results from TLR2 signaling upon recognition of staphylococcal PGN-embedded molecules and activation of PI3K-Akt signaling to stimulate IL-10 production [80, 148, 156]. Moreover, down-regulation of the co-stimulatory molecules CD86 and up-regulation of the immunoregulatory PD-L1 may provide complementary effects to limit the development of an adaptive immune response [81]. Interestingly, monocytes and macrophages are more potent at activating this response than dendritic cells [160]. Altogether, the anti-inflammatory TLR2 signaling may promote an environment of disease tolerance to *S. aureus* and support commensalism by this microbe [82].

It has been assumed that both pro- and anti-inflammatory responses to TLR2 engagement emanate coordinately and simultaneously from this receptor. If this paradigm is correct, then one would expect that both types of responses result at the same ratio upon receptor engagement. In contrast to this paradigm, we report here that the pro- and anti-inflammatory responses to *S. aureus* are uncoupled, i.e., independent of one another. Such an uncoupling can be observed in the analysis of responses to nasal isolates of *S. aureus* from community carriers of this microbe, suggesting an ongoing selective process for these properties. We show that the human anti-inflammatory response to these *S. aureus* isolates is mediated by the PI3K-Akt-mTOR and ERK pathways and does not require internalization of *S. aureus* whereas the pro-inflammatory response utilizes the p38 pathway and is dependent on phagocytosis of this microbe. Moreover, the magnitude of the IL-10-inducing response translates into different regulation of adaptive T cell responses to *S. aureus*. Based on these data, we propose that the cell wall of *S. aureus* contains two sets of TLR2 ligands: one that induces predominantly pro-inflammatory responses, and a second set that induces predominantly anti-inflammatory responses.

2.3. Materials & Methods

Cells. Human peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of healthy volunteers by Ficoll-Hypaque density gradient centrifugation. Volunteers gave their informed consent in compliance with the Research Ethics Office at McGill University. PBMCs were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin-streptomycin, L-glutamine, non-essential amino acids and sodium pyruvate. For experiments comparing clindamycin-treated and heat-killed *S. aureus*, clindamycin (1 μg mL^{-1}) was substituted for

penicillin-streptomycin in supplemented RPMI 1640. Peripheral blood neutrophils were isolated by red blood cell lysis of the pellet following Ficoll-Hypaque centrifugation.

Bacteria. *S. aureus* isolates were obtained from the nostrils of individuals attending an Ear/Nose/Throat clinic. All the isolates were confirmed as *S. aureus* using PCR with primers specific for the *16S* ribosomal RNA gene common to all bacteria (5'-AGAGTTTGATCATGGCTCAG-3'; 5'-GGACTACCAGGGTATCTAAT-3') and the *S. aureus nuc* gene (5'-GCGATTGATGGTGATACGGTT-3'; 5'-ACGCAAGCCTTGACGAACTAAAGC-3') (**Supplementary Fig. 2.1**). In addition, full genome sequencing and multi-locus sequence typing (MLST) of isolates further confirmed they were *S. aureus* [372]. The clonality of these isolates is representative of *S. aureus* strains in the community (**Supplementary Table 2.1**). Bacteria were grown overnight to stationary phase in tryptic soy broth (TSB), washed, and resuspended in sterile PBS. Culture supernatants were collected, filtered through a 0.2 μm filter, and stored at -20^{0}C. Bacteria (~10^{10} CFUs) were plate counted and heat-killed for 1 h at 100^{0}C in a heating block. For bacterial internalization experiments, *S. aureus* (10^9 CFUs) were stained with 5 μg/mL of TAMRA (Sigma-Aldrich) in 50 μL of PBS for 1 h at room temperature and washed and re-suspended in sterile PBS. For bacterial fractionation experiments, *S. aureus* isolates were digested with 1 mg/mL lysozyme and 0.1 mg/mL mutanolysin for 1 h in TES buffer (10 mM Tris, 1 mM EDTA, 25% sucrose, pH 8). Cell wall fractions were separated from protoplast fractions by differential centrifugation at 2,500 x *g* for 10 min and precipitated with 10% Trichloroacetic acid and resuspended in PBS. Protoplast fractions were resuspended in TE buffer (10 mM Tris, 1 mM EDTA, 2% SDS) and boiled for 5 min to reduce viscosity. For clindamycin-treated experiments, *S. aureus* was grown overnight in TSB followed by culture in TSB-containing clindamycin (1 μg mL^{-1}) for an additional 6 h period.

Reagents. Clindamycin, cytochalasin D, dynasore, PD-98059, rapamycin, SB-203580, staphylococcal peptidoglycan, and wortmannin were purchased from Sigma-Aldrich. The PI3K p110 isoform specific inhibitors PIK-75, TGX-221, AS-604850 and IC-87114 were purchased from EMB-Millipore. BIRB-0796 was purchased from Cayman Chemicals. Antibodies to phosphorylated Akt at Ser473 (clone 193H12), pan Akt (clone 11E7), phosphorylated p38 (Thr180/Tyr182; clone 12F8), pan p38 (9212), phosphorylated Erk1/2 (Thr202/Tyr204; clone 197G2), and pan Erk1/2 (clone 137F5) were purchased from Cell Signaling Technology. Conjugated antibodies used for flow cytometry were CD3-APC-eF780 from eBioscience, and CD14-PerCP-Cy5.5, CD19-APC, IL-10-PE, TNF-α-AlexaFluor 700, phospho-Akt(S473)-AlexaFluor 488 and pan Akt-BV421 from BD Biosciences.

Functional assays. PBMCs were seeded in 96-well plates (200,000 cells in a volume of 200µl per well) and stimulated under the conditions indicated in the corresponding figure legends. When inhibitors were used, cells were incubated for 1 h prior to stimulation, using 0.1 % DMSO as a control. Cell-free supernatants were collected and stored at -20^{0}C until analyzed for accumulation of cytokines by ELISA (eBioscience).

Flow cytometry. PBMCs (1 x 10^6 cells per group) were stimulated with the *S. aureus* isolates under the conditions indicated in the respective figure legends. For intracellular cytokine staining, 3 µg/ml of brefeldin A (eBioscience) was added after 6 h of stimulation, and the stimulation was continued for an additional 12 h. Dead cells were excluded from the analysis using Zombie Aqua fixable viability kit (BioLegend). Cells were washed in PBS containing 2% FBS and 2 mM EDTA, blocked with 10% normal human serum, and stained for CD3, CD14, and CD19. Cells were fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) and stained for IL-10 and TNF-α. For phospho-flow, PBMCs were stimulated for 30 min, fixed with Fix Buffer I (BD

Biosciences), stained for extracellular markers, permeabilized using Perm/Wash Buffer I (BD Biosciences) and stained for the intracellular molecules of interest. Events were acquired on a LSRII Fortessa (BD) and doublets were excluded based on FSC-A/FSC-H. Data analysis was performed using FlowJo version 10.x (Tree Star Inc.).

Western blotting. PBMCs (5 x 10^6 cells per group) were resuspended in 100 μL of media and rested for 5 min at 37^0C. When used, inhibitors were added at a 1:1 (v/v) ratio to the cells at twice the concentration indicated in the figure legends for 1 h. Next, the stimulants were added for 30 min, and cell lysates were prepared, run on 10% acrylamide gels and immunoblotted as described [80, 373].

Statistics. Statistical analysis of intra-group differences was performed using ANOVA and the Student's t-test or analysis of variance with post hoc Bonferroni test on Prism GraphPad and a *P* value of < 0.05 was deemed significant.

2.4. Results

Uncoupling of pro- and anti-inflammatory responses to nasal isolates of S. aureus

S. aureus recognition by host TLR2 induces both pro-inflammatory and anti-inflammatory cytokine production [369]. However, the mechanisms governing these responses have been studied only in the context of laboratory isolates, crude staphylococcal preparations, or synthetic TLR2 ligands [80, 160]. These experimental systems may not account for the diversity of interactions that the human immune system has with *S. aureus* and the subsequent heterogeneity of responses to this microbe. Indeed, *S. aureus* is capable of causing a spectrum of diseases, in addition to being a part of the human commensal flora. To determine the variation of the host responses to *S. aureus*, we stimulated human PBMCs with 16 *S. aureus* isolates obtained from the nostrils of human

carriers. These isolates were representative of a cross-section of *S. aureus* found in the community as indicated by MLST (**Table 2.1**) [374]. We then measured TNF-α and IL-10 production to assess their pro- and anti-inflammatory properties, respectively. We found up to a 3-fold difference in the IL-10 production in response to these nasal *S. aureus* isolates by PBMCs (**Figure 2.1A**). This response was reproducible and consistent for multiple PBMC donors, and largely determined by the bacterial isolate. A similar heterogeneity was seen in the capacity of these isolates to induce a pro-inflammatory TNF-α response (**Figure 2.1B**). However, when we compared the capacity for an isolate of *S. aureus* to induce both IL-10 and TNF-α, we observed no correlation between these two responses ($r^2 = 0.07586$, $p = 0.3019$; **Figure 2.1C**). From these results, we postulated that the pro- and anti-inflammatory responses to *S. aureus* can be uncoupled.

Human PBMCs are a heterogeneous population consisting mostly of T cells, B cells and monocytes, which can all produce IL-10 and TNF-α under different conditions [375]. One possible explanation for uncoupling of the pro- and anti-inflammatory responses is a different cellular source of these cytokines. To address which population(s) were producing IL-10 and TNF-α, we stimulated human PBMCs with isolates that induced a high or a low IL-10 response, and used intracellular flow cytometry to identify the PBMC population producing these cytokines. For simplicity and from here onward, we are showing the results obtained with S8 and S5 isolates as representative of the results obtained of high and low IL-10 inducers. Monocytes mounted a robust TNF-α and IL-10 response to both *S. aureus* isolates (**Figure 2.1D**). We detected monocytes producing only TNF-α or IL-10, or both TNF-α and IL-10. More monocytes responded to the S8 *S. aureus* isolate and produced more IL-10 on a per cell basis compared to the S5 *S. aureus* isolate, whereas TNF-α production to each isolate was similar. We observed little to no contribution of T cells or B cells to either of these responses (**Supplementary Figure 2.2A,B**). In addition, human

peripheral blood neutrophils did not produce IL-10 or TNF-α to *S. aureus* when mixed with autologous PBMCs (**Supplementary Figure 2.2C**). Thus, nasal *S. aureus* isolates have differential capacities to independently induce pro- and anti-inflammatory cytokine production by human monocytes.

Nasal S. aureus isolates contain qualitatively different IL-10-inducing TLR2 ligands in their cell walls

We have previously shown that the IL-10 response to *S. aureus* is primarily initiated by TLR2 engagement [80]. These ligands could be membrane-bound, cell wall anchored, or secreted molecules. To determine where the IL-10-inducing ligand(s) are located in nasal *S. aureus* isolates, we fractionated a high IL-10-inducing *S. aureus* isolate (S8) into its cell wall and protoplasm and tested the IL-10-inducing capacity of these fractions. We observed that the IL-10 and TNF-α responses were almost exclusively induced by the staphylococcal cell wall (**Figure 2.2A,B**). Moreover, culture supernatants of *S. aureus*, which contain the secreted toxins and shedded components of the staphylococcal cell wall, minimally induced IL-10 and TNF-α production by PBMCs (< 10% of heat-killed *S. aureus*; **Figure 2.2C,D**). Together, these findings conclude that the pro- and anti-inflammatory TLR2 ligands are largely restricted to the staphylococcal cell wall. We therefore focused subsequent experiments on the pro- and anti-inflammatory properties of heat-killed *S. aureus*.

Next, we wanted to determine if high IL-10-inducing *S. aureus* isolates contain quantitatively more IL-10-inducing ligands or qualitatively different ligands than the low IL-10-inducing counterparts. To test this, we performed an extensive titration of *S. aureus* isolates representative of a high and a low IL-10-inducing capacity (shown here for S8 and S5 isolates) to

determine the multiplicity of infection (MOI) that induced maximal pro- and anti-inflammatory responses. IL-10 was already detectable at an MOI of 1 and peaked at an MOI of 6 for both isolates (**Figure 2.2E**). At all MOIs tested, the IL-10 production to *S. aureus* S8 was at least 2-fold greater than the response to S5. Importantly, TNF-α production to the isolates did not differ (**Figure 2.2F**). These results suggest that the anti-inflammatory response is due to qualitatively different ligand(s) than those responsible for the pro-inflammatory response and, therefore, the anti-inflammatory response to *S. aureus* can be uncoupled from the pro-inflammatory response.

Pro- and anti-inflammatory responses to S. aureus are uncoupled at the signaling level

Given that *S. aureus* may contain multiple IL-10-inducing ligands that act on TLR2, we next asked if these ligands were activating the same pathway(s). The PI3K-Akt pathway has previously been shown to be essential for the anti-inflammatory response to TLR2 [160] and TLR4 [376] ligands. Thus, we examined activation of this pathway in response to several nasal *S. aureus* isolates. As shown in **Figure 2.3A**, for two representative isolates, we found that phosphorylation of Akt at S473 correlated with the IL-10-inducing capacity of nasal *S. aureus* isolates. Similar results were observed using intracellular staining of phospho-Akt^{S473} in monocytes **(Figure 2.3B)**. Interestingly, we also observed Akt phosphorylation in a subset of B cells (~ 20%) but this did not differ between high and low IL-10-inducing isolates **(Supplementary Figure 2.3)**. Inhibition of signaling through this pathway using the pan-PI3K inhibitor wortmannin significantly reduced IL-10 production to both *S. aureus* S5 and S8, but did not significantly affect the TNF-α response (**Figure 2.3C**). Furthermore, using PI3K p110 isoform-specific inhibitors, we observed that p110δ was the dominant isoform mediating the IL-10 response, with a minor contribution from p110β (**Figure 2.3D**).

To further corroborate the selective participation of the PI3K-Akt pathway in the anti-inflammatory response, we examined signaling steps further downstream in this cascade. We found that the mTOR inhibitor rapamycin significantly decreased the IL-10 response to *S. aureus* S5 and S8 (**Figure 2.3E**). Interestingly, we observed a small but significant increase in TNF-α, which is most likely a reflection of the antagonistic properties IL-10 has on TNF-α production [377]. Together, these results show that the PI3K-Akt-mTOR pathway is differentially activated by nasal *S. aureus* isolates, regulating the anti-inflammatory but not the pro-inflammatory response to this microbe.

The importance of MAPK signaling in the cytokine response to MAMPs is well documented [378]. Moreover, p38 and ERK have both been documented to regulate IL-10 production to various stimuli [375]. Consistent with this, we observed that both p38 and ERK were activated in response to the nasal *S. aureus* isolates (**Figure 2.4A**). To elucidate the specific roles of these members of the MAPK family in the response to *S. aureus*, we used the selective inhibitors PD-98059 for ERK1/2, and SB-203580 and BIRB-0796 for p38. PD-98059 slightly but significantly decreased the IL-10 response to *S. aureus* (**Figure 2.4B**) and had no effect on the TNF-α response (**Figure 2.4C**). In contrast, the p38 inhibitors SB-203580 and BIRB-0796 significantly decreased the TNF-α response without affecting the IL-10 response (**Figure 2.4D, E**). These results further document the uncoupling of pro- and anti-inflammatory responses to *S. aureus* at the level of MAPK signaling by showing the selective dependence of the pro-inflammatory response on the p38 MAPK signaling pathway.

Pro- and anti-inflammatory responses to S. aureus have differential requirements for microbial internalization and phagosome maturation

It has been recently shown that the anti-inflammatory IL-10 response to Gram-negative *E. coli* LPS requires PI3K-dependent internalization of TLR4 [376]. Thus, we examined whether a similar mechanism could be involved in the anti-inflammatory response to *S. aureus* through TLR2. First, we tested if inhibition of PI3K could prevent internalization of *S. aureus*. As shown in **Figure 2.5A**, this was not the case as *S. aureus* was still internalized in the presence of wortmanin suggesting that PI3K signaling is not required for *S. aureus* phagocytosis. Inhibition of phagocytosis with either the actin inhibitor cytochalasin D or the dynamin inhibitor dynasore did not prevent *S. aureus*-induced phospho-Akt (**Figure 2.5B**), which is required for the anti-inflammatory response to *S. aureus*. Unfortunately, due to cytotoxicity of these inhibitors, we were unable to examine their effects on cytokine production (data not shown). Altogether, these findings implied that the IL-10 response to *S. aureus* occurred at the cell surface and did not require *S. aureus* internalization. Consistent with this claim, we saw that the IL-10 response to *S. aureus* was not affected by inhibition of endophagosome acidification whereas the pro-inflammatory response was significantly inhibited by chloroquine and NH_4Cl (**Figure 2.5C, D**). These results demonstrate a differential requirement for microbe internalization on pro- and anti-inflammatory responses, further documenting the spontaneous uncoupling of these properties in *S. aureus*.

Uncoupling of pro- and anti-inflammatory properties of S. aureus translates into differential modulation of adaptive immunity to staphylococcal superantigens

We have previously reported that the IL-10 response to *S. aureus* suppresses superantigen-induced T cell activation and may be protective against staphylococcal TSS [80]. We therefore predicted that those isolates able to induce a high IL-10 response are better at suppressing this T cell activation. We found that this was the case: even though all *S. aureus* isolates induced enough IL-

10 to decrease the SAg-induced T cell activation as measured by IL-2 production (**Figure 2.6A**), the high IL-10-inducing *S. aureus* isolates (e.g., S8) were significantly better suppressors than the low IL-10-inducing isolates (e.g. S3).

Bacteriostatic antibiotics, such as clindamycin, have been recommended for the management of staphylococcal TSS [171]. The effectiveness of clindamycin to treat staphylococcal TSS is attributed to blocking the translation of staphylococcal SAg [173]. We postulated that, in addition to this mechanism, clindamycin may also maintain the integrity of the staphylococcal cell wall to induce IL-10 production. To test this hypothesis, we cultured *S. aureus* in TSB containing clindamycin for 6 h and compared its immunostimulatory capacity to heat-killed *S. aureus*. We observed that clindamycin-treated *S. aureus* induced significantly more IL-10 than heat-killed *S. aureus*, but did not significantly change TNF-α production (**Figure 2.6B**). This result reveals a potential biological implication of the uncoupling of pro-inflammatory and anti-inflammatory properties and suggests that clindamycin may be effective for treating staphylococcal TSS by promoting an anti-inflammatory to *S. aureus*, in addition to inhibiting SAg production.

To further characterize the modulatory effect of the community isolates of *S. aureus* on the adaptive immune response to staphylococcal superantigens, we performed a multiplex analysis of the cytokine response to high and low IL-10-inducing nasal *S. aureus* isolates (**Figure 2.6C**). We observed no difference in the production of pro-inflammatory IL-1β, IL-6 or TNF-α cytokines among these isolates (**Figure 2.6D**), suggesting that these cytokines are similarly regulated in response to *S. aureus*. However, the Th1 cytokines IL-12p70 and IFN-γ were induced to a greater extent by *S. aureus* isolates that had less anti-inflammatory properties (**Figure 2.6E**). Altogether, these results imply that the low IL-10-inducing capacity of an isolate of *S. aureus* imprints adaptive

immunity to a Th1 profile and thus influence the development of protective pro-inflammatory responses in the context of staphylococcal diseases.

2.5. Discussion

TLR signaling leading to the production of IL-10 and other anti-inflammatory mediators in response to MAMPs has been previously reported [79, 80, 160, 376]. However, its qualitative and quantitative relationship to the pro-inflammatory cytokine response (i.e. IL-1β, IL-6, TNF-α, etc.) triggered by PRR signaling has not yet been studied. Specifically, it is not known whether both types of responses occur in parallel and to a similar extent or alternatively, pro- and anti-inflammatory responses are the result of qualitatively different MAMP:PRR recognition events that can be uncoupled. Using multiple nasal isolates of *S. aureus* from chronic human carriers of this microbe, we report here that, for TLR2, these responses are mechanistically distinct and are naturally uncoupled.

Such an uncoupling was seen in all the isolates of a representative sample of community *S. aureus* independently of the magnitude of IL-10 response they induced. Our findings support the idea that the pro- and anti-inflammatory properties of a given *S. aureus* isolate are the result of qualitatively different responses to recognition of MAMPs on the staphylococcal cell wall. In support of this conclusion, we found that IL-10 production to *S. aureus* was dependent on PI3K-Akt-mTOR and ERK signaling whereas the TNF-α response was dependent on p38. Furthermore, internalization and phagosome maturation were required only for the pro-inflammatory response but not the anti-inflammatory response. Lastly, natural differences in the capacity of nasal *S. aureus* isolates to induce an IL-10 response in the host, independently of the pro-inflammatory response they can induce, differentially imprints the adaptive immune response to *S. aureus*.

Whether the uncoupling of pro- and anti-inflammatory properties of a given *S. aureus* isolate is the result of multiple ligands on the staphylococcal cell wall recognized by one PRR or by different PRRs is still unclear. Our previous data suggested that the pro-inflammatory response occur from multiple PRRs including TLR2, TLR9 and, NOD1/2, whereas the anti-inflammatory response to *S. aureus* is predominantly secondary to TLR2 signaling [80]. NOD1/2 was ruled out as a player in the anti-inflammatory IL-10 response because ultra-pure staphylococcal PGN (PGN-Sandi), which lacks TLR2 stimulating capacity and thus can only activate NOD1/2, did not induce IL-10 production, despite giving the same pro-inflammatory profile as crude staphylococcal PGN [160]. Both NODs and TLR9 can also be excluded by the fact that IL-10 production in response to *S. aureus* is independent of phagocytosis. Thus, our data support that the uncoupling occurs, in part, by several ligands acting on TLR2. Indeed, previous work from our lab has shown that the TLR2 accessory molecules CD14 and CD36 are only required for the pro-inflammatory response to staphylococcal PGN preparations [160]. Moreover, CD36 is required for *S. aureus* internalization [379] and would explain the dependency of this molecule only for the pro-inflammatory response [160]. Together, these findings point to structural differences in pro- and anti-inflammatory TLR2 ligands. The molecular nature of these ligands is unknown at the moment although it may be diverse and present in other microbial species (e.g., polysaccharide A in *Bacillus fragilis* [156]).

TLR2-based uncoupling of pro- and anti-inflammatory responses may stem from differential TLR2 oligomer formation. For example, TLR2 dimerization with TLR6 has been linked to anti-inflammatory responses, whereas TLR2/1 complexes promote pro-inflammatory responses [380-382]. The molecular definition of the resulting signalosomes is uncertain [165, 383]. It is plausible to suggest that, in response to *S. aureus*, the availability of TLR2 on the cell

surface is a limiting factor of the response. If so, the relative abundance of the pro- vs. anti-inflammatory ligands would dictate the outcome of the response. We cannot rule out that, under some circumstances (e.g., *S. aureus* isolates lacking TLR2 stimulatory capacity [384] an IL-10 response may occur through an alternative, less efficient mechanism.

Phagocytosis is an important defense mechanism to mount an effective inflammatory response against *S. aureus* [146, 371]. We have corroborated this fact using nasal *S. aureus* isolates. Interestingly though, the anti-inflammatory response did not require phagocytosis by monocytes/macrophages. Since we used heat-killed bacteria as well as cell wall preparations, our findings indicate that the anti-inflammatory ligands are present in the staphylococcal cell wall in a recognizable conformation unlike their pro-inflammatory counterparts. Such an arrangement may ensure an anti-inflammatory response that down-plays the Th1 response to *S. aureus* and promotes a state of disease tolerance to this microbe [6].

The mechanism of TLR2-dependent anti-inflammatory response is different to that reported recently for the TLR4-dependent anti-inflammatory response. The anti-inflammatory response to TLR4 signaling by LPS requires activation of PI3K p110δ for endosomal translocation and switch from the TIRAP-MyD88 pathway to TRAM-TRIF pathway for anti-inflammatory signaling to occur [376]. In contrast, for TLR2 signaling, PI3K p110δ, although required for the IL-10 response, is not involved in endosomal trafficking of TLR2. We found that inhibition of PI3K did not affect *S. aureus* internalization by monocytes and the IL-10 response was independent of phagocytosis and phagosome maturation. Furthermore, although the switch to anti-inflammatory TLR4 signaling resulted in a diminished pro-inflammatory response [376], we did not observe such an effect with nasal *S. aureus* isolates. Altogether, our findings are consistent

with those showing that the PI3K-Akt-mTOR pathway directly leads to IL-10 production [79, 385, 386].

We have previously shown that the IL-10 response to *S. aureus* is predominantly mounted by monocytes/macrophages. This is in contrast to the IL-10 response to other MAMPs [156, 159, 387, 388]. Monocytes/macrophages are early responders to *S. aureus* and their phenotype would be highly influential in establishing the microenvironment that sets up subsequent adaptive responses. Previous work from our laboratory has shown that the IL-10-producing monocytes/macrophages are classically activated and not alternatively activated macrophages, because of the robust inflammatory response simultaneously observed and the inability to show IL-4/IL-13 during the generation of this response [160]. Moreover, a high proportion of these monocytes acquire a dual phenotype (i.e. IL-10^+ TNF-α^+), a phenotype not reported for M2 macrophages (reviewed in [389]). The characterization of the different macrophage subsets responding to nasal *S. aureus* isolates based on their cytokine production profiles is a matter for future studies. It is likely that these cells show a phenotype similar to inflammatory monocytes seen in the gut during *Toxoplasma gondii* infection [390] in which simultaneous expression of pro-inflammatory and anti-inflammatory mediators is observed. Proper balance of this expression may determine pathogen elimination while limiting tissue damage and deleterious effects on commensals.

The findings reported here have clinical implications. The balance between pro- and anti-inflammatory responses to a given *S. aureus* isolate may determine the outcome of the encounter, i.e. commensalism vs. disease. *S. aureus* isolates with a high capacity to induce IL-10 would be better at colonizing the upper respiratory tract as the heighten IL-10 levels may provide a tolerogenic environment and be less likely to cause staphylococcal TSS. Alternatively, a

predominance of inflammatory cytokine production may exacerbate mucosal injury [391]. Lastly, high IL-10 induction by a given isolate may be detrimental during staphylococcal bacteremia by dampening adaptive immunity [8].

In conclusion, our findings have revealed that the pro- and anti-inflammatory properties of nasal *S. aureus* isolates are independent of each other, i.e., they can be uncoupled. Such an uncoupling obeys to different mechanistic requirements. The ability to naturally uncouple these two types of responses suggests that *S. aureus* contains two sets of MAMPs: ones that preferentially induce a pro-inflammatory response and minimal anti-inflammatory mediators; and a second set that induces a robust anti-inflammatory response, with little pro-inflammatory cytokines. Balance between the ensuing responses may determine colonization and disease tolerance vs. pathogenicity and disease by *S. aureus*.

2.6. Acknowledgements

This work was supported by the Canadian Institutes for Health Research (to J.M.). A.P. is a Fonds de la Recherche en Santé du Québec Research Scholar. J.M. holds a Tier I Canada Research Chair in Human Immunology. We thank Dr. Mark Hancock and the McGill Multiplexing Facility (McGill University) for assistance with multiplexing and the Canadian Foundation for Innovation for infrastructure support, and Drs. David E. Heinrichs and Martin McGavin (University of Western Ontario, London, ON, Canada) and members of the Madrenas Laboratory for helpful comments and criticisms.

2.7. Table

Table 2.1. Multi-Locus Sequence Typing of some of the S. aureus isolates used in this study

	***S. aureus* isolate number**					
Gene	**S1**	**S4**	**S5**	**S8**	**S12**	**S33**
arcC	**13**	**3**	**1**	**2**	**1**	**7**
aroE	**13**	**3**	**4**	**2**	**1**	**6**
glpF	**1**	**1**	**1**	**2**	**1**	**1**
gmk	**1**	**1**	**4**	**2**	**1**	**5**
pta	**12**	**NF***	**12**	**6**	**1**	**8**
tpi	**11**	**4**	**1**	**3**	**1**	**8**
yqiL	**13**	**3**	**10**	**2**	**1**	**6**
MLST	**15**	**N/A***	**5**	**30**	**1**	**22**

***NF = Not found; N/A = Not applicable**

2.8. Figures

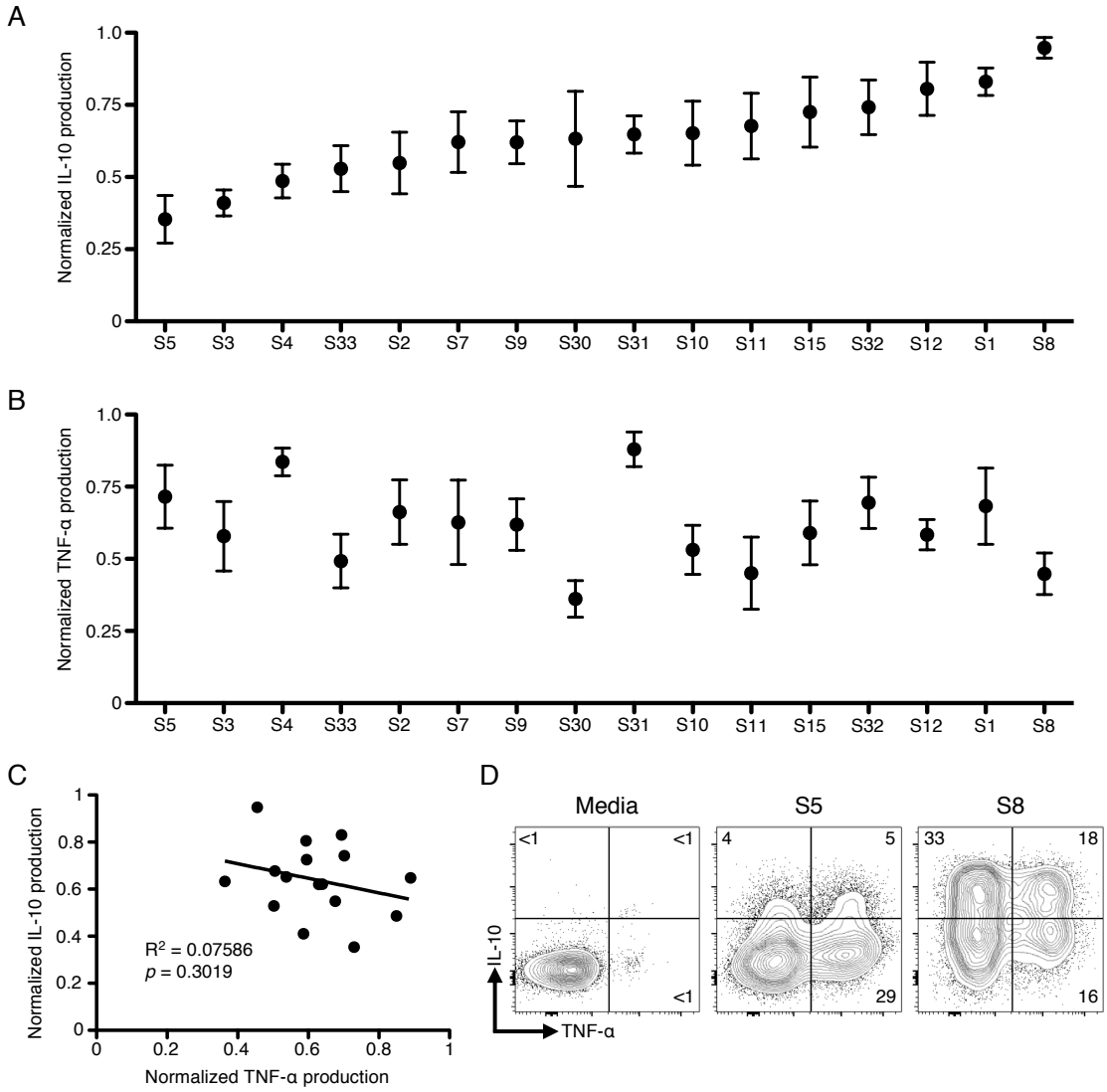


Figure 2.1. Uncoupling of pro- and anti-inflammatory PBMC responses to nasal *S. aureus* isolates. (A,B) Human PBMCs were stimulated with 16 nasal *S. aureus* isolates for 18 h and accumulation of IL-10 (A) and TNF-α (B) in the supernatants was measured by ELISA. Normalized data is plotted as mean ± SEM of triplicates of 4-5 independent experiments from 5 different donors. (C) Scattered plot of IL-10 vs. TNF-α production by human PBMCs in response to nasal *S. aureus* isolates. (D) Intracellular cytokine staining of IL-10 and TNF-α in human monocytes stimulated with two representative *S. aureus* isolates inducing low (S5) or high (S8) IL-10 responses (MOI = 5) for 18 h. Stained PBMCs were gated on single, live, $CD14^+$ cells. Plots are representative of three independent experiments from three different donors. Intracellular cytokine staining of T cells, B cells, and neutrophils are shown in Supplementary Figure 2.1.

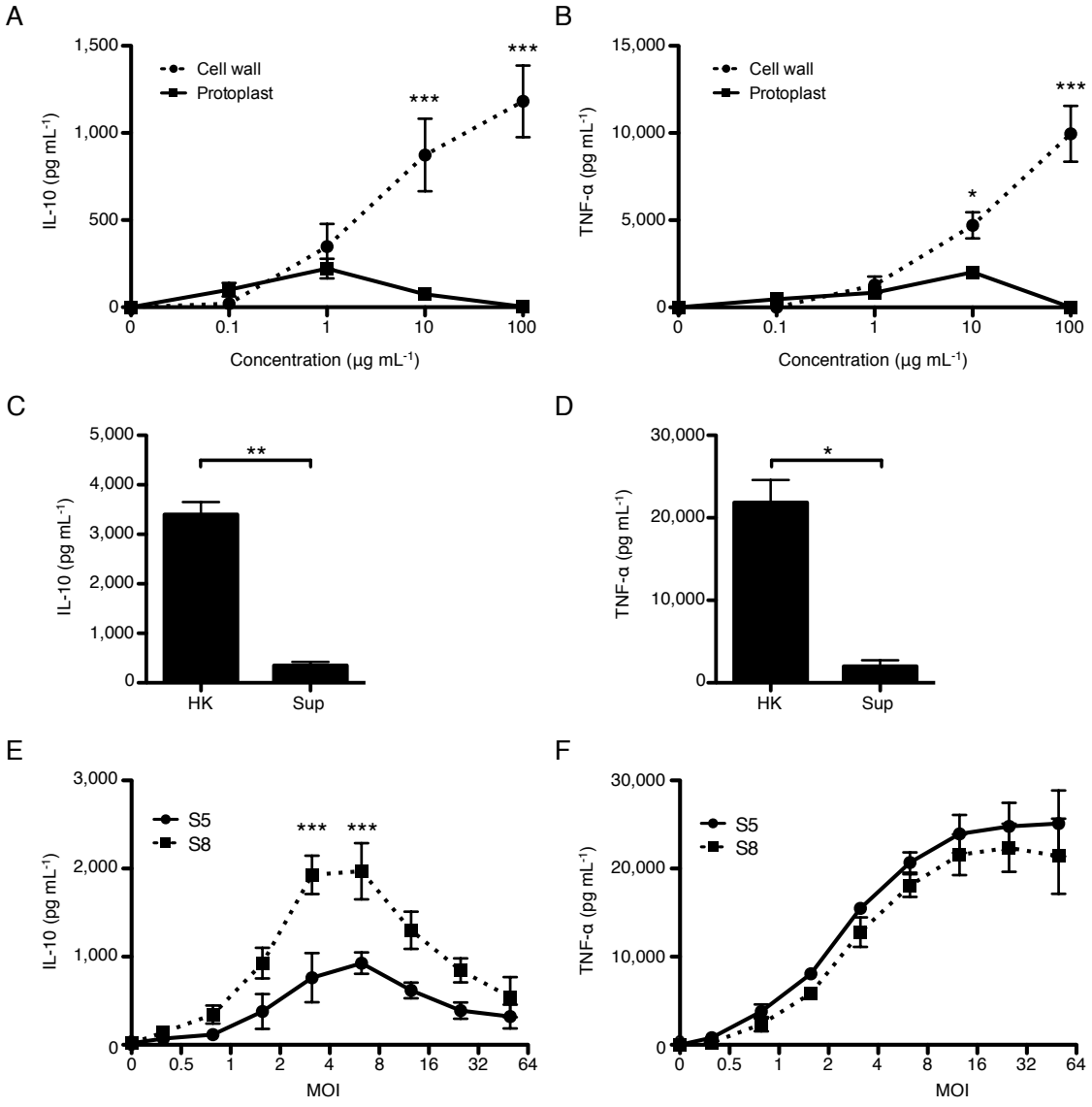


Figure 2.2. Nasal *S. aureus* isolates have qualitatively different IL-10-inducing ligands embedded in their cell walls. (A,B) Human PBMCs were stimulated with protoplasm (PP) or cell wall (CW) fractions of the S8 *S. aureus* isolate for 18 h. (C,D) PBMC response to heat-killed (MOI = 5) or supernatants (1% of overnight culture). (E,F) Human PBMCs were stimulated with increasing amounts of two representative *S. aureus* isolates inducing low (S5, circles and solid line) or high (S8, squares and dashed line) IL-10 responses for 18 h. Quantification of IL-10 (A,C,E) and TNF-α (B,D,F) in the supernatants was performed by ELISA. Data are plotted as mean ± SEM of 3-5 different donors performed in triplicates. Statistical analysis was performed using ANOVA with post hoc Boniferroni test (A,B,E,F) or Student's t-test (C,D).

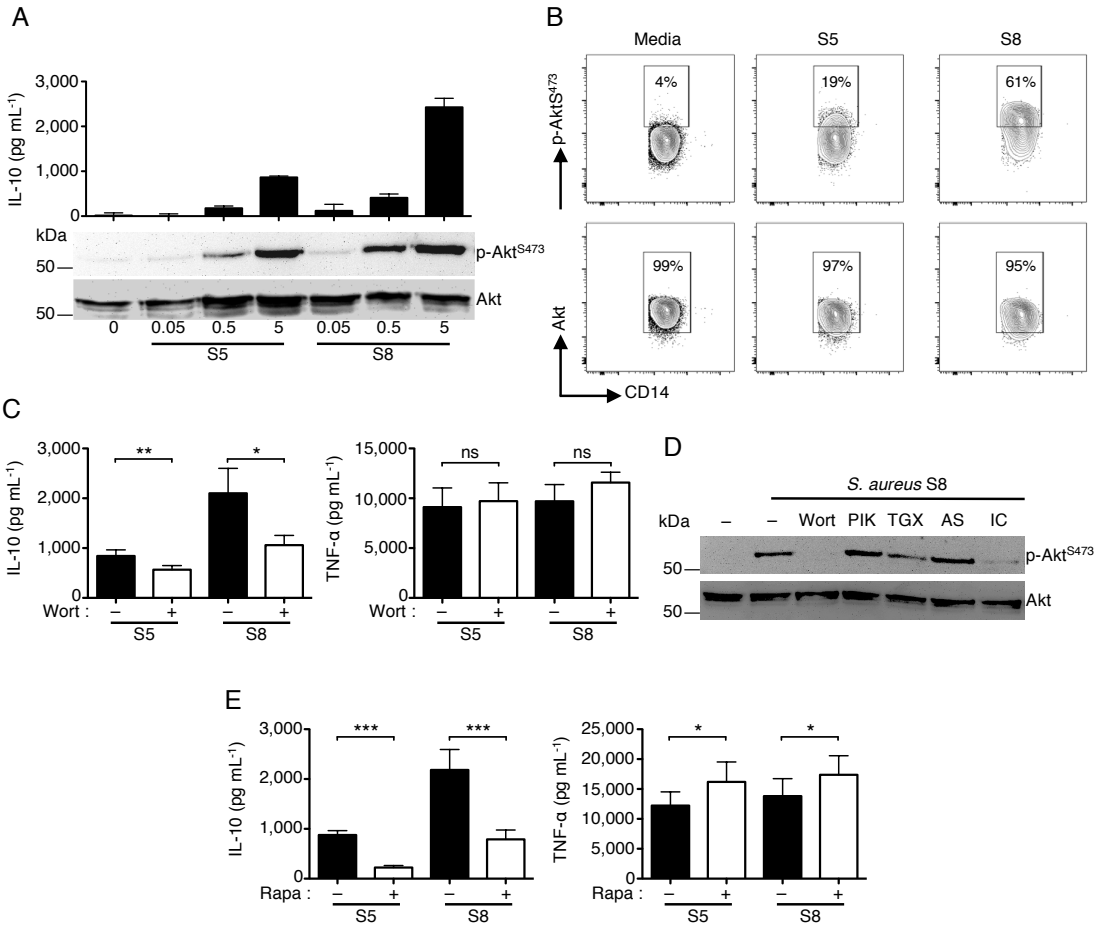


Figure 2.3. The PI3K/Akt/mTOR pathway mediates the IL-10 response to nasal *S. aureus* isolates. (A) PBMCs were stimulated with two *S. aureus* isolates at the indicated MOIs for 30 min for western blot experiments or for 18 h in experiments looking at IL-10 accumulation by ELISA. (B) Flow cytometric analysis of Akt phosphorylation (S473) in $CD14^+$ gated human PBMCs stimulated with two representative *S. aureus* isolates inducing low (S5) or high (S8) IL-10 responses. (C) PBMCs were pre-treated with wortmannin (1 μM) then stimulated with *S. aureus* (MOI = 5) for 18 h. Quantification of IL-10 and TNF-α accumulation in the supernatants was done by ELISA. (D) Western blot of PBMCs pre-treated with the pan PI3K-p110 inhibitor wortmannin (Wort) or p110 isoform inhibitors PIK-75 (PIK: p110α inhibitor; 100 nM), TGX-221 (TGX: p110β inhibitor; 500 nM), AS-604580 (AS: p110γ inhibitor; 10 μM) or IC-87114 (IC: p110δ inhibitor; 5 μM) for 1 h then stimulated with *S. aureus* S8 (MOI: 5) for 30 min. (E) PBMCs were pre-treated with rapamycin (10 nM) then stimulated with *S. aureus* (MOI: 5) for 18 h. Quantification of IL-10 and TNF-α accumulation in the supernatants was determined by ELISA. Data in A, B and D are representative of three independent experiments from three different donors. Data in C and E are plotted as mean ± S.E.M. of eight individual donors performed in triplicate. Statistical analysis was performed using Student's t-test. *P < 0.05, ** P < 0.01, *** P < 0.001.

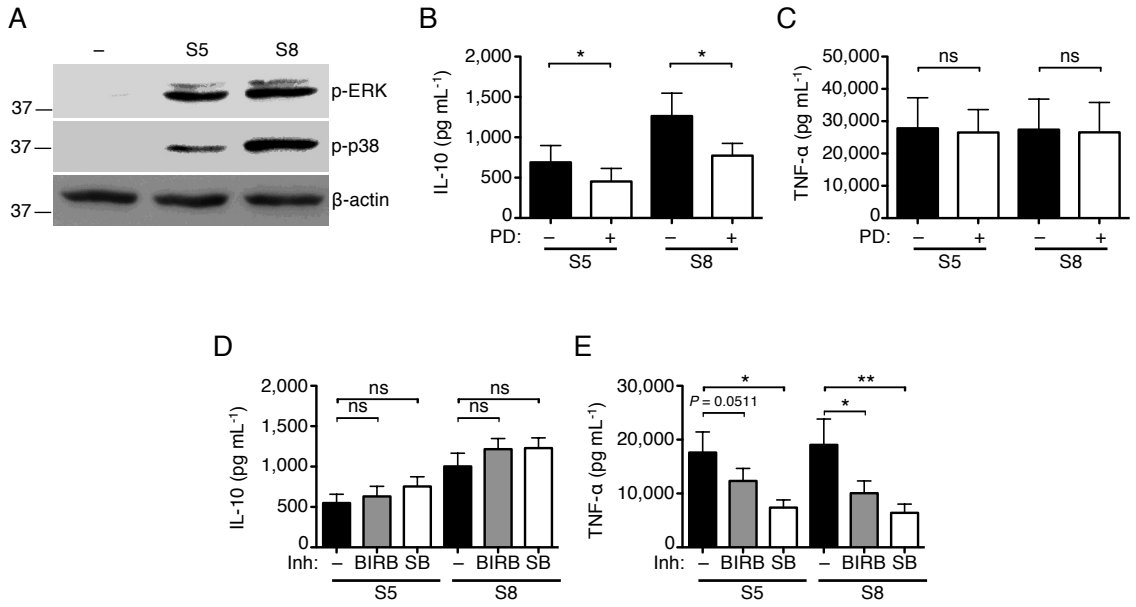


Figure 2.4. The MAPK p38 mediates the pro-inflammatory response to nasal *S. aureus* isolates. (A) Western blot of phospho-ERK and -p38 from human PBMCs stimulated with two representative *S. aureus* isolates inducing low (S5) or high (S8) IL-10 responses (MOI = 5) for 30 min. Blots are representative of three independent experiments from three different donors. (B-E) PBMCs were pre-treated with the ERK inhibitor PD-98059 (B,C) or the p38 inhibitors SB-203580 or BIRB-0796 (D,E) for 1 h then stimulated with two representative *S. aureus* isolates inducing low (S5) or high (S8) IL-10 responses (MOI = 5) for 18 h. Quantification of accumulation of IL-10 (B,D), TNF-α (C,E) in the supernatants was done by ELISA. Bar graphs represent mean ± S.E.M. of data from five different donors performed in triplicates. Statistical analysis was performed using Student's t-test. *P < 0.05.

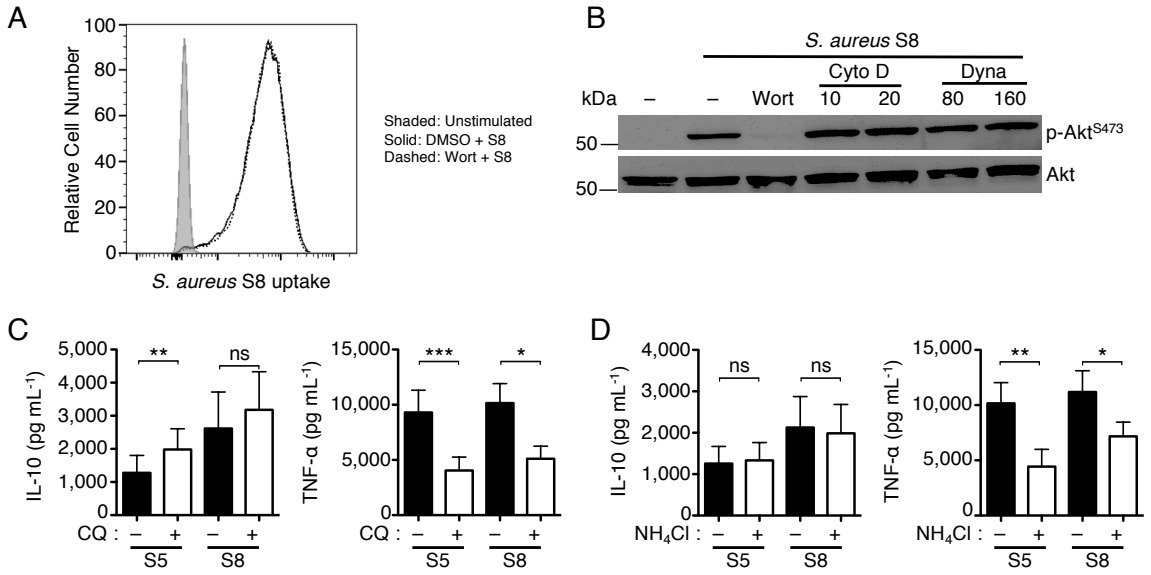


Figure 2.5. Pro- and anti-inflammatory responses to *S. aureus* have differential requirements for microbial internalization and processing. (A) Human PBMCs were pre-treated with DMSO or wortmannin (1 μM), and cultured with TAMRA-labelled *S. aureus* (MOI = 5) for 30 min. Uptake of *S. aureus* by $CD14^+$ monocytes was determined by flow cytometry. Plot is representative of three independent experiments. (B) phospho-Akt western blot of human PBMCs stimulated with *S. aureus* S8 for 30 min with or without pre-treatment with wortmannin or the internalization inhibitors cytochalasin D or dynasore. Blots are representative of three independent experiments from three different donors. (C,D) PBMCs were pre-treated for 1 h with chloroquine (CQ; C) or NH_4Cl (D) then stimulated with two representative *S. aureus* isolates inducing low (S5) or high (S8) IL-10 responses (MOI = 5) for 18 h. Accumulation of IL-10 or TNF-α was quantified by ELISA. Bar graphs represent mean ± S.E.M. of five donors performed in triplicates. Statistical analysis was performed using Student's t-test. *$P < 0.05$, ** $P < 0.01$.

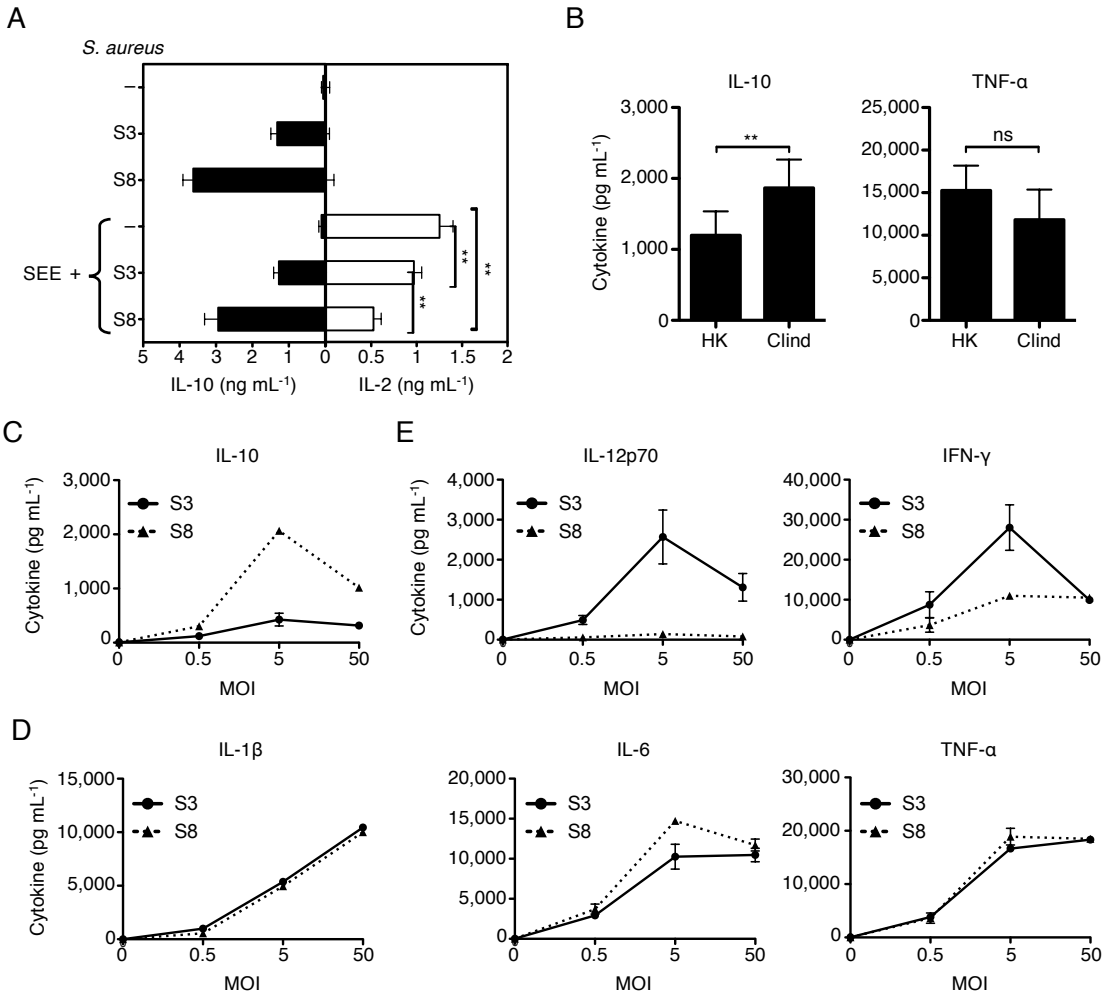
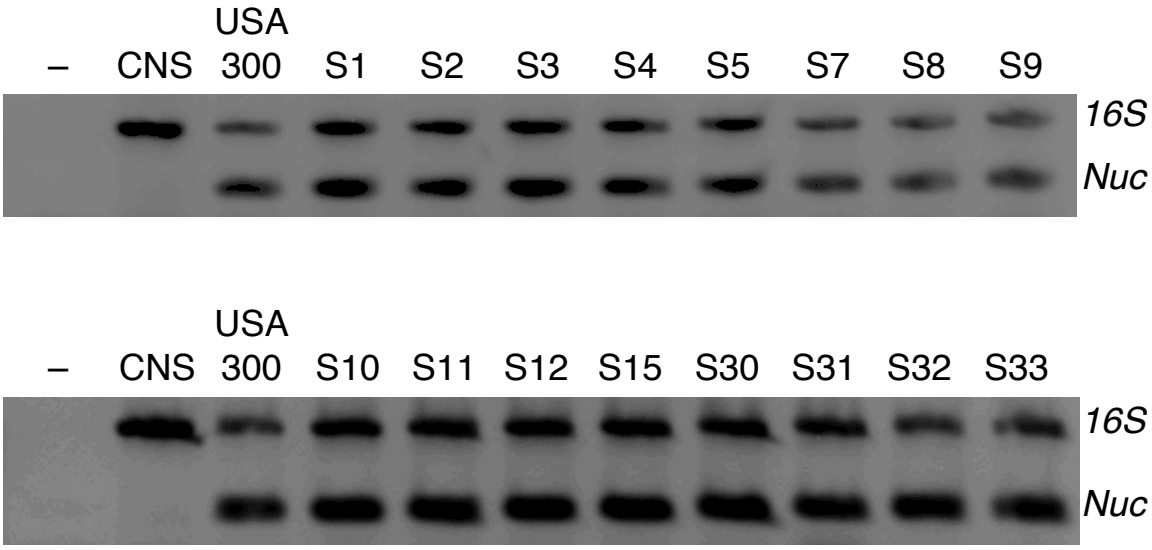
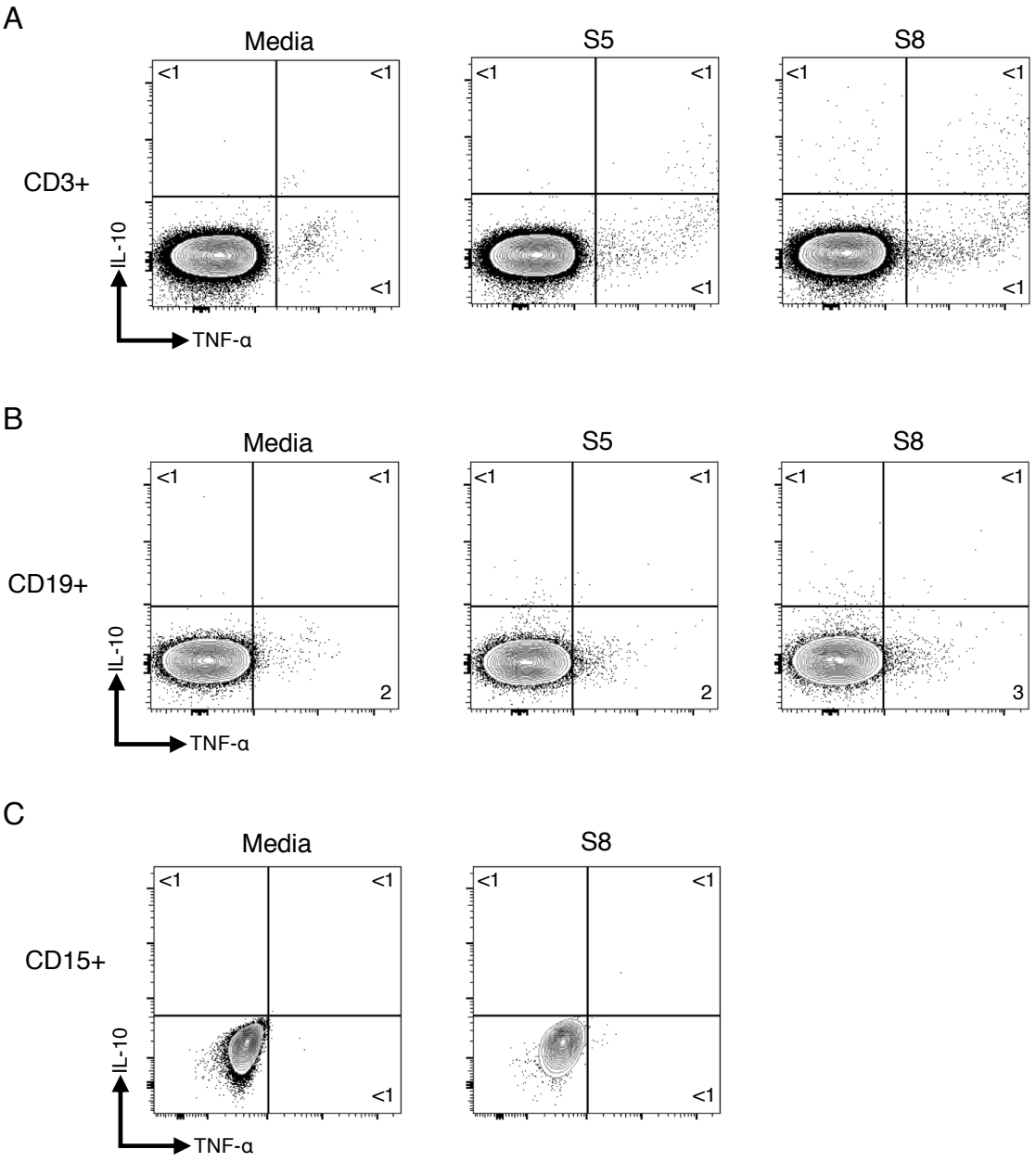


Figure 2.6. The IL-10-inducing capacity of nasal *S. aureus* isolates correlates with the modulation of adaptive immunity. (A) PBMCs were stimulated with SEE (10 ng mL^{-1}) for 18 h in the presence or absence of representative *S. aureus* isolate inducing low (S3) or high (S8) IL-10 responses (MOI = 5). Quantification of IL-2 and IL-10 in the supernatants was done by ELISA. Data are plotted as mean ± S.D. and representative of three independent experiments from three different donors. (B) IL-10 and TNF-α response by PBMCs to heat-killed (HK) or clindamycin-treated (Clind) *S. aureus* S8 (MOI = 5). Data are plotted as mean ± S.E.M. of 5 donors performed in triplicates. Statistical analysis was performed using Student's t-test. (C-E) Profile of cytokine response of PBMCs to *S. aureus*. PBMCs were stimulated for 18 h wih *S. aureus* S3 or S8 and IL-10 (C), the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (D), and the Th1 cytokines IL-12p70, and IFN-γ (E), were measured. Data are plotted as mean ± S.D. and representative of two independent experiments from two different donors. **P < 0.01

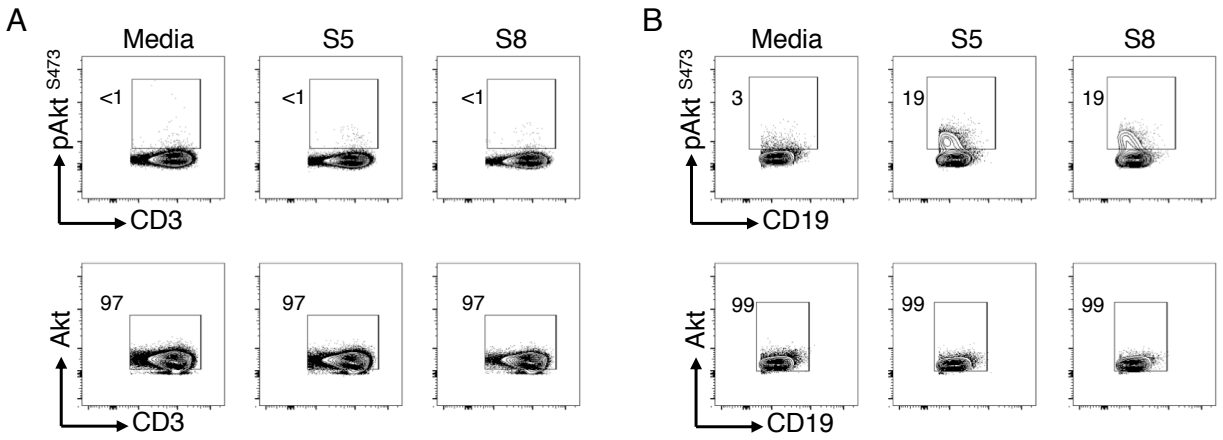
2. 9 Supplemental Figures



Supplementary Figure 2.1. PCR confirmation of nasal *S. aureus* isolates. Primers were designed to amplify the staphylococcal *nuc* gene specific for *S. aureus* (bottom band) and the *16S* ribosomal RNA common to all bacteria (top band). PCR products were run in a 1% agarose gel. Coagulase-negative *Staphylococcus* (CNS) and *S. aureus* USA300 were used as controls.



Supplementary Figure 2.2. Intracellular cytokine staining of human peripheral blood immune cells stimulated with nasal *S. aureus* isolates. (A,B) PBMCs were stimulated as in **Figure 2.1C** and events were gated on single, live (A) CD3+ or (B) CD19+ cells. (C) Human neutrophils were mixed with autologous PBMCs and stimulated as in **Figure 2.1C**. Events are gated on single, live $CD15^{+}$ cells. (A-C) Plots are representative of three independent experiments from three different donors.



Supplementary Figure 2.3. Intracellular phospho-Akt staining of human PBMCs stimulated with nasal *S. aureus* isolates. PBMCs were stimulated as in **Figure 2.2B** and events were gated on single (A) CD3+ or (B) CD19+ cells. Plots are representative of three independent experiments from three different donors.

Preface to Chapter 3

In Chapter 2, we demonstrated that *S. aureus* isolates differed in their ability to induce the anti-inflammatory cytokine IL-10. In Chapter 3, we switched our focus to a second key regulator of the inflammatory response, AHR. Specifically, we explored how the negative regulators of AHR were changing during monocyte/macrophage activation by PAMPs. Given that murine macrophages lacking AHR produce excessive amounts of pro-inflammatory cytokines in response to PAMPs [343, 392], we reasoned that *Cyp1* and *Ahrr*, the two inducible regulators of AHR, would be inhibited following PRR activation. Such an effect would enhance AHR activation and limit the inflammatory response by monocytes and macrophages.

Chapter 3 : Suppression of CYP1 Members of the AHR Response by Pathogen-Associated Molecular Patterns

Adam G. Peres[1], Robert Zamboni[2], Irah L. King[1], and Joaquín Madrenas[1,3]

the [1]Microbiome and Disease Tolerance Centre and Department of Microbiology and Immunology, and [2]Department of Chemistry, McGill University, Montréal, QC, Canada; and [3]Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance CA

This chapter contains the following published manuscript:

Peres AG, Zamboni R, King IL, and Madrenas J. 2017. Suppression of CYP1 members of the AHR response by pathogen-associated molecular patterns. *J Leukoc Biol* 102:1471-1480. *doi: 10.1189/jlb.4A0617-218RR*

3.1 Abstract

AHR is a ligand-activated transcription factor that triggers a broad response, which includes the regulation of pro-inflammatory cytokine production by monocytes and macrophages. AHR is negatively regulated by a set of genes it transcriptionally activates, including the *Ahrr* and the *Cyp1* family, which are critical for preventing exacerbated AHR activity. An imbalance in these regulatory mechanisms has been shown to cause severe defects in lymphoid cells. We therefore wanted to assess how AHR activation is regulated in monocytes and macrophages in the context of innate immune responses induced by pathogen-associated molecular patterns (PAMPs). We found that concomitant stimulation of primary human monocytes with PAMPs and the AHR agonist FICZ led to a selective dose-dependent inhibition of *Cyp1* family members induction. Two other AHR-dependent genes, *Ahrr* and *Nqo1*, were not affected under these conditions, suggesting a split in AHR regulation by PAMPs. This down-regulation of *Cyp1* family members did not require *de novo* protein production nor signaling through p38, ERK or PI3K-Akt-mTOR pathways. Furthermore, such a split regulation of the AHR response was more apparent in GM-CSF-derived macrophages, a finding corroborated at the functional level by decreased CYP1 activity and decreased pro-inflammatory cytokine production in response to FICZ and LPS. Collectively, our findings identify a role for PRR signaling in regulating the AHR response through selective down-regulation of *Cyp1* expression in human monocytes and macrophages.

3.2 Introduction

AHR is a ligand-activated transcription factor initially identified as the receptor mediating the toxic effects of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) [241]. Its ligands are certain polycyclic aromatic compounds, including TCDD and 6-formylindolo(3,2-b)carbazole (FICZ), as well as tryptophan metabolites such as kynurenine (Kyn) and indoles [269]. In homeostasis, AHR is predominately found in the cytoplasm tethered to the cytoskeleton by an inactivation complex that includes HSP-90 [393, 394], the AHR-interacting protein (AIP) [297, 298], and p23 [296]. Following ligand binding, AHR dissociates from the cytoskeleton, sheds the majority of its inactivation complex, and translocates into the nucleus in a β–importin-dependent mechanism [303]. In the nucleus, AHR dimerizes with its DNA binding partner ARNT (also known as HIF-1β) [395, 396] and transcriptionally activates a set of genes collectively known as the AHR gene battery. The best characterized AHR gene battery members are the cytochrome P450 family 1 (*Cyp1a1*, *Cyp1a2*, and *Cyp1b1*), and the aryl hydrocarbon receptor repressor (*Ahrr*), but may also include other genes such as *Fmo3*, *Nqo1*, *Npxt1*, *Tiparp,* and *Ugt1a6* [307].

Critical for an AHR response is the ability to turn off the signal when it is no longer required. The problem of having enhanced or prolonged AHR activation is best exemplified by the toxicity of TCDD, a contaminant in Agent Orange that causes birth defects in children and other health problems due to its high resistance to metabolism by CYP1 [241]. AHR signaling can be turned off by three mechanisms: 1) proteasome degradation following nuclear export and ubiquitination [302, 397]; 2) disruption of AHR-ARNT dimers by AHRR [315]; and 3) metabolism of ligands by CYP1 [398] and other enzymes [282]. With regards to this last mechanism, it has been reported that inhibiting CYP1 in keratinocytes was sufficient to prolong AHR activation and preserve extracellular ligand concentrations [319]. It has also been observed

that *Cyp1*-deficient zebrafish treated with FICZ had developmental defects similar to those caused by TCDD [399]. Moreover, some molecules initially described as AHR ligands were later determined to be antagonists of CYP1s [319].

In addition to its role in clearing dioxins, AHR is also an effective regulator of the development and function of the immune system [321]. AHR activation can enhance T helper 17 cell (Th17) or regulatory T cell (Treg) differentiation and augment or protect against experimental autoimmune encephalitis, respectively [292, 293]. AHR can also regulate innate immunity by controlling pro-inflammatory responses in both macrophages [344] and dendritic cells [283]. In macrophages, AHR activation limits the production of pro-inflammatory cytokines (e.g. IL-6, IL-12p70, and TNF-α) by blocking the NF-κB-dependent transcription through a mechanism that involves STAT1 [344]. Interestingly, this regulation was not observed for the anti-inflammatory cytokine IL-10, despite NF-κB signaling at least partially regulating its transcription [375]. The significance of these findings is that AHR is involved in the mechanism of endotoxin tolerance and could protect against subsequent infections [232]. Moreover, it was recently shown that AHR can regulate anti-viral responses and type I interferon production by blocking TBK1 activity through its target gene TiPARP [345]. Collectively, these findings highlight the impact of AHR and its ligands on the development of an innate immune response.

What are beginning to gain attention are the implications of the negative feedback regulators of AHR signaling on the development and progression of an immune response. For example, it was recently reported that the AHRR is highly expressed in barrier immune cells and works in concert with AHR to decrease inflammation at these sites [400]. In contrast, during systemic inflammation, the AHRR augments the hyper-inflammatory state in endotoxin shock, likely through blocking AHR and causing enhanced NF-κB signaling. Similarly, transgenic

overexpression of AHRR in mice also protects against acute TCDD toxicity by decreasing the production of pro-inflammatory cytokines [401]. Dysregulated *Cyp1a1* expression can also have a profound effect on the immune system in mice. Mice constitutively overexpressing *Cyp1a1* had depleted AHR ligands levels, particularly at mucosal sites, and acquired a quasi-AHR-deficient phenotype, characterized by low numbers of intestinal Th17 and group 3 innate lymphoid cells, and were highly susceptible to *C. rodentium* infections [274]. This phenotype could be reversed by supplementing with dietary indoles, a source of AHR ligands, highlighting the importance of AHR ligand availability in regulating the gut immune responses.

Monocytes and macrophages play an important role in the recognition of microbes through sensing the presence of pathogen-associated molecular patterns (PAMPs, such as LPS) through pattern recognition receptors (PRRs, such as TLR4) and triggering an inflammatory response. Given the importance of AHR activation in regulating this inflammatory response, we sought to determine the profile of expression and the regulation of the AHR gene battery in monocytes and macrophages. We found that, in primary human monocytes, concomitant stimulation with an AHR ligand (e.g. FICZ or Kyn) and a TLR4 ligand (e.g. LPS) selectively suppressed the expression and function of CYP1 family members, paramount members of the AHR response, but not other genes of the AHR gene battery. Interestingly, this effect was most apparent in GM-CSF-differentiated macrophages. Such an effect was likely acquired during the differentiation of monocytes to macrophages under GM-CSF, rather than a direct effect of GM-CSF stimulation. Our results reveal a novel regulatory step of AHR function that may determine the rate of AHR ligand metabolism and influence AHR functions during innate immune responses.

3.3 Materials & Methods

Human Cells. Human peripheral blood mononuclear cells (PBMCs) were isolated from venous blood of healthy donors using Ficoll-Paque Density Centrifugation (GE Healthcare). All individuals gave their informed consent in compliance with the McGill University Ethics Review Board. Human primary monocytes were enriched (>90% purity) from PBMCs by negative-selection using the EasySep Human Monocyte Isolation Kit (StemCell). To obtain monocyte-derived macrophages (MDMs), monocytes were differentiated in either 20 ng/mL of M-CSF (M-MDM) or GM-CSF (GM-MDM). The phenotype of these cells has been characterized elsewhere [402]. All cells were cultured in RPMI-1640 (HyClone) supplemented with 10% heat-inactivated FBS, sodium pyruvate, non-essential amino acids, L-glutamine, HEPES buffer (pH 8), and penicillin-streptamycin.

Reagents. Cycloheximide, *Escherichia coli* LPS, wortmannin, rapamycin, PD-184352, polyinosinc-polycytidylic acid (Poly I:C), and staphylococcal peptidoglycan (PGN) were purchased from Sigma-Aldrich. BIRB-0796, SB-203580, Bay 11-7082, and kynurenine were purchased from Cayman Chemicals. Resiquimoid (R848), zymosan, depleted zymosan, staphylococcal PGN-SAndi, and CpG ODN2216 were purchased from InvivoGen. *S. aureus* strain S8 was isolated from the nostrils of a chronic carrier individual, and cultured and prepared as previously described [1]. FICZ was synthesized in-house as previously described [403].

Cytokine Production. Human GM-MDMs (100,000 cells per well) were seeded in 96-well round-bottom, tissue culture-treated plates and stimulants were added at twice the desired concentration at a volume of 1:1. Cell-free supernatants were collected and cytokine production was measured by ELISA (eBioscience). When inhibitors were used, cells were pre-treated for 1 hour prior to stimulation. Unless indicated otherwise, DMSO (0.1%) was used as a vehicle control.

RT-qPCR. RNA from human monocytes (1 x 10^6 per group) was harvested using RNA Minipreps Super Kit (BioBasic) and reverse-transcribed using the High Capacity Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed using SsoAdvanced SYBR Green SuperMix Kit (BioRad) and run on a CFX-96 (BioRad). Primers used in this study were designed using IDT PrimerQuest and can be found in **Supplementary Table S1**.

CYP1 activity. CYP1 activity was determined using the 7-ethoxyresorufin-*O*-deethylase (EROD) assay as described [319]. Briefly, cells were stimulated as indicated in the respective figure legends, washed once in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$, and re-suspended in 150 µL of 7-ethoxyresorufin (2 µM in PBS + 1 mM $MgCl_2$ + 1 mM $CaCl_2$) and incubated for 30 minutes at 37°C. Supernatants (100 µL) were then transferred to black fluorescent plates and stored at -20°C until read at 560/590 nm excitation/emission [404] on an EnSpire Plate Reader (PerkinElmer). A standard curve of resorufin was used to determine the amount of resorufin produced. Following the EROD assay, cells were washed in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and lysed in 25-50 µL of RIPA buffer to determine protein amount (BCA assay, ThermoFisher). Data were plotted as pmol of resorufin produced per mg of protein.

Statistics. Statistical analysis was performed with GraphPad Prism 6 and a P value of < 0.05 was deemed as significant.

3.4 Results

Pattern Recognition Receptor (PRR) signaling inhibits the induction of Cyp1 family members by AHR ligands

AHR has recently been shown to be an effective regulator of pro-inflammatory cytokine production by monocytes and macrophage [232, 392]. However, the mechanisms of expression and regulation of the AHR gene battery in primary human monocytes and macrophages have not yet been explored. To investigate these mechanisms, we stimulated primary human monocytes isolated from venous blood of healthy donors with the AHR ligand FICZ alone, or concomitantly with the TLR4 ligand LPS or the Gram-positive bacterium *S. aureus*, which principally signals through TLR2 [405], and screened a panel of AHR gene battery members for expression using RT-qPCR. As expected, AHR activation by FICZ led to a robust increase in the expression of the AHR response genes *Cyp1a1*, *Cyp1b1*, *Ahrr*, and *Nqo1* (**Figure 3.1A-D**), but not other members tested (e.g. *Cyp1a2, Fmo3, Gstu1, Nptx1, Serpine1, Ugt1a6*; data not shown). PRR signaling by itself either through TLR4 by LPS or through TLR2/NOD by *S. aureus* did not induce AHR activation, although we observed mild activation of AHR genes, predominantly *Ahrr*, in response to *S. aureus* in monocytes from some individuals. However, when primary human monocytes were stimulated concomitantly with both FICZ and LPS or *S. aureus*, we observed a significant decrease in the induction of *Cyp1a1* and *Cyp1b1* (**Figure 3.1A,B**). Such an inhibition was not observed with the other two AHR gene battery members, *Ahrr* and *Nqo1* (**Figure 3.1C,D**), implying that the inhibition of AHR activation was selective for the *Cyp1* family genes.

To verify this finding, we performed an extensive dose titration of both FICZ (0.01-100 nM) and LPS (0.01-1 ng/mL) on primary human monocytes and assessed *Cyp1a1* and *Ahrr* mRNA levels. We observed that LPS inhibited the induction of Cyp1a1 by FICZ across all concentrations

tested in a dose-dependent manner whereas, as expected, *Ahrr* was not suppressed by any LPS concentration tested (**Figure 3.1E**). This inhibition was also not unique to FICZ, as a similar down-regulation of *Cyp1a1* was also observed in response to Kyn (**Figure 3.1F**), an AHR ligand generated from tryptophan catabolism that is increased during systemic infections [232]. Based on these results, we concluded that TLR4 and TLR2 signaling selectively regulate the induction of the *Cyp1* family by AHR activation.

We next investigated if this selective down-regulation of the *Cyp1* members upon AHR activation was unique to TLR4 and TLR2 signaling or was applicable to other PRR signaling. To do this, we tested the *Cyp1a1*-regulatory capacity of *E. coli* LPS (TLR4 ligand), staphylococcal PGN (TLR2 and NOD1/2 ligands), ultra-pure staphylococcal PGN SANDI (NOD1/2 ligand), zymosan (TLR2 and dectin-1 ligands), depleted zymosan (dectin-1 ligand), poly I:C (TLR3 ligand), resiquimod (R848; TLR8 ligand), and CpG ODN2216 (TLR9 ligand). We found that all pathogen-associated molecule patterns (PAMPs) tested were able to significantly inhibit the induction of *Cyp1a1* by FICZ (**Figure 3.2)**. Thus, down-regulation of the *Cyp1* family members by PRR signaling upon AHR activation is likely applicable to all PRRs.

We next examined the mechanism by which PRR signaling down-regulates *Cyp1* family induction by AHR. It has previously been reported that pro-inflammatory cytokines such as IL-1β and TNF-α can inhibit TCDD-induced *Cyp1a1* expression in hepatocytes [406, 407]. To assess if cytokine production in response to PRR signaling was required for PRR-mediated down-regulation of *Cyp1*, we blocked cytokine production by inhibiting protein translation with cycloheximide (CHX) and assessed if LPS was still able to suppress *Cyp1a1* induction. Similar to a previous report [408], CHX super-induced *Cyp1a1* expression with and without FICZ stimulation (**Figure 3.3A**). However, even in the presence of CHX, where cytokine protein production would not occur,

LPS was still able to suppress *Cyp1a1* induction to a proportionally similar extent as in the absence of CHX. Interestingly, LPS was also able to block in part the super-inducing effects of CHX on *Cyp1a1* expression. This result suggests that protein synthesis, and therefore *de novo* cytokine production, is not required for the down-regulation of *Cyp1* by PRRs.

As CHX treatment did not prevent *Cyp1* down-regulation by LPS, we hypothesized that PRR signaling was directly blocking *Cyp1* induction by AHR activation. PRRs signal through three major pathways: PI3K-Akt-mTOR, MAPK, and NF-κB [370]. To identify which of these pathways was required for *Cyp1* inhibition, we used small molecules that specifically target each pathway [409]. We first assessed the PI3K-Akt-mTOR pathways using the pan-PI3K inhibitor wortmannin and the mTOR inhibitor rapamycin (**Figure 3.3B**). We observed that PI3K-Akt-mTOR inhibition significantly reduced the *Cyp1a1*, but not *Ahrr*, induction by FICZ. However, neither inhibitor reversed the down-regulatory effects of LPS on *Cyp1a1* expression. Next, we blocked the MAPK p38 using SB-203580 or BIRB-0796 (**Figure 3.3C**). Again, we observed no effect on LPS-mediated down-regulation of *Cyp1a1*, but we did find a significant reduction in *Cyp1a1* and *Ahrr* induction by FICZ, perhaps by regulating AHR nuclear localization [311, 312]. Moreover, blocking ERK-MAPK signaling using PD-184352 also failed to reverse the effect of LPS stimulation on *Cyp1a1* expression or block induction of the AHR gene battery by FICZ (**Figure 3.3D**). Lastly, we blocked the NF-κB using BAY 11-7082. However, BAY 11-7082 completely prevented the upregulation of the *Cyp1a1* and *Ahrr* by FICZ precluding our ability to assess whether LPS stimulation could down-regulate the expression of *Cyp1a1* (**Figure 3.3E**). Collectively, these results suggest that PRRs likely regulate *Cyp1* induction through mechanisms different from the PI3K-Akt and the p38 and ERK MAPK pathways, leaving open the possibility that it is through activation of the NF-κB pathway.

Selective suppression of the CYP1 members by PAMPs is most apparent in GM-CSF-differentiated MDMs

We next wanted to corroborate our findings by investigating if the suppression of the *Cyp1* family by PAMPs translated into reduced expression and enzymatic activity of CYP1. To test this, we stimulated human primary monocytes with FICZ and/or LPS for 24 hours and assayed for CYP1 activity using the EROD assay. In primary human monocytes, the levels of CYP1 activity upon FICZ stimulation were below the sensitivity of the assay (**Figure 3.4A**). During an infection, monocytes migrate to inflamed tissues and differentiate into macrophages, playing important roles in cytokine production, pathogen clearance, and resolution of inflammation [12, 21]. We therefore asked if monocyte-derived macrophages (MDMs) expressed CYP1. *In vitro,* monocytes can be differentiated into macrophages using either GM-CSF (GM-MDMs) or M-CSF (M-MDMs). GM-MDMs have a more pro-inflammatory M1 macrophage phenotype (e.g. classically-activated macrophages) whereas M-MDMs are more of an anti-inflammatory M2-type macrophage (e.g. alternatively-activated macrophages) [402]. When we stimulated MDMs for 24 hours with FICZ, we found an increase in CYP1 activity in GM-MDMs while the CYP1 activity in M-MDM remained unchanged (**Figure 3.4B**). Importantly, in GM-MDMs, concomitant stimulation with FICZ and LPS led to a significant reduction of CYP1 activity compared to FICZ stimulation alone, corroborating what was observed at the mRNA level in monocytes.

The induction of CYP1 activity in macrophages, particularly in GM-MDM, was not the result of direct up-regulation of CYP1 activity by GM-CSF but was due to the differentiation state of the macrophages. This was illustrated by the observations that a 1-hour pre-treatment with GM-CSF did not induce CYP1 activity in human primary monocytes in resting conditions or after FICZ

or LPS stimulation (**Figure 3.5A**), and by the minimal effect of GM-CSF once MDM had been generated in the presence of M-CSF (**Figure 3.5B**). Importantly, in all conditions, LPS significantly inhibited CYP1 activity induced by FICZ (**Figure 3.5B**). Collectively, these data suggest that GM-CSF cannot directly drive CYP1 expression, but rather differentiates monocytes into macrophages that express CYP1 protein upon AHR activation, and that LPS can inhibit this expression.

AHR activation regulates the pro-inflammatory cytokine response of GM-CSF-differentiated MDMs

Given the selective down-regulation of *Cyp1* members in the AHR gene battery by PRR signaling, we tested the effect of AHR activation on PRR-induced cytokine production by GM-MDMs. This was important because the AHR pathway has been linked to LPS tolerance and inhibition of pro-inflammatory responses [344]. So, we stimulated GM-MDMs with LPS alone, or in combination with the AHR ligand FICZ and determined the production of pro-inflammatory cytokines and chemokines, and of the anti-inflammatory cytokine IL-10. We found that the secretion of the pro-inflammatory cytokine IL-6 (**Figure 3.6A**) and chemokine CCL3 (**Figure 3.6B**) were significantly reduced by AHR activation but that the production of the anti-inflammatory cytokine IL-10 was not affected (**Figure 3.6C**). In human moDCs, AHR has been reported to enhance the production of IL-1β and IL-8 [346], contrary to its inhibitory effect on other pro-inflammatory cytokines [392]. Although we were unable to detect significant production of IL-1β in GM-MDMs (data not shown), we too found that AHR activation by FICZ significantly enhanced the production of IL-8 in GM-MDMs stimulated with LPS (**Figure 3.6D**).

3.5 Discussion

It is now well-established that the AHR is an effective regulator of the immune system [321]. In particular, it has been shown that AHR activation can influence the differentiation and function of Th17 [292, 293, 327] and Tr1 cells [330]), and suppress the production of inflammatory cytokines in monocytes and macrophages [344]. These AHR functions have been associated with its ability to directly interact with known transcription factors of the immune system (e.g. NF-κB [344], c-Maf [330], and ROR-γt [339]). However, the expression and functional implications of classical AHR gene battery members in the immune system is understudied. Similar to what is observed in the liver by inflammatory stimuli [221, 410, 411], we report that PRR signaling can selectively inhibit the induction of the *Cyp1* family by AHR ligands in primary human monocytes and macrophages. This could potentially lead to enhanced AHR activation by decreasing the metabolism of AHR ligands through CYP1. Such a claim is consistent with the previous observation that pharmacological inhibition of CYP1 augments and prolongs AHR activation [319]. Therefore, the down-regulation of the *Cyp1* family by PAMPs may function as a feedback mechanism to weaken the inflammatory response in monocytes and macrophages by enhancing AHR activation.

When monocytes egress from the blood and enter into peripheral tissues, they differentiate into macrophages or DCs [12, 21]. *In vitro*, human monocytes can be differentiated into macrophages using either M-CSF or GM-CSF [402]. M-CSF is constitutively expressed *in vivo* and regulates macrophage functions during homeostasis whereas GM-CSF is produced in a variety of infections and chronic inflammatory conditions and is a potent activator of inflammatory responses in macrophages [412]. Interestingly, we were only able to observe substantial CYP1 activity in GM-CSF-derived macrophages. These macrophages are much more pro-inflammatory

than their M-CSF-derived counterparts, and the enhanced activity of CYP1 may serve as a mechanism to help regulate this phenotype. The molecular mechanisms of how GM-CSF induces CYP1 activity in macrophages are unknown. GM-CSF was unable to increase CYP1 activity in monocytes, and continuous exposure to GM-CSF was required for full CYP1 activity in GM-CSF-derived macrophages. Moreover, we observed a slight increase in CYP1 activity in M-CSF-derived macrophages treated with GM-CSF for 2 days. Therefore, our data suggests that GM-CSF primes human macrophages for CYP1 protein expression following AHR activation, and that persistent GM-CSF exposure is required to maintain this expression in differentiated macrophages.

Monocytes can also differentiate into DCs during inflammation and tissue infiltration [413]. A recent study found that LPS stimulation in human moDCs augmented the expression of AHR and enhanced the induction of *Cyp1a1* by the AHR ligand TCDD [414]. Using U927-derived DCs, the authors determined that LPS caused NF-κB binding to the promoter of *Ahr* and transactivation of the *Ahr* gene. Interestingly, we did not observe the same effect in human monocytes or monocyte-derived macrophages. To the contrary, our data showed that LPS could not induce *Ahr* expression (data not shown), and that AHR-dependent *Cyp1a1* induction was inhibited in these cells. However, similar to what has been reported in moDCs [346], we also observed that AHR antagonized some pro-inflammatory cytokines (e.g. IL-6, CCL3), but enhanced the production of others (e.g. IL-8). Similarly, AHR activation increased the expression of IL-23 in M-CSF-derived macrophages [415]. It therefore appears that the effects of AHR on PRR-induced cytokine production by myeloid cells may be more complex than what was originally reported [283, 344]. Further work is required to fully understand the discrepancies, and similarities, in AHR-NF-κB interactions between monocytes, MDMs, and moDCs, and their implications *in vivo*.

PRRs activate several signaling pathways upon ligand binding, including NF-κB, MAPK, and PI3K-Akt-mTOR (**Figure 3.3B, C and E**). Using pathway-specific small molecule inhibitors [409], we found that the PI3K-Akt-mTOR, and p38 and ERK MAPKs signaling pathways were dispensable for *Cyp1* down-regulation by PAMPs. In addition, we found that blocking NF-κB signaling with Bay 11-7082 resulted in the complete suppression of AHR gene battery induction by FICZ, and that this could not be further reduced by LPS. Although not entire conclusive, these data suggest that NF-κB signaling could be responsible for the down-regulation of *Cyp1* by PAMPs. This is supported by a previous study showing that overexpression of NF-κB in a hepatocyte cell line blocked TCDD-induced AHR activation [406]. Binding interactions between AHR and NF-κB have also been reported [392, 416, 417]. These studies have uncovered a function for AHR in regulating NF-κB-dependent responses to LPS, such as inhibition of IL-6 production [344]. Our data extends this model to include AHR-dependent genes, and suggests that the AHR-NF-κB interaction in monocytes and macrophages is a mutually inhibitory event that suppresses both AHR-dependent genes (e.g. *Cyp1a1*, *Cyp1a2*), and NF-κB-dependent genes (e.g. IL-6, CCL3, etc.). Why this interaction does not affect other AHR gene battery members, e.g. *Ahrr* or *Nqo1*, remains unclear. One possibility is that some of these genes, such as *Ahrr* [418], contain an NF-κB binding site in their promoter. In these cases, the ability of either AHR or NF-κB to transcriptionally activate the gene may supersede the inhibitory mechanism. Important for this model, the AHR-NF-κB binding interaction does not prevent the DNA binding capacity of each other [344, 407].

Our experiments show that AHR is regulated by signaling pathways used by PRRs, even in the absence of exogenous activation of these receptors. Specifically, we found that inhibitors of the p38, PI3K-Akt-mTOR, and NF-κB pathways significantly decreased the induction of *Cyp1a1*

and *Ahrr* by FICZ. Although we cannot completely rule out off-target effects of these inhibitors on AHR signaling, we think this is an unlikely explanation for two reasons. First, although most of the inhibitors used contain ring-like chemical structures, none of them contain the successive polyaromatic rings seen in conventional AHR ligands, such as TCDD and FICZ. Moreover, the two p38 inhibitors used in this study, BIRB-0796 and SB-203580, have substantially different chemical structures, but both were able to decrease *Cyp1a1* induction by FICZ. Second, wortmannin [419] and rapamycin [420] have previously been used in other cells types without reducing the transcriptional activity of AHR. In fact, in a hepatocyte luciferase-reporter cell line, rapamycin augmented AHR activation by TCDD [420]. It is therefore more plausible that these signaling cascades can regulate AHR function. It has already been established that p38 regulates AHR nuclear localization by phosphorylating Ser68 in its nuclear export sequence (NES) [311, 312, 421] preventing CRM1 recognition of the NES and blocking subsequent nuclear export, with the net effect of increased nuclear accumulation of AHR. How PI3K-Akt-mTOR and NF-κB signaling regulates AHR signaling is not known. Collectively, these pathways may function to promote AHR activation in an attempt to counter-balance the inflammatory response induced by PAMPs.

In conclusion, our work reveals a mechanism of PRR signaling in regulating AHR-dependent responses in human monocytes and macrophages. This mechanism is selective for the *Cyp1* family and likely evolved as a mechanism to limit hyperinflammatory states such as sepsis. Future studies should explore the molecular mechanisms that are required for this inhibition to identify novel therapeutic targets for hyper-inflammatory disorders.

3.6 Acknowledgements

We would like to thank members of the Madrenas Laboratory for many helpful discussions and feedback on this research. This work was supported by the Canadian Institutes for Health Research (to J.M.). A.P. is a Fonds de la Recherché en Santé du Québec Research Scholar. I.L.K. holds a Tier II Canada Research Chair in Humoral Immunity. J.M. holds a Tier I Canada Research Chair in Human Immunology.

3.7 Figures

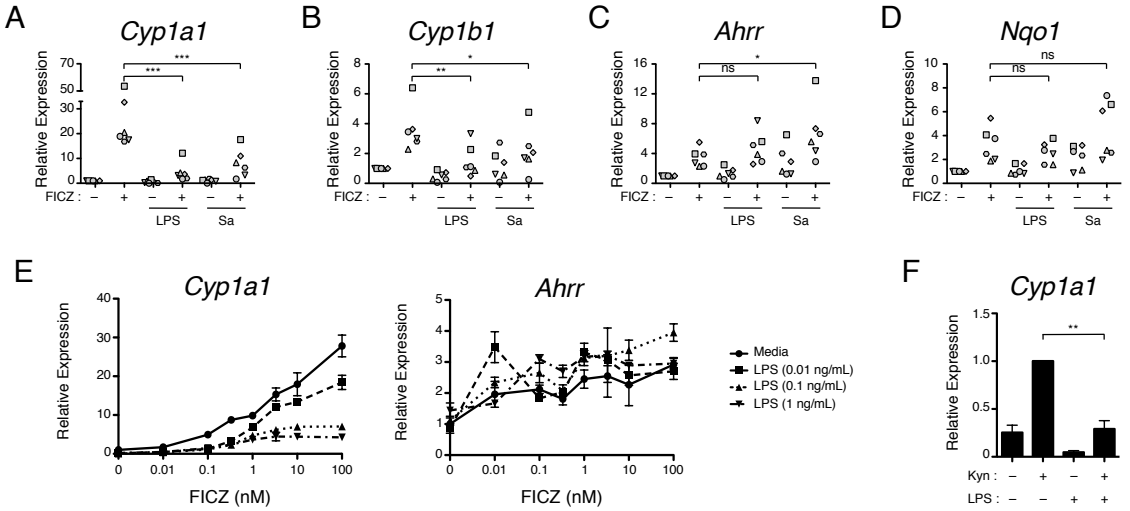


Figure 3.1. PRR signaling suppresses the induction of the *Cyp1* family by AHR ligands in primary human monocytes. (A-D) RNA expression of AHR gene battery members (A) *Cyp1a1*, (B) *Cyp1b1*, (C) *Ahrr*, or (D) *Nqo1* in primary human monocytes stimulated with FICZ (300 nM) and/or LPS (10 ng/mL) or *S. aureus* (Sa) for 6 hours as measured by RT-qPCR. Graphs show means of technical duplicates of 6 individual donors labeled by different symbols. *P < 0.05, **P < 0.01, ***P < 0.001 as calculated by One-way ANOVA with Bonferroni's post-hoc analysis. (E) LPS dose-dependent inhibition of AHR-induced *Cyp1a1*, but not *Ahrr*. Data are representative of two independent experiments from two separate donors. (F) Expression of *Cyp1a1* in primary human monocytes stimulated with kynurenine (Kyn; 100 μM) and/or LPS for 6 hours as measured by RT-qPCR.

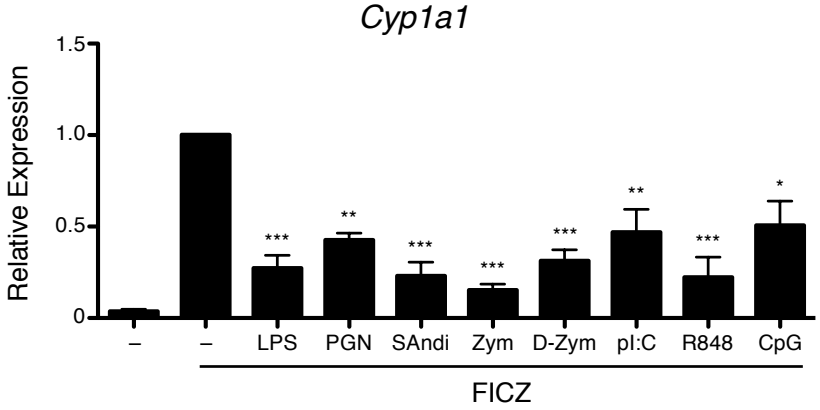


Figure 3.2. A broad range of PAMPs suppress *Cyp1a1* induction by FICZ in primary human monocytes. *Cyp1a1* RNA expression in human monocytes stimulated with FICZ and various PAMPs for 6 hours, as measured by RT-qPCR. PGN: staphylococcal PGN (10 μg/mL); SAndi: staphylococcal PGN SANDI (10 μg/mL); Zym: zymosan (10 μg/mL); D-Zym: depleted zymosan (10 μg/mL); PIC: poly I:C (10 μg/mL); R848: resiquimod (10 μg/mL); CpG: OD2216 (1 μM). Data is plotted as means ± S.E.M of three independent experiments from three different donors, each performed in duplicates.

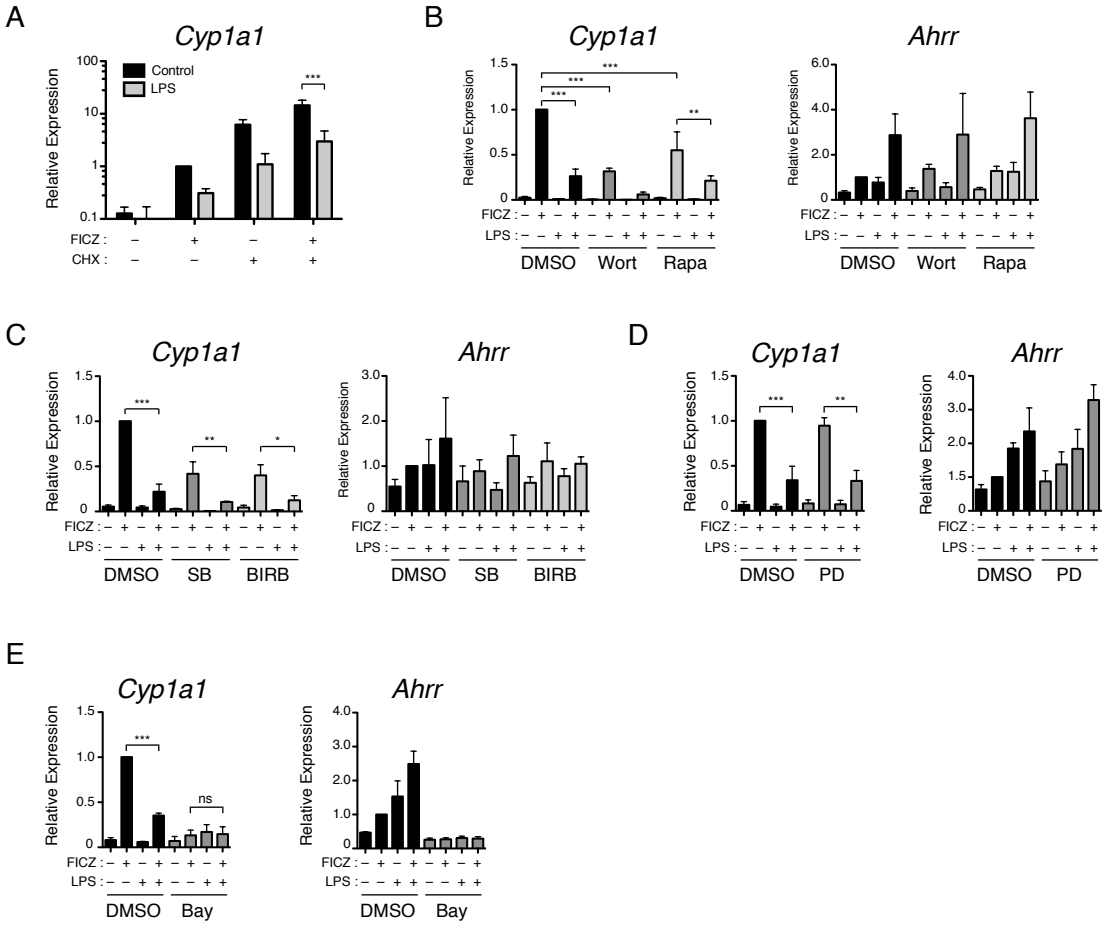


Figure 3.3. PRR signaling regulates AHR gene battery members at the transcriptional level in primary human monocytes. (A) Human monocytes were pre-treated with cycloheximide (10 μg/mL) for 1 hour before stimulated with FICZ (300 nM) and/or LPS (10 ng/mL) for 6 hours. Expression of *Cyp1a1* and *Ahrr* was determined by RT-qPCR. Effect of PI3K/mTOR inhibitors (B), p38 MAPK inhibitors (C), ERK MAPK inhibitors (D), and NF-κB inhibitors (E) on LPS-down-regulation of AHR activation. All data are plotted as means ± S.E.M of three independent experiments from three different donors, each performed in duplicates.

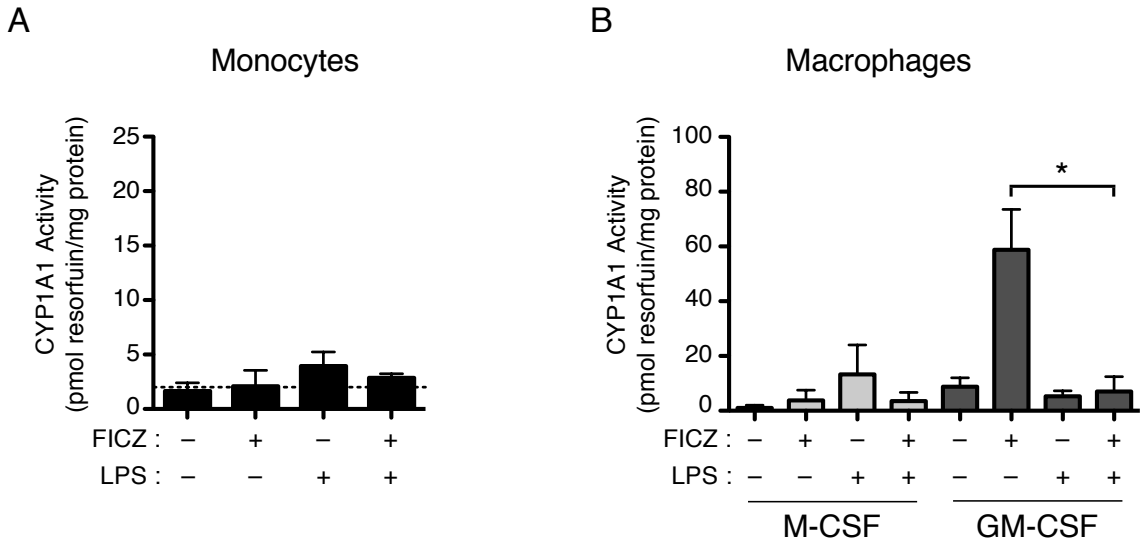


Figure 3.4. LPS regulates CYP1 family expression and function in GM-CSF-derived macrophages. (A) EROD activity in monocytes stimulated with FICZ (300 nM) and/or LPS (10 ng/mL) for 24 hours. Data are plotted as mean ± S.E.M of three donors performed in triplicates. (B) EROD activity in M-MDM and GM-MDM stimulated with FICZ (300 nM) and/or LPS (10 ng/mL) for 24 hours. Data are plotted as means ± S.E.M of four donors, each performed in triplicates. *$P < 0.05$ as determined by paired Student's t-test.

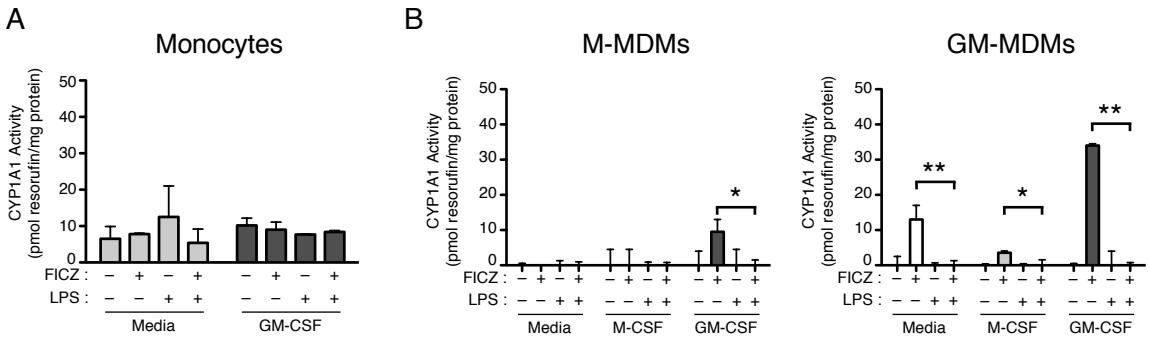


Figure 3.5. LPS-dependent regulation of CYP1 activity depends on macrophage differentiation conditions. (A) EROD activity in primary human monocytes stimulated with FICZ and/or LPS for 24 hours. (B) EROD activity in monocyte-derived macrophages in the presence of M-CSF or GM-CSF for 7 days, then stimulated with FICZ and/or LPS for 24 hours. *P < 0.05 as determined by paired Student's t-test.

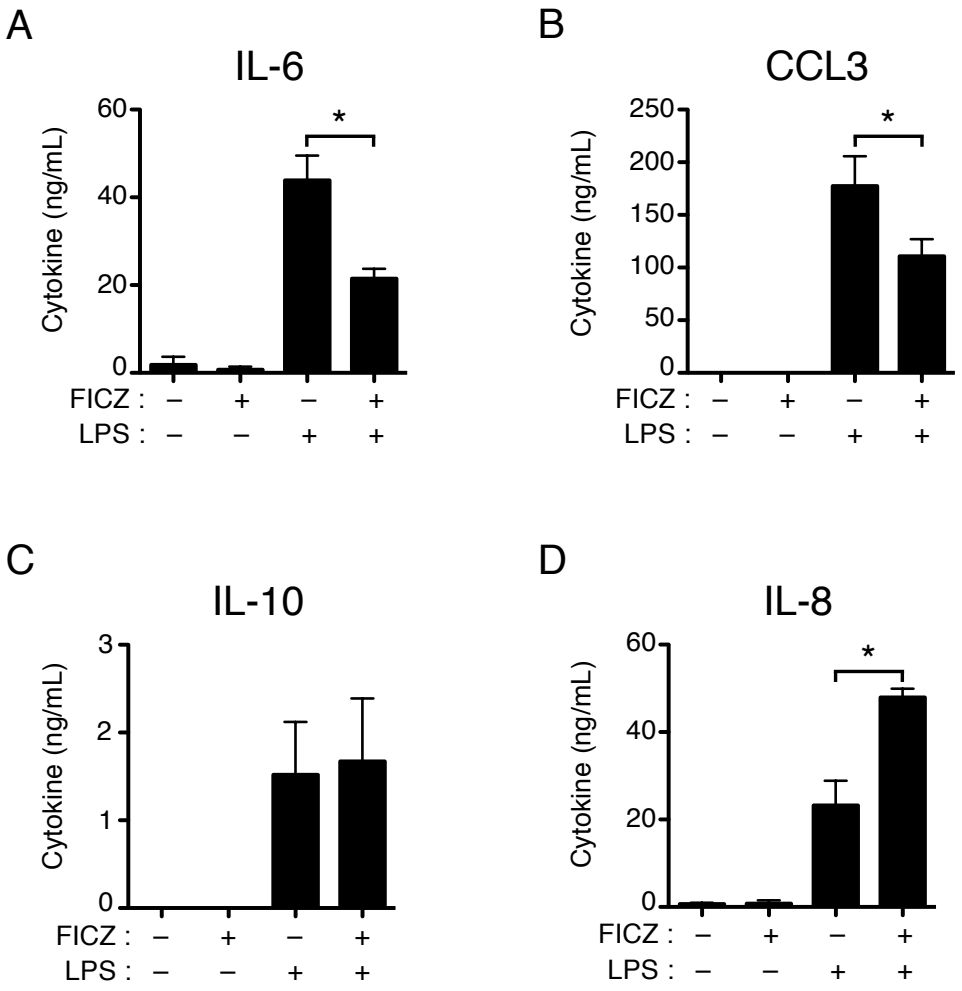


Figure 3.6. AHR activation diminishes pro-inflammatory cytokine production by GM-MDMs. GM-CSF-differentiated MDMs were stimulated with LPS (10 ng/mL) and/or FICZ (300 nM) for 24 hours, and accumulation of IL-6 (A), CCL3 (B), IL-10 (C), and IL-8 (D) in the supernatants was quantified by ELISA. Data are means ± S.E.M of three different donors from three independent experiments, each performed in triplicates. *$P < 0.05$ as determined by paired Student's t-test.

Preface to Chapter 4

In Chapter 3, we showed that PAMPs stimulation of human monocytes and monocyte-derived macrophages selectively down-regulated the expression of the *Cyp1* family, but not other members of the AHR gene battery. Although we were able to detect CYP1 activity in human GM-MDMs, the highest *Cyp1* family expression is found in non-hematopoietic cells. We therefore reasoned that the down-regulation of *Cyp1* in these cells would be the most impactful on the degree of AHR function. During a systemic infection, such as staphylococcal bacteremia, the liver plays an essential role in the acute phase response and clearance of toxins. Moreover, the liver constitutively expresses *Cyp1a2* that is down-regulated during systemic inflammation [422]. We therefore hypothesized that decreased hepatic *Cyp1a2* expression during endotoxemia would increase serum AHR ligand levels and limit the inflammatory response to LPS by enhancing AHR signaling in macrophages.

Chapter 4 : The Role of the CYP1 Family Members and the AHR Gene Program in Endotoxemia and the Establishment of Endotoxin Tolerance

Adam G. Peres[1], Vladimir-Andrey Gimenez-Rivera[1], Irah L. King[1,2], Joaquín Madrenas[1,3]

[1]Microbiome and Disease Tolerance Centre and Department of Microbiology and Immunology, and [2]Meakins-Christie Laboratories, McGill University, Montréal, QC, Canada; and [3]Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance CA 90502

Manuscript in Preparation

4.1 Abstract

AHR is a potent regulator of monocyte and macrophage cytokine production. It has been recently suggested that AHR mediates immunosuppression of these innate leukocytes following endotoxin exposure, a phenomenon known as endotoxin tolerance. However, the regulation of AHR signaling itself and its contribution to endotoxemia and endotoxin tolerance are unclear. The CYP1 family members are enzymes that metabolize AHR ligands and are therefore important regulators of AHR signaling. We hypothesized that modulation of CYP1 expression in the liver would alter the availability of AHR ligands in the serum and subsequently influence AHR-dependent regulation of monocytes/macrophages. Using both murine and primary human monocyte models of endotoxin tolerance, we found that AHR can regulate the monocyte/macrophage pro-inflammatory cytokine response to LPS during both primary endotoxemia and in the state of endotoxin tolerance. However, AHR was not required for the establishment of endotoxin tolerance in mice or humans, as AHR-deficient mice were tolerized to LPS, and the addition of AHR ligands did not change the magnitude of endotoxin tolerance in human monocytes. During primary endotoxemia, we also found that the liver CYP1A2 expression and activity was significantly reduced in an AHR-independent manner. However, CYP1A2-deficient mice were actually more susceptible to primary endotoxemia, suggesting that CYP1A2 plays a protective role during endotoxemia through a mechanism that likely does not involve AHR. Future work will focus on assessing the immune response, tissue pathology, and AHR activation in the absence of CYP1A2 expression to better understand the role of this protein in primary endotoxemia and endotoxin tolerance.

4.2 Introduction

AHR is emerging as a critical regulator of numerous immune responses [321, 322]. In particular, AHR has been shown to intrinsically modulate the PAMP-triggered production of pro-inflammatory cytokines in macrophages through blocking NF-κB-dependent transcription [344]. By limiting this macrophage inflammatory response, AHR protects against excessive immunopathology caused by the systemic inflammatory response in endotoxemia, a murine model of the early hyper-inflammatory phase of sepsis [343, 344].

Recently, AHR has also been proposed to be essential for the establishment of endotoxin tolerance in mice [232]. Endotoxin tolerance is a state of unresponsiveness of APCs following repeated exposure of LPS [199]. The initial exposure to LPS causes epigenetic changes and chromatin remodeling of a select group of inflammatory genes that prevents expression following re-challenge. Moreover, decreased expression of TLR4 or its co-receptors, impaired activation of NF-κB signaling, and up-regulation of negative regulators of TLR signaling are also contributing factors to this state of unresponsiveness [199]. Endotoxin tolerance can be induced *in vivo* or *in vitro* and lasts upwards of 7 days [199]. Monocytes and macrophages tolerized with LPS have many of the hallmark features of refractory monocytes from patients with sepsis [198], including impaired cytokine production and expression of antigen-presentation machinery [235], but maintain other functions, such as phagocytosis [234], anti-microbial peptide production [227], and tissue repair [198]. AHR is proposed to contribute to this unresponsive state through a mechanism that involves a Src kinase and phosphorylated IDO1 [232], but how this complex contributes to the establishment of endotoxin tolerance is unknown.

Despite its integral role in regulating immunity, exacerbated AHR activation can have detrimental physiological effects. As such, multiple negative regulatory mechanisms of AHR

signaling have evolved. First, following ligand binding and induction of transcription, AHR is exported from the nuclear and ubiquitin-targeted for proteasome degradation [313]. Second, AHRR disrupts dimerization of AHR with ARNT and prevents transcriptional activation by AHR [315]. Third, induction of the CYP1 members of the cytochrome P450 family by AHR negatively feedbacks on the pathway by metabolizing the AHR ligands and preventing further AHR activation [319]. It is now apparent that these negative regulators have important physiological roles. For example, over-expression of CYP1A1 depletes AHR ligands in the murine gut and consequently impairs Th17-like type 3 immunity that renders mice susceptible to *C. rodentium* infections [274]. Moreover, deletion of AHRR protects mice from endotoxemia by decreasing pro-inflammatory cytokine production, likely through prolonged AHR activation in macrophages [400]. However, whether the CYP1 family members can similarly control AHR activation during systemic inflammation has not been explored.

Dysregulated cytochrome P450 expression during systemic inflammation is well-documented [221, 423]. However, the involvement of individual cytochrome P450 genes or families in the host response during systemic inflammation has never been properly assessed. Given the importance of the CYP1 family in regulating AHR activation, and the importance of AHR in endotoxemia and endotoxin tolerance [232], we therefore wanted to explore the role of the CYP1 family in systemic inflammation using the endotoxemia mouse model.

4.3 Materials & Methods

Mice. C57BL/6 *Ahr*$^{-/-}$ mice [278] and 129S *Cyp1a2*$^{-/-}$ mice [424] were kindly provided by Dr. Frank Gonzalez (National Cancer Institute, National Institutes of Health, Bethesda MD, USA) and were housed on a 12-hour dark/light schedule under specific-pathogen free conditions. All experiments used mice 6-12 weeks of age and were approved by the McGill University Animal Care Committee.

Cells. Human peripheral blood mononuclear cells (PBMCs) from the venous blood of healthy donors were isolated by Ficoll-Hypaque centrifugation (GE Healthcare). Donors gave their informed consent in accordance with the McGill University Research Ethics Office. Monocytes (> 95% purity) were isolated from PBMCs by negative selection using the EasySep Human Monocyte Isolation Kit (STEMCELL). All cells were cultured in RPMI-1640 containing 10% PBS, penicillin-streptomycin, non-essential amino acids, L-glutamine, HEPES buffer (pH 7), and sodium pyruvate.

Reagents. *Escherichia coli* O111:B4 lipopolysaccharide, 7-methoxyresorufin, resorufin, and NADPH were purchased from Sigma-Aldrich. FICZ was generated in-house as previously described [2].

Murine Models of Endotoxemia and Endotoxin Tolerance. For endotoxemia, mice were treated with a LD50 dose of LPS (5 or 10 mg/kg for C57BL/6 mice, and 30 mg/kg for 129S mice) for the duration indicated in the figure legends. To establish endotoxin tolerance, mice were treated with a sub-lethal dose of LPS (0.5 mg/kg, intraperitoneal) and seven days later treated with a lethal dose of LPS (15 mg/kg for C57BL/6 mice). For survival experiments, mice were monitored every 12 hours for signs of severe distress.

Monocyte Endotoxin Tolerance Model. To induce endotoxin tolerance, purified human monocytes (1 x 10^6 cells per mL) were stimulated with LPS (1 ng/ml) for 6 hours, then washed 3 times, and rested for 18 hours. Naïve monocytes were treated as tolerized monocytes but were cultured only in media. After the resting period, the cells were recounted and seeded in a 96-well round bottom plate (1 x 10^5 cells per well), and were then restimulated with LPS (10 ng/mL) and/or FICZ (300 nM) for 3 hours for RT-qPCR or 18 hours for ELISA.

Liver Microsome Preparation. Murine liver microsomes were prepared as described previously [425]. Briefly, 0.4-0.6 g of flash frozen liver was homogenized in 4 mL of PBS and centrifuged at 10,000 *g* for 10 minutes, twice. The supernatant, containing cytosolic and microsomal proteins, was collected and centrifuged at 100,000 *g* for 1 hour. The microsome-containing pellet was then re-suspended in 1 mL of PBS and quantified for protein by BCA assay (ThermoFischer).

CYP1 Activity. MROD assays was performed as described previously [319]. Each reaction contained 10 μg of freshly prepared liver microsome protein, 2 μM of 7-methoxyresorufin, and 0.5 M NADPH in 100 μL. The production of resorufin was detected over 15 minutes by the absorbance at 560/590 nm using an EnSpire Plate Reader (Perkin-Elmer), and compared to a standard curve of resorufin. Data was plotted as pmol of resorufin produced per mg of protein.

RT-qPCR. RNA was extracted from murine liver tissue (75-100 mg) by TRIzol® (Life Technologies), per the manufactures conditions, then passed through a RNA Minipreps Super Kit (BioBasic) column. For human monocytes, cells were lysed in RLT buffer and RNA was harvested using the RNA Minipreps Super Kit (BioBasic). All RNA was reverse-transcribed using the High Capacity Reverse Transcription Kit (Applied Biosystems) and quantitative PCR was performed using the SensiFAST™ SYBR® No-ROX Kit (BioLine) on a CFX96 System (BioRad). IDT PrimerQuest was used to design primers and can be found in **Supplementary Tables S1 & S2**.

Serum Cytokine Levels. Blood from mice treated as indicated in the figure legend was collected by cardiac puncture following euthanasia. Blood was left to coagulate for 30 minutes at room temperature and serum was obtained after centrifugation at 2,000 *g* at 4°C. Cytokines in the serum were quantified by Ready-SET-Go! ELISA (eBioscience) following the manufacture's recommended protocol.

Statistics. Data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison post-hoc test using GraphPad Prism. A difference with $P < 0.05$ was considered significant.

4.4 Results

AHR down-regulates the inflammatory response to LPS but is not required for LPS tolerance

AHR is emerging as an effective regulator of the monocyte/macrophage pro-inflammatory response to PAMPs, such as LPS. Similar to what has previously been reported [232, 343, 344], we found that *Ahr*$^{-/-}$ mice were highly susceptible to endotoxemia (**Figure 4.1A**). This susceptibility correlated with higher serum levels of the pro-inflammatory cytokines TNF-α and IFNγ (**Figure 4.1B**) and lower levels of the anti-inflammatory cytokine IL-10 (**Figure 4.1C**). Therefore, AHR is protective against primary endotoxemia by limiting the pro-inflammatory cytokine response and enhancing IL-10 production.

We next examined if AHR is required for the induction of endotoxin tolerance in mice [232]. To test this, we primed wild-type or *Ahr*$^{-/-}$ C57BL/6 mice with a sublethal dose of LPS (0.5 mg/kg) and one week later re-challenged the mice with a lethal dose of LPS (15 mg/kg). As expected, primed wild-type mice were completely protected from endotoxemia-induced mortality (**Figure 4.2A)**. We also found that priming *Ahr*$^{-/-}$ mice significantly reduced the mortality caused

by endotoxemia, in contrast to a previous report that had claimed that AHR was required for LPS tolerance [232]. In support of our observation, we documented a similar reduction in serum IL-6 and TNF-α levels at 3 hours after re-challenge in both primed wild-type and *Ahr*$^{-/-}$ mice (**Figure 4.2B**). Therefore, our data indicated that AHR is not required for the establishment of endotoxin tolerance.

AHR ligands do not enhance endotoxin tolerance of human monocytes in vitro

We next wanted to test if AHR activation influences the development or function of endotoxin tolerized monocytes. Human monocytes were tolerized with LPS for 6 hours and allowed to rest. This protocol resulted in an almost 90% reduction in cytokine production upon re-challenge (**Figure 4.3A**). As expected, AHR suppressed the induction of IL-1β and IL-6 by LPS in naïve monocytes, but did not affect the production of IL-10, indicating that the AHR program is maintained in tolerized monocytes. In support of this finding, we showed that the transcriptional activity of AHR was unchanged, as treatment with FICZ upregulated *Cyp1a1* and *Cyp1b1* to similar levels in both cellular states (**Figure 4.3B**). We next asked if AHR activation alters the establishment of endotoxin tolerance in monocytes. For this, we treated monocytes with LPS, FICZ or concomitantly with both LPS and FICZ and examined the degree of tolerance by measuring cytokine production after re-stimulation with LPS. FICZ alone did not induce a state of refractory response to LPS in monocytes, nor did it affect the magnitude of tolerance driven by LPS alone (**Figure 4.3C**). Collectively, these results indicate that AHR may further exacerbate the immunoparalysis of monocytes during sepsis, but that the presence of an AHR ligand during tolerization by endotoxin does not augment monocyte unresponsiveness.

LPS decreases Cyp1a2 expression and activity in mouse liver

AHR signaling is regulated in part by the metabolism of its ligands by the CYP1 family [221, 423]. How individual cytochrome P450 enzymes contribute to this regulation *in vivo* has not been explored. We hypothesized that any change in the CYP1 expression during inflammation, such as endotoxemia, would affect AHR activation and responses. The liver expresses the highest levels of CYP1 of any visceral organs and also plays a crucial role in the pathogenesis of sepsis [217]. We therefore monitored the expression and activity of the CYP1 family in the liver during endotoxemia. We observed that the expression of liver *Cyp1a1* and *Cyp1a2* began to decrease 6 hours after the administration of LPS and was sustained for at least 36 hours (**Figure 4.4A**). Interestingly, *Cyp1b1* expression was increased starting at 12 hours after LPS administration. The net effect of these changes was a significant reduction in the activity of CYP1 (**Figure 4.4B**), as measured by the MROD assay, likely because the major CYP1 isoform in the liver is *Cyp1a2* (**Figure 4.4A** and [317]). From these results we hypothesize that reduced *Cyp1a2* expression slows down the rate of AHR ligand metabolism and enhances AHR activation during endotoxemia.

AHR is not required for the Cyp1a2 downregulation during endotoxemia

The *Cyp1a1* and *Cyp1a2* expression is primarily regulated by AHR and is greatly upregulated following administration of its ligands [278, 280]. We therefore asked if the decreased *Cyp1a2* expression observed during endotoxemia was because of reduced AHR expression or signaling. To test this, we assessed *Cyp1a2* expression and activity in $Ahr^{-/-}$ mice 24 hours after LPS administration. As previously reported [278], $Ahr^{-/-}$ mice had lower basal liver *Cyp1a2* expression (**Figure 4.5A**) and activity (**Figure 4.5B**) compared to wild-type C57BL/6 mice. We observed a similar reduction in *Cyp1a2* expression and activity in wild-type and $Ahr^{-/-}$ mice 24 hours after

LPS injection (**Figure 4.5A,B**), indicating that downregulation of *Cyp1a2* occurs through a mechanism that is independent of AHR. In support of this result, we found that liver *Ahr* expression remained unchanged during the first 36 hours of endotoxemia in wild-type mice (**Figure 4.5C**). Therefore, AHR is not required for the decreased *Cyp1a2* expression in the murine liver following LPS administration.

Cyp1a2$^{-/-}$ mice are more susceptible to endotoxemia than wild-type mice

Given that the CYP1 family can negatively regulate AHR through the metabolism of its ligands [274, 319], we hypothesized that decreased CYP1A2 activity during endotoxemia would increase the availability of AHR ligands, thereby reducing the inflammatory response of macrophages and limiting immunopathology. To test this hypothesis, we administered a lethal dose of LPS (30 mg/kg) to *Cyp1a2$^{-/-}$* 129S mice and monitored their survival over 5 days (**Figure 4.6**) in comparison to wild-type controls. Interestingly, similar to mice lacking AHR, CYP1A2-deficient mice were also more susceptible to endotoxemia than wild-type 129S mice. Further work is required to determine the increased mortality in *Cyp1a2$^{-/-}$* mice including careful assessment of the immune response, immunopathology, and AHR activation. However, these preliminary results suggest that CYP1A2 and AHR may function separately to promote host survival during endotoxemia.

4.5 Discussion

AHR is emerging as a critical regulator of the pro-inflammatory response of monocytes and macrophages [2, 232, 343, 344]. In this report, we confirmed that AHR-deficient mice display a hyper-inflammatory phenotype. However, we found that AHR is not required to establish

endotoxin tolerance *in vivo*. We also showed that hepatic expression and activity of a negative regulatory of AHR, CYP1A2, was significantly reduced shortly after endotoxemia, in an AHR-independent manner. Intriguingly, CYP1A2 deficiency likely did not enhance AHR activation and limit immunopathology from endotoxemia, as *Cyp1a2*$^{-/-}$ mice were more susceptible to endotoxemia than wild-type mice. The mechanistic basis of such an observation remains to be determined. However, these data reveal that there is a component of *Cyp1a2* expression that does not regulate nor is dependent on AHR. This section will outline some of the future directions to complete this work.

It has already been previously reported that *Ahr*$^{-/-}$ mice are hyper-sensitive to LPS [232, 343, 344]. Similar to these groups, we found that AHR-deficient mice were more susceptible to a primary LPS challenge and that this susceptibility was associated with higher serum pro-inflammatory cytokines levels and less IL-10 secretion. The excessive production of pro-inflammatory cytokines and impaired IL-10 response is likely from an intrinsic AHR mechanism in peritoneal macrophages. Peritoneal macrophages are the initial responding cell subset in the intraperitoneal LPS model and AHR limits pro-inflammatory cytokine production in these cells *in vitro* by blocking NF-κB-dependent transcription [344]. In support of this, a macrophage-specific AHR deletion using LysM-cre^{+}*Ahr*$^{fl/fl}$ mice were also susceptible to LPS [343] and we demonstrated that the AHR ligand FICZ suppressed pro-inflammatory cytokine production by human monocytes (**Figure 4.3**) and GM-MDM [2]. Therefore, AHR-deficient mice are likely susceptible to LPS because of lethal immunopathology caused by excessive production of pro-inflammatory cytokines by peritoneal macrophages.

In addition to being critical for the limiting pathology to a primary LPS challenge, Bessede *et al* (2014) reported that AHR was also required for endotoxin tolerance. Using both AHR-

deficient mice and the AHR antagonist CH-223191, they found that in the absence or inhibition of AHR, LPS primed mice were as susceptible to a lethal dose of LPS as naïve mice [232]. However, in our hands AHR-deficient mice showed no impairment in the development of endotoxin tolerance (**Figure 4.2**). Instead, we found that LPS primed *Ahr*$^{-/-}$ mice were protected from a subsequent lethal LPS dose similar to wild-type B6 mice. We used the same 7-day priming protocol as Bessede *et al* (2014), but a different type of *E. coli* LPS (O55:B5 vs. O111B4). LPS can be a highly variable molecule with different responses observed even between different batches of the same type and this is a variable that should be controlled for between our experiments. However, it is important to note that Bessede *et al.* (2014) did observe almost 40% survival in primed *Ahr*$^{-/-}$ mice, compared to 100% mortality in naïve *Ahr*$^{-/-}$ mice, and did not report the serum cytokine levels in these mice [232]. We supported our survival experiments by assessing the serum levels of TNF-α and IL-6 and showed a similar decrease in these cytokines in primed wild-type and *Ahr*$^{-/-}$ mice. Moreover, we also found that the AHR ligand FICZ did not impact the endotoxin tolerization of human primary monocytes *in vitro*. Bessede *et al.* (2014) were able to completely block the endotoxin tolerance by treating mice with the small molecule AHR antagonist CH-223191 for four days after LPS priming [232]. It is unclear why an inhibitor of AHR would be more effective at preventing endotoxin tolerance than a complete genetic deletion but it may be due to the lack of selectivity of small molecule inhibitors [409]. To this point, one could test whether CH-223191 can also prevent endotoxin tolerance in *Ahr*$^{-/-}$ mice.

We observed that hepatic CYP1A2 expression and activity was significantly reduced during primary endotoxemia (**Figure 4.4**). Previous reports have observed a similar inhibition of TCDD-induced *Cyp1a1* in hepatocyte cell line [406, 407] and *Cyp1a2* in rats [422]. The inhibition of *Cyp1a1* occurred following stimulation with either LPS and TNF-α and was mediated by NF-

κB p65 blocking AHR-dependent gene transcription. Interestingly, using AHR-deficient mice, we observed that *Cyp1a2* was down-regulated during primary endotoxemia occurred independently of AHR (**Figure 4.5**). Thus, it appears that the regulation of *Cyp1a1* and *Cyp1a2* by inflammatory stimuli may use distinct mechanisms. The *Cyp1a1* and *Cyp1a2* genes are found in a head-to-head structure on chromosome, separated by a 13.9 kb in mice (23.3 kb in humans [426]) segment containing no additional open reading frames [427]. Unfortunately, because AHR regulates *Cyp1a2* expression through the DRE located adjacent to the *Cyp1a1* promoter site [428], little work has been done to identify if any regulatory elements are located directly upstream of the *Cyp1a2* promoter. However, even in AHR-deficient mice, hepatic *Cyp1a2* expression and MROD activity were still half that of wild-type mice, indicating other factors regulate *Cyp1a2* expression. We also do not know the inflammatory meditator(s) that are required for downregulation of *Cyp1a2*. Similar to *Cyp1a1*, LPS could either directly activate hepatocytes or induce production of a cytokine that is responsible for the *Cyp1a2* down-regulation. These possibilities can be tested in mice by administering recombinant cytokines or blocking antibodies that target these molecules.

The CYP1 family is a group of cytochrome P450 enzymes that metabolize polycyclic molecules and many of these same molecules are also ligands of AHR [320]. We therefore hypothesized that the decreased *Cyp1a2* expression we observed during endotoxemia would increase the availability of AHR ligands to control the pro-inflammatory response by peritoneal macrophages and protect against endotoxin-induced immunopathology. To test this hypothesis, we used mice lacking CYP1A2 and predicted these mice would be resistant to endotoxin administration. Intriguingly, we found the opposite result: *Cyp1a2*$^{-/-}$ mice were more susceptible to endotoxemia than wild-type 129S mice. Since both AHR- and *Cyp1a2*$^{-/-}$ mice are susceptible to endotoxemia, our preliminary results indicate that AHR and CYP1A2 may not work in a linear

axis as we predicted, however further work is required to support this conclusion. In particular, verifying that the loss of *Cyp1a2* does impair liver MROD activity and elevates serum AHR ligand levels should be investigated. One possibility is that a compensatory mechanism has occurred in these mice through the up-regulation of *Cyp1a1* and/or *Cyp1b1*. If so, the *Cyp1a1*/*Cyp1a2/Cyp1b1* triple knockout mice could be used as an alternative approach [429]. Nevertheless, we are currently working on elucidating the mechanism of how *Cyp1a2* contributes to protection from endotoxemia and are testing two hypotheses.

The first hypothesis to explain the increased susceptibility of *Cyp1a2*$^{-/-}$ mice is that elevated basal levels of AHR ligands accumulate in the absence of *Cyp1a2* leading to a state of AHR unresponsiveness and loss of the immune-regulatory effects of AHR during endotoxemia. We will first have to confirm that AHR ligands are indeed elevated in *Cyp1a2*$^{-/-}$ mice in the steady state and following LPS challenge. If correct, then we would have to assess the immune response in these mice by quantifying the serum cytokine levels, phenotyping the peritoneal macrophages, and assessing the damage and inflammation in the lung, liver, and kidney using histology. If our hypothesis is correct, *Cyp1a2*$^{-/-}$ mice should have a hyper-inflammatory phenotype and increased immunopathology similar to *Ahr*$^{-/-}$ mice. To directly test if *Cyp1a2*$^{-/-}$ mice are unresponsive to AHR stimulation, we would harvest peritoneal macrophages from naïve WT or *Cyp1a2*$^{-/-}$ mice and stimulate them *in vitro* with FICZ and measure induction of *Cyp1a1*, *Cyp1b1*, and *Ahrr* by RT-qPCR. In addition, we would measure AHR expression in these cells using western blot as AHR activation leads to its degradation [308, 309].

Our second hypothesis is that, instead of metabolizing AHR ligands, CYP1A2 is required to clear other toxins in the blood that cause organ damage and failure, irrespective of AHR and inflammation. Similar to our first hypothesis, we would assess the immune response,

immunopathology and serum AHR ligand levels during endotoxemia. Lastly, we would like to assess if *Cyp1a2* is required for the establishment of endotoxemia. If our hypothesis is true, and CYP1A2 protects against endotoxemia independently of the AHR and the macrophage pro-inflammatory response, it should not participate in endotoxin tolerance, and primed and naïve *Cyp1a2*$^{-/-}$ mice should be equally susceptible to endotoxemia because toxin build-up in these mice would be similar.

In summary, the work in this chapter suggest that CYP1A2 and AHR are not in a linear pathway during the host response to endotoxemia, as initially hypothesized. However, further work is required to determine the protective mechanism of CYP1A2 to endotoxemia and explore its role in endotoxin tolerance.

4.6 Acknowledgements

We thank members of the Madrenas and King laboratories for thoughtful feedback on the manuscript. We also would like to thank Dr. Robert Zamboni (McGill University) for generating FICZ, and Dr. Frank Gonzalez (NCI) for the *Ahr*$^{-/-}$ and *Cyp1a2*$^{-/-}$ mice.

4.7 Figures

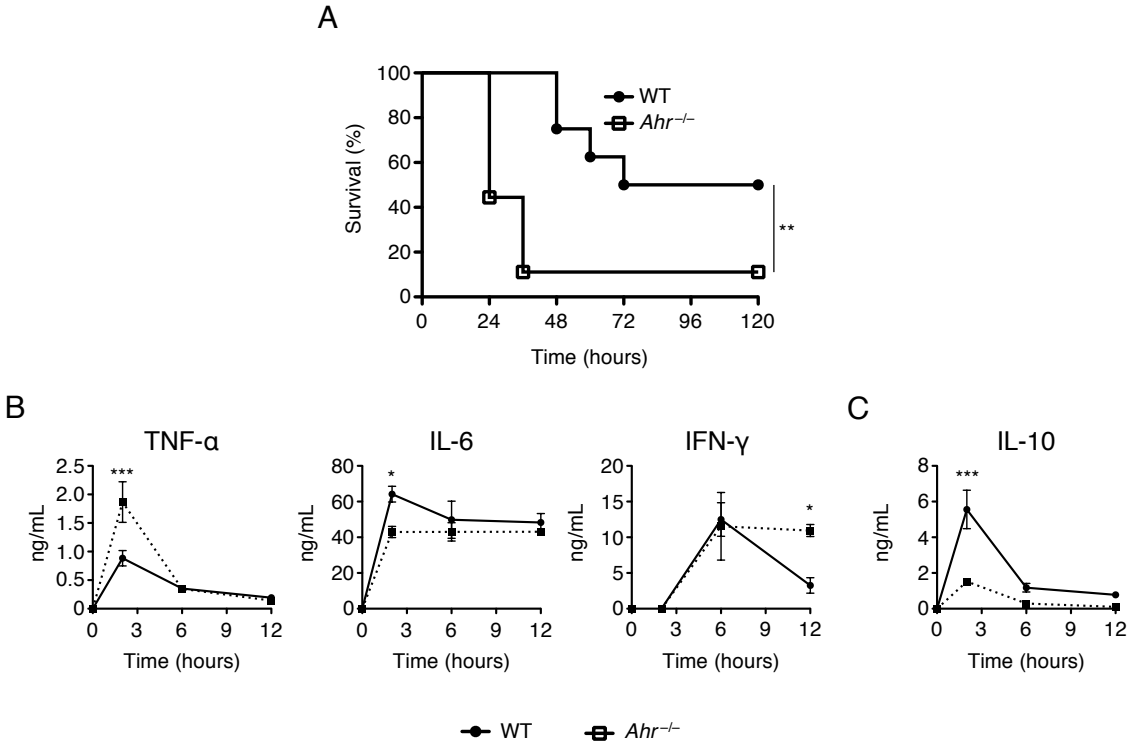


Figure 4.1. AHR-deficient mice are more susceptible to primary endotoxemia. (A) Kaplan-Meier survival curve of wild-type and *Ahr*$^{-/-}$ C57BL/6 mice treated with LPS (7.5 mg/kg). Data are representative of two independent experiments (n=5-7 mice per group per experiment). (B,C) Serum levels of pro-inflammatory cytokines (B) or IL-10 (C) from mice treated as in (A). Data are plotted as mean ± S.E.M. and representative of two independent experiments (n=3 mice per group per experiment). Statistics was performed using a one-way ANOVA with a Bonferonni's post-hoc test. *P < 0.05, *** P < 0.001

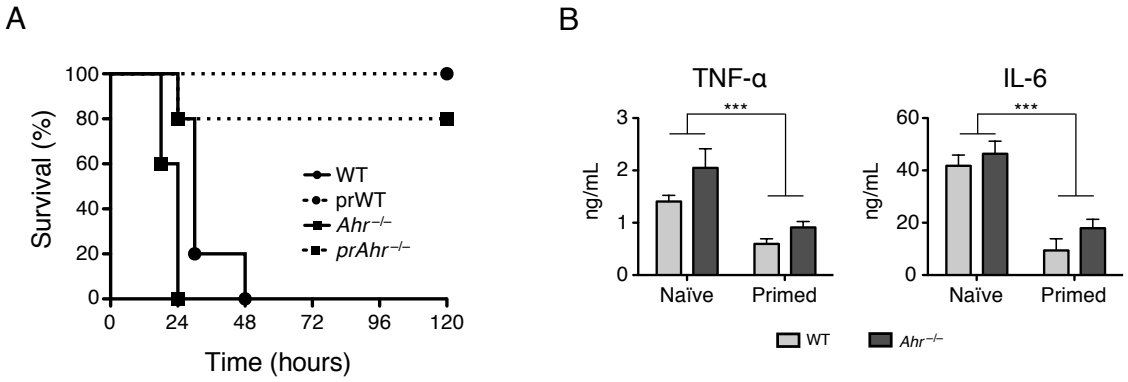


Figure 4.2. AHR is not required for the establishment of endotoxin tolerance. C57BL/6 wild-type or *Ahr*$^{-/-}$ mice (n=5 mice per group per experiment) were primed with PBS or a sublethal dose of LPS (0.5 mg/kg), and 7 days later treated with a lethal LPS dose (15 mg/kg). (A) Kaplan-Meier survival curve of mice. Data are representative of two independent experiments. (B) Serum TNF-α and IL-6 levels at 3 hours after second LPS administration. Data are representative of three independent experiments (n=3-4 mice per group per experiment). Statistics was performed using a one-way ANOVA with a Bonferonni's post-hoc test. *** $P < 0.001$

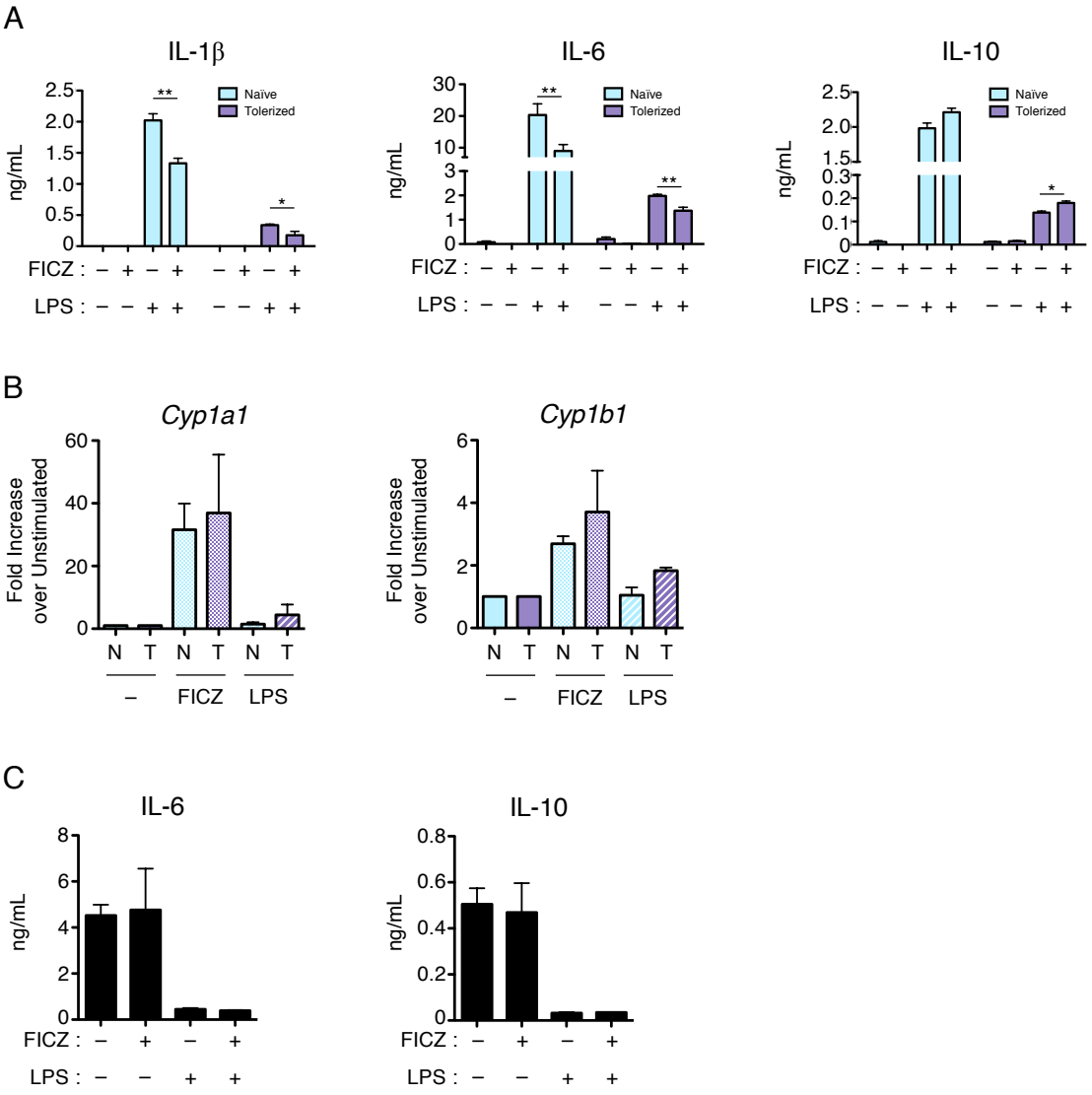


Figure 4.3. AHR activation does not enhance endotoxin tolerance in human monocytes. (A) Regulation of cytokine production to LPS by FICZ in naïve and tolerized human monocytes generated as described in the Materials & Methods section. IL-1β IL-6 and IL-10 accumulation in the supernatants after 18 hours was measured by ELISA. (B) Induction of *Cyp1a1* and *Cyp1b1* by FICZ in naïve (N) and tolerized (T) human monocytes as measured by RT-qPCR. (C) Monocytes were treated with LPS, FICZ, or both for 6 hours, then rested for 18 hours, and subsequently re-stimulated with LPS. IL-6 and IL-10 accumulation in the supernatants after 18 hours was measured by ELISA. In all graphs, x-axis indicates primary treatment of the monocytes. All data are representative of at 2-3 independent experiments from 2-3 different donors. *P < 0.05, ** P < 0. 01, *** P < 0.001

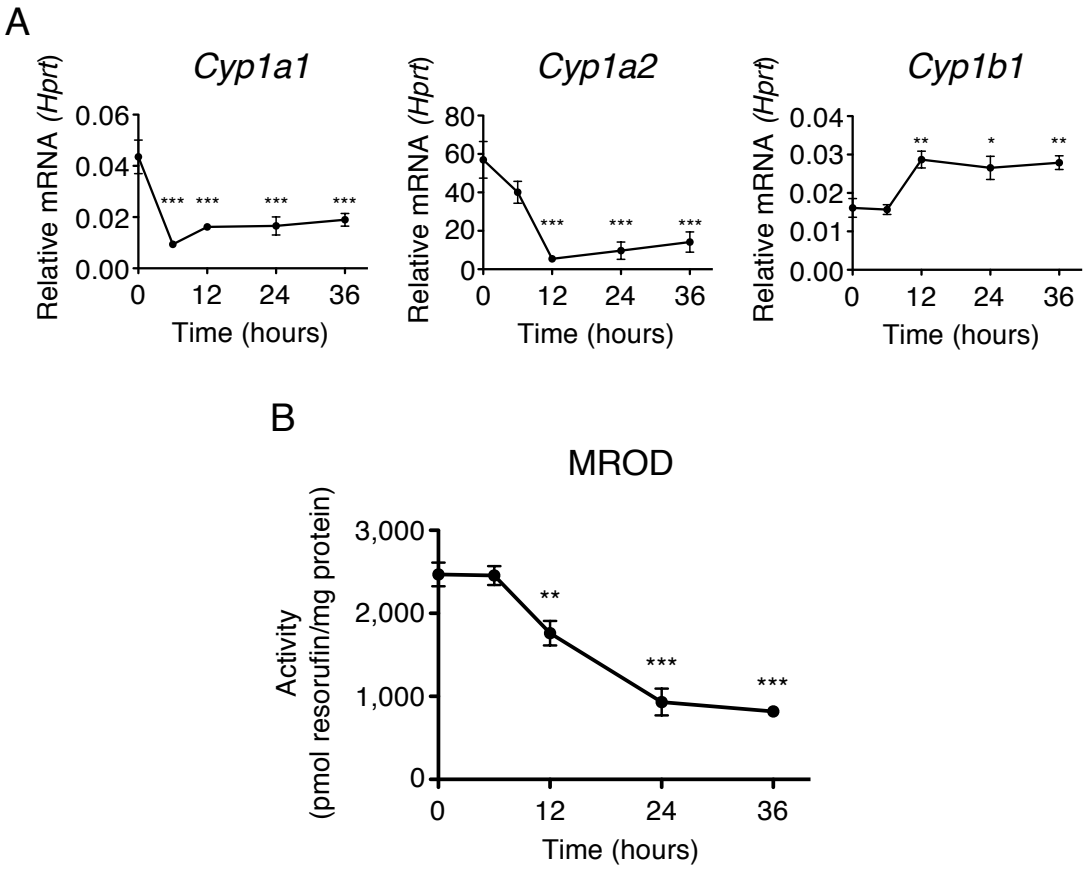


Figure 4.4. Dysregulation of liver *Cyp1* family expression during murine endotoxemia. C57BL/6 wild-type mice were treated with LPS (5 mg/kg) and liver (A) *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* mRNA, or (B) microsomal MROD activity was assessed 0, 6, 12, 24, or 36 hours later. Data are plotted as mean ± S.E.M. and representative of 2 independent experiments (n=6-8 mice per group per experiment). Statistics is in comparison to $t = 0$ and was performed using a one-way ANOVA with a Bonferonni's post-hoc test. *P < 0.05, ** P < 0.01, *** P < 0.001

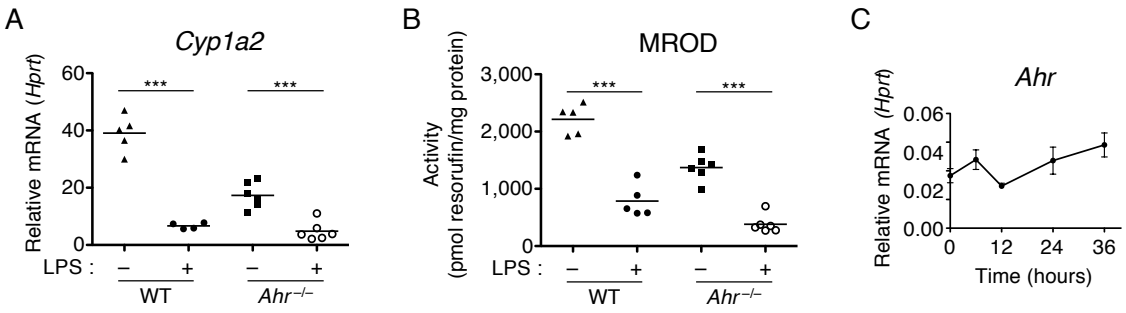


Figure 4.5. Down-regulation of liver *Cyp1a2* is not dependent on AHR. (*A,B*) C57BL/6 wild-type or *Ahr*$^{-/-}$ mice were treated with LPS (5 mg/kg) for 24 hours and liver (*A*) *Cyp1a2* mRNA, or (*B*) microsomal MROD activity was quantified. Data are plotted as mean ± S.E.M. and representative of 2 independent experiments (n=4-6 mice per group per experiment). (*C*) Liver *Ahr* expression in wild-type C57BL/6 mice treated with LPS (5 mg/kg) for 0-36 hours. Data are plotted as mean ± S.E.M. and representative of 2 independent experiments (n=6-8 mice per group per experiment). Statistics was performed using a one-way ANOVA with a Bonferonni's post-hoc test. *P < 0.05, ** P < 0.01, *** P < 0.001

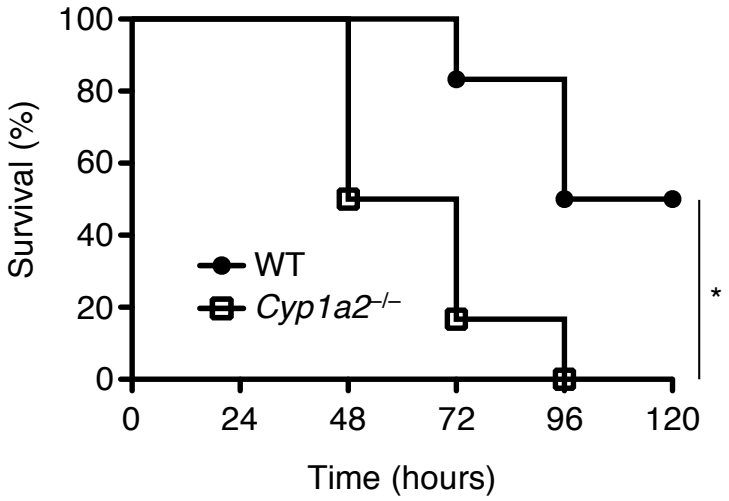


Figure 4.6. CYP1A2-deficient mice are more susceptible to primary endotoxemia than wild-type mice. Kaplan-Meier survival curve of 129S wild-type or *Cyp1a2*$^{-/-}$ mice treated with LPS (30 mg/kg). Data are representative of two independent experiments (n=4-6 mice per group per experiment). *$P < 0.05$

Chapter 5 : Conclusion and Future Directions

The immune system has evolved multiple mechanisms that aim to eliminate infection and combat the development of disease. In parallel to these critical effector mechanisms, a network of regulatory mechanisms has evolved to prevent exacerbated and potentially damaging responses leading to immunopathology. The work in this thesis describes two such immune-regulatory mechanisms: 1) Chapter 2 described the mechanism of IL-10 induction by natural-occurring isolates of *S. aureus*, and 2) Chapters 3 and 4 detailed the intrinsic and extrinsic effects of the CYP1-AHR axis in regulating monocyte pro-inflammatory functions. This chapter will discuss the implications and limitations of this work, and how it can be expanded into future research avenues.

S. aureus is both part of the normal human microbiome and a lethal pathogen [3]. Interestingly, there is no difference in the clonal origin of *S. aureus* isolates from carriers or patients with invasive staphylococcal infections [430], indicating that the same strain of *S. aureus* can cause both severe infection and asymptomatically colonize. For example, USA300 was found in over 80% of MRSA-colonized noses [431], but has also caused epidemics of skin and soft tissue infections in the USA, Canada, and Europe [432]. The duality in the human-*S. aureus* relationship indicates a constantly evolving interaction between the microbe and host. We hypothesize that one mechanism central to this evolving host-microbe interaction is IL-10. Using human PBMCs, we found that nasal *S. aureus* isolates induce a robust IL-10 response in monocytes. This response requires TLR2 [80] and activation of the PI3K-Akt-mTOR pathway [1, 160]. Although the identification of these PGN-embedded, IL-10-inducing molecule(s) is unknown, we predict that they are being constantly selected to promote a symbiotic interaction between *S. aureus* and the human immune system [433]. The IL-10 response to these ligands would generate an immune regulatory environment in the nose to promote *S. aureus* asymptomatic colonization.

One interesting future avenue that stems from this work is the involvement of AHR in the nasal colonization by *S. aureus*. It is known that bacterial species of the intestinal microbiota can produce AHR ligands that contribute to gut homeostasis [272, 434], but if a similar occurrence happens in the nose has not yet been explored. AHR-deficient mice do have a different skin microbiota composition compared to wild-type littermate controls [435], including a higher abundance of species of the *Corynebacterium* genus, which is a component of the normal human nasal microbiome. *Corynebacterium* species compete with *S. aureus* for nasal colonization and have been used as an effective strategy to eradicate *S. aureus* in chronic nasal carriers [107]. AHR may thus promote *S. aureus* colonization indirectly by decreasing the presence of *Corynebacterium* in the nose. Therefore, if AHR contributes to the colonization of *S. aureus* in the nose, through either direct or indirect mechanisms, should be investigated.

Our work on both IL-10 and AHR have important implications for the development of sepsis-induced immunosuppression and, in particular, the function of monocytes during this phase (**Figure 5.1**). Our results from the 16 nasal *S. aureus* isolates suggest that strains of this bacterium may cause varying degrees of sepsis-induced immunosuppression depending on their IL-10-inducting capacity. Since IL-10 is partially responsible for the establishment of endotoxin tolerance in monocytes *in vitro* [229], we would predict that high IL-10-inducing *S. aureus* strains would cause a more severe state of sepsis-induced immunosuppression, and would be associated with worse outcomes from sepsis. In support of this prediction, patients with elevated IL-10 serum levels at the time of hospitalization with staphylococcal bacteremia are at a higher risk of mortality [8, 168, 169]. Furthermore, IL-10 is a predictor of mortality from sepsis [200], and an *Il10* single nucleotide polymorphism (SNP) associated with higher IL-10 production correlated with higher severity of worse pneumococcal septic shock [436].

AHR, however, is likely not involved in the development of monocyte immunosuppression during sepsis *per se*, as we did not observe a function for it in inducing endotoxin tolerance in either human monocytes or mice. However, AHR did suppress the pro-inflammatory cytokine production in both naïve and tolerized monocytes. We also found that AHR ligands were able to up-regulate member of the AHR gene battery in tolerized monocytes *in vitro*, indicating that AHR is still operational in these cells. Therefore, AHR would likely be beneficial during the early inflammatory phase of sepsis by limiting the excessive pro-inflammatory cytokine production, but would also exacerbate the refractory phenotype in monocytes by further suppressing pro-inflammatory cytokine production (**Figure 5.1**). Indeed, there is a correlation between serum Kyn levels, a proposed AHR ligand [232], and the severity and mortality of sepsis [437, 438]. Whether Kyn signals through AHR during human sepsis still needs to be verified. Moreover, the impact of CYP1 on the availability of AHR ligands during sepsis is a critical future avenue of this thesis, and a comparison of the contribution of CYP1 expression from myeloid cells and hepatocytes should be addressed specifically.

The identification of the immunosuppressive phase of sepsis and the understanding of the immune-regulatory mechanisms that drive it has also led to a re-evaluation of how to treat septic patients. Historically, most treatment strategies have focused on controlling the early hyper-inflammatory phase, however, over 100 trials targeting the mediators of this phase have failed [439]. These trials have targeted multiple aspects of the sepsis-associated inflammatory response, including TLRs, CD14, and inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α), but it remains unknown why these trials have continuously failed. One possibility is that our fundamental understanding of the drivers of pathology during sepsis is incomplete. Although valuable, mouse models of sepsis are imperfect and may lack some of the key components of the human disease

[440]. Another possibility is that the window of opportunity to interrupt the action of inflammatory mediators during sepsis has passed by the time of diagnosis and initiation of treatment. As such, the cytokine storm is already well on its way to causing tissue immunopathology. It therefore may be required to look at late stage effector molecules downstream of the early inflammatory response. Lastly, redundancy in many of these inflammatory mechanisms makes it challenging to design a one-drug solution for treating sepsis.

The failure of drugs designed against the inflammatory mediators of sepsis has also led to an alternative approach to treating sepsis by targeting the immunosuppressive phase [441]. For example, GM-CSF and IFNγ can revert the immunoparalysis of tolerized monocytes [442, 443] and would provide an effective way to jump-start the immune response during sepsis-induced immunosuppression. Small clinical studies have deemed these cytokines as safe for use [444-446] and there is currently an ongoing multi-center double-blind phase 3 trial to examine the efficacy of GM-CSF to treat severe sepsis [447]. In addition to re-activating immunoparalyzed sepsis monocytes, targeting the immune regulatory mechanisms, including IL-10 production and AHR signaling, may be a complementary approach. Targeting these molecules may prove to be a similarly successful approach or, at the very least, make the biologics more effective. Indeed, AHR blocks STAT1 and STAT5 [327, 392], key components of IFNγ and GM-CSF receptor signaling, respectively, and IL-10 blocks macrophages activation by IFNγ [448]. Therefore, treating sepsis with GM-CSF and/or IFNγ in combination with drugs that block the AHR or IL-10 pathways may provide a successful strategy to treat sepsis.

Mechanistically, the induction of IL-10 and regulation of *Cyp1a1* requires different PRR signaling pathways. We have previously reported that IL-10 production in response to the cell wall of *S. aureus* was dependent on PI3K-Akt signaling [160]. This finding was corroborated in nasal

isolates of *S. aureus* and we showed that phosphorylation of Akt in monocytes correlates with an isolates capacity to induce IL-10 production. Moreover, inhibition of the PI3K-Akt-mTOR pathway with either wortmannin or rapamycin, or the ERK pathway with PD-98059, significantly reduced IL-10 induction by nasal *S. aureus* isolates. The identification of the PI3K-Akt-mTOR pathway in the IL-10 response to *S. aureus* also opens up the possibility for therapeutic interventions to invasive diseases caused by this bacterium, and drugs targeting this pathway are already approved for use in other diseases [449, 450]. However, mTOR is used by every cell for multiple different reasons and unwanted consequences may occur. For example, administration of the mTOR inhibitor metformin to mice increased mortality to sepsis caused by *Candida albicans*, likely by preventing an adaptive T cell response [228]. As such, to properly exploit this pathway for therapeutic intervention, delivery methods that selectively target monocytes (e.g. liposomes) should be employed.

On the other hand, the PI3K-Akt-mTOR, p38 and ERK pathways were all dispensable for down-regulation of *Cyp1a1* and *Cyp1b1* in human monocytes. Although we were unable to definitively determine the PRR signaling pathway required for this down-regulation, another group has reported that NF-κB p65 blocks *Cyp1a1* induction by 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in hepatocyte cell lines [406], and we predict a similar mechanism in human monocytes. Unfortunately, in our hands, the NF-κB inhibitor BAY 11-7082 completely abrogated the up-regulation of *Cyp1a1* to FICZ alone and we were therefore unable to properly assess the involvement of NF-κB. Interestingly, deletion of *Rela*, the gene encoding the NF-κB p65 subunit, in mouse embryonic fibroblasts also impaired *Cyp1a1* induction by TCDD by reducing the protein levels of AHR [414]. Collectively, these results reveal a complex interaction between AHR and NF-κB, whereby NF-κB may be both required for *Cyp1a1* induction by AHR ligands and

suppression by PAMPs. This highlights the necessity for further work to explain the dichotomy in these outcomes.

One of the most striking observation in this thesis is that CYP1 activity following AHR activation was restricted to GM-MDMs and not observed in primary monocytes or M-MDMs (**Figure 3.4**). The former macrophages display a greater "pro-inflammatory" phenotype than monocytes and M-MDMs [402], but what human tissue-resident macrophages do these cells resemble, and under what conditions and diseases do they arise, is unknown. Moreover, the re-shaping of the macrophage biology field over the past decade following the identification of bone marrow- and embryonic-derived macrophages with distinct phenotypes and functions further compounds this issue [21]. For example, GM-CSF is required for the development of self-maintaining alveolar macrophages during fetal development [451]. Alveolar macrophages are only mildly inflammatory and often involved in tissue repair mechanisms [452], and thus, despite their dependence on GM-CSF, more similarly resemble M-MDMs. Therefore, GM-CSF may still enable macrophages to express CYP1 protein independently of a programmed pro-inflammatory phenotype. To fully put these findings into context, a complete phenotyping of human tissue macrophages (e.g. alveolar macrophages, Kupffer cells, microglia, osteoblasts, etc.) during homeostasis and disease is required.

The tissue microenvironment of a macrophage may also influence its AHR responsiveness and CYP1 expression. Indeed, although AHR controls the pro-inflammatory response in peritoneal macrophages [344], it enhances the alveolar macrophage response during pulmonary infections [275], and this is likely due to environmental factors that control the functions of these cells. Similarly, macrophages may also receive cues from the tissue environment that enables or blocks the expression of the CYP1 family. It is intriguing to speculate that, given the importance of AHR

at barrier sites and as a link between the environment and the immune system [326], macrophages found at barrier sites (e.g. alveolar macrophages) would have higher CYP1 expression than those found in visceral organs (e.g. Kupffer cells). Changes in the tissue environment during an infection or injury may also modulate CYP1 expression in a spatial or temporal fashion.

Dendritic cells are innate immune cell that can also be regulated by AHR [283] and can be derived from monocytes [413]. It would therefore be interesting to test if DC are capable of displaying CYP1A1 activity and if it is regulated by PAMPs. In contrast to our results with monocytes and macrophages, LPS stimulation of human moDCs enhanced the expression of *Cyp1a1* mRNA induced by FICZ or TCDD [346]. This up-regulation was attributed to an increase in *Ahr* expression by NF-κB p65. However, we did not observe a change in *Ahr* expression during the differentiation or LPS activation of M-MDMs or GM-MDMs (Peres & Madrenas, unpublished data 2017). Collectively, these results indicate a fundamental difference in the regulation of *Ahr* and *Cyp1a1* in human macrophages and DCs that should be explored further.

A single cell analysis recently revealed that murine bone marrow differentiated with GM-CSF, a classic protocol to obtain bone marrow-derived DCs, actually produces a heterogeneous population of macrophages and DCs of equal proportions [453]. These two populations differed in their response to LPS and the ability to present antigen to naïve T cells. A similar assessment has not been performed on GM-CSF-cultured human monocytes, but given that other cytokine cocktails can produce heterogeneous macrophage and DC cultures [349], such an analysis is warranted. At the very least, at the population level, GM-MDMs do transcriptionally resemble macrophages [402]. If there is DC contamination in our GM-MDM cultures, they are unlikely to be responsible for the decrease CYP1A1 activity we observed because moDCs, differentiated with GM-CSF plus IL-4, had increased *Cyp1a1* expression following LPS stimulation [346].

An important observation raised by Chapters 3 & 4 is the necessity of limiting AHR activation. AHR is negatively regulated by three distinct mechanisms [320], each of which are operational in a cell-specific manner. For example, CYP1 activity is highest in non-hematopoietic cells and, even in GM-MDMs, the CYP1 activity seen in leukocytes is substantially lower than what is observed in the former cell population [454]. We therefore propose a model whereby AHR activation in leukocytes is principally regulated by intrinsic mechanisms (e.g. AHR proteasome degradation and/or blocking of AHR:ARNT dimerization by AHRR), while the availability of AHR ligands is controlled by the structural cells of the tissue through CYP1 family expression. Such mechanisms may have evolved as a means of communication to ensure a coordinated immune response in the local environment, and would also generate a gradient of AHR ligands that could sequentially modulate leukocyte function as they migrate into the inflamed tissue. This hypothesis is supported by the work of another group studying the murine gut. Even though Th17 cells possess CYP1A1 activity in culture, the availability and depletion of AHR ligands in the murine gut was controlled by the intestinal epithelial cells [274]. Whether CYP1 function is modulated during inflammation at other barrier sites where AHR also influences the immune response, such as the skin [291] and lung [275], should also be investigated.

In conclusion, the work presented here advances our understanding of the immune regulatory mechanisms orchestrated and/or mediated by monocytes and monocyte-derived macrophages, especially in the context of *S. aureus* pathobiosis and sepsis-induced immunosuppression. Through the coordination of these mechanisms, TLR2 ligands embedded in the cell wall of *S. aureus* may contribute to the establishment of a balance between commensalism and pathogenicity of this microbe. This contribution would be mediated by a predominant IL-10 response and the development of an immune regulatory environment. Such a mechanism could be

complemented by the effect of AHR ligands on controlling monocyte differentiation and function during sepsis.

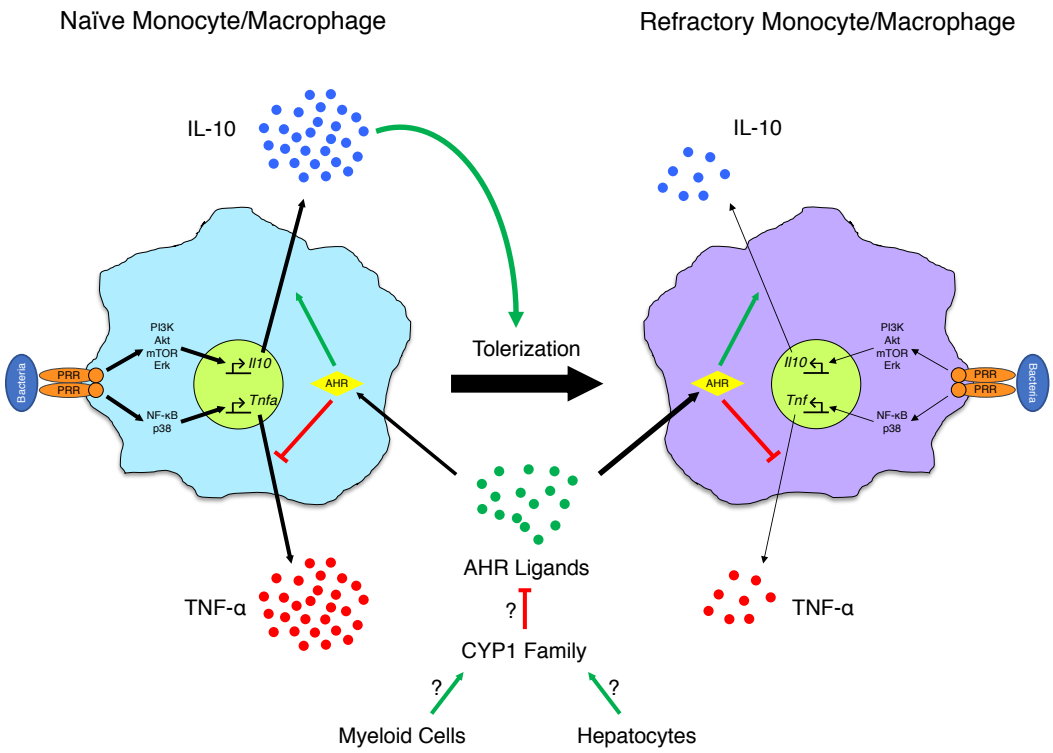


Figure 5.1. Proposed mechanism of IL-10 and AHR in the immune regulation of monocytes/macrophages during sepsis. Recognition of pathogens by PRRs on monocytes or macrophages leads to activation of distinct signaling pathways that drive the production of pro-inflammatory cytokines (exemplified by TNF-α in this figure) or the anti-inflammatory cytokine IL-10. The magnitude of the IL-10 response would dictate, in part, the degree of unresponsiveness of refractory monocytes/macrophages during sepsis. In addition, AHR activation would suppress TNF-α and promote IL-10 production in both naïve and refractory monocytes/macrophages, thereby exacerbating sepsis-induced immunosuppression. However, AHR is not directly involved in the development of refractory monocytes/macrophages. The availability of AHR ligands may be controlled by CYP1 family members expressed by hepatocytes or myeloid cells.

References

1. Peres, A.G., et al., *Uncoupling of pro- and anti-inflammatory properties of Staphylococcus aureus.* Infect Immun, 2015. **83**(4): p. 1587-97.

2. Peres, A.G., et al., *Suppression of CYP1 members of the AHR response by pathogen-associated molecular patterns.* J Leukoc Biol, 2017. **102**(6): p. 1471-1480.

3. Peres, A.G. and J. Madrenas, *The broad landscape of immune interactions with Staphylococcus aureus: from commensalism to lethal infections.* Burns, 2013. **39**(3): p. 380-8.

4. Iwasaki, A. and R. Medzhitov, *Regulation of adaptive immunity by the innate immune system.* Science, 2010. **327**(5963): p. 291-5.

5. Kamada, N., et al., *Control of pathogens and pathobionts by the gut microbiota.* Nat Immunol, 2013. **14**(7): p. 685-90.

6. Medzhitov, R., D.S. Schneider, and M.P. Soares, *Disease tolerance as a defense strategy.* Science, 2012. **335**(6071): p. 936-41.

7. Boomer, J.S., et al., *Immunosuppression in patients who die of sepsis and multiple organ failure.* JAMA, 2011. **306**(23): p. 2594-605.

8. Rose, W.E., et al., *Elevated serum interleukin-10 at time of hospital admission is predictive of mortality in patients with Staphylococcus aureus bacteremia.* J Infect Dis, 2012. **206**(10): p. 1604-11.

9. Rabinovich, G.A., D. Gabrilovich, and E.M. Sotomayor, *Immunosuppressive strategies that are mediated by tumor cells.* Annu Rev Immunol, 2007. **25**: p. 267-96.

10. Eberl, G., *Immunity by equilibrium.* Nat Rev Immunol, 2016. **16**(8): p. 524-32.

11. van Furth, R. and Z.A. Cohn, *The origin and kinetics of mononuclear phagocytes.* J Exp Med, 1968. **128**(3): p. 415-35.

12. Ginhoux, F. and S. Jung, *Monocytes and macrophages: developmental pathways and tissue homeostasis.* Nat Rev Immunol, 2014. **14**(6): p. 392-404.

13. Geissmann, F., et al., *Development of monocytes, macrophages, and dendritic cells.* Science, 2010. **327**(5966): p. 656-61.

14. Ziegler-Heitbrock, L., *Blood Monocytes and Their Subsets: Established Features and Open Questions.* Front Immunol, 2015. **6**: p. 423.

15. Serbina, N.V., et al., *Monocyte-mediated defense against microbial pathogens.* Annu Rev Immunol, 2008. **26**: p. 421-52.

16. Thomas, G., et al., *Nonclassical patrolling monocyte function in the vasculature.* Arterioscler Thromb Vasc Biol, 2015. **35**(6): p. 1306-16.

17. Sunderkotter, C., et al., *Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response.* J Immunol, 2004. **172**(7): p. 4410-7.

18. Shi, C. and E.G. Pamer, *Monocyte recruitment during infection and inflammation.* Nat Rev Immunol, 2011. **11**(11): p. 762-74.

19. Geissmann, F., S. Jung, and D.R. Littman, *Blood monocytes consist of two principal subsets with distinct migratory properties.* Immunity, 2003. **19**(1): p. 71-82.

20. Bogunovic, M., et al., *Origin of the lamina propria dendritic cell network.* Immunity, 2009. **31**(3): p. 513-25.

21. Varol, C., A. Mildner, and S. Jung, *Macrophages: development and tissue specialization.* Annu Rev Immunol, 2015. **33**: p. 643-75.

22. Alliot, F., I. Godin, and B. Pessac, *Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain.* Brain Res Dev Brain Res, 1999. **117**(2): p. 145-52.

23. Ginhoux, F., et al., *Fate mapping analysis reveals that adult microglia derive from primitive macrophages.* Science, 2010. **330**(6005): p. 841-5.

24. Schulz, C., et al., *A lineage of myeloid cells independent of Myb and hematopoietic stem cells.* Science, 2012. **336**(6077): p. 86-90.

25. Hoeffel, G., et al., *Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages.* J Exp Med, 2012. **209**(6): p. 1167-81.

26. Lavin, Y., et al., *Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment.* Cell, 2014. **159**: p. 1312-26.

27. Gosselin, D., et al., *Environment Drives Selection and Function of Enhancers Controlling Tissue-Specific Macrophage Identity.* Cell, 2014. **159**(1327-40).

28. Roberts, A.W., et al., *Tissue-Resident Macrophages Are Locally Programmed for Silent Clearance of Apoptotic Cells.* Immunity, 2017. **47**(5): p. 913-927 e6.

29. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.

30. Plato, A., S.E. Hardison, and G.D. Brown, *Pattern recognition receptors in antifungal immunity.* Semin Immunopathol, 2015. **37**(2): p. 97-106.

31. Loo, Y.M. and M. Gale, Jr., *Immune signaling by RIG-I-like receptors.* Immunity, 2011. **34**(5): p. 680-92.

32. Guo, H., J.B. Callaway, and J.P. Ting, *Inflammasomes: mechanism of action, role in disease, and therapeutics.* Nat Med, 2015. **21**(7): p. 677-87.

33. Alexander, C. and E.T. Rietschel, *Bacterial lipopolysaccharides and innate immunity.* J Endotoxin Res, 2001. **7**(3): p. 167-202.

34. Steimle, A., I.B. Autenrieth, and J.S. Frick, *Structure and function: Lipid A modifications in commensals and pathogens.* Int J Med Microbiol, 2016. **306**(5): p. 290-301.

35. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.

36. Maeshima, N. and R.C. Fernandez, *Recognition of lipid A variants by the TLR4-MD-2 receptor complex.* Front Cell Infect Microbiol, 2013. **3**: p. 3.

37. Miyake, K., *Roles for accessory molecules in microbial recognition by Toll-like receptors.* J Endotoxin Res, 2006. **12**(4): p. 195-204.

38. Shimazu, R., et al., *MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4.* J Exp Med, 1999. **189**(11): p. 1777-82.

39. Park, B.S., et al., *The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex.* Nature, 2009. **458**(7242): p. 1191-5.

40. Park, B.S. and J.O. Lee, *Recognition of lipopolysaccharide pattern by TLR4 complexes.* Exp Mol Med, 2013. **45**: p. e66.

41. Bell, J.K., et al., *Leucine-rich repeats and pathogen recognition in Toll-like receptors.* Trends Immunol, 2003. **24**(10): p. 528-33.

42. Botos, I., D.M. Segal, and D.R. Davies, *The structural biology of Toll-like receptors.* Structure, 2011. **19**(4): p. 447-59.

43. Jin, M.S., et al., *Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide.* Cell, 2007. **130**(6): p. 1071-82.

44. Kang, J.Y., et al., *Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer.* Immunity, 2009. **31**(6): p. 873-84.

45. Guan, Y., et al., *Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling.* J Immunol, 2010. **184**(9): p. 5094-103.

46. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.* Science, 1998. **282**(5396): p. 2085-8.

47. Qureshi, S.T., et al., *Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4).* J Exp Med, 1999. **189**(4): p. 615-25.

48. Medzhitov, R., et al., *MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways.* Mol Cell, 1998. **2**(2): p. 253-8.

49. Kawai, T., et al., *Unresponsiveness of MyD88-deficient mice to endotoxin.* Immunity, 1999. **11**(1): p. 115-22.

50. Fitzgerald, K.A., et al., *Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction.* Nature, 2001. **413**(6851): p. 78-83.

51. Horng, T., G.M. Barton, and R. Medzhitov, *TIRAP: an adapter molecule in the Toll signaling pathway.* Nat Immunol, 2001. **2**(9): p. 835-41.

52. Horng, T., et al., *The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors.* Nature, 2002. **420**(6913): p. 329-33.

53. Yamamoto, M., et al., *Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4.* Nature, 2002. **420**(6913): p. 324-9.

54. Cao, Z., W.J. Henzel, and X. Gao, *IRAK: a kinase associated with the interleukin-1 receptor.* Science, 1996. **271**(5252): p. 1128-31.

55. Muzio, M., et al., *IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling.* Science, 1997. **278**(5343): p. 1612-5.

56. Li, S., et al., *IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase.* Proc Natl Acad Sci U S A, 2002. **99**(8): p. 5567-72.

57. Cao, Z., et al., *TRAF6 is a signal transducer for interleukin-1.* Nature, 1996. **383**(6599): p. 443-6.

58. Irie, T., T. Muta, and K. Takeshige, *TAK1 mediates an activation signal from toll-like receptor(s) to nuclear factor-kappaB in lipopolysaccharide-stimulated macrophages.* FEBS Lett, 2000. **467**(2-3): p. 160-4.

59. Zhang, Q., M.J. Lenardo, and D. Baltimore, *30 Years of NF-kappaB: A Blossoming of Relevance to Human Pathobiology.* Cell, 2017. **168**(1-2): p. 37-57.

60. Yamamoto, M., et al., *Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway.* Science, 2003. **301**(5633): p. 640-3.

61. Hoebe, K., et al., *Identification of Lps2 as a key transducer of MyD88-independent TIR signalling.* Nature, 2003. **424**(6950): p. 743-8.

62. Zanoni, I., et al., *CD14 controls the LPS-induced endocytosis of Toll-like receptor 4.* Cell, 2011. **147**(4): p. 868-80.

63. Yamamoto, M., et al., *TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway.* Nat Immunol, 2003. **4**(11): p. 1144-50.

64. Oganesyan, G., et al., *Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response.* Nature, 2006. **439**(7073): p. 208-11.

65. Sato, M., et al., *Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the IFN-beta gene.* FEBS Lett, 1998. **425**(1): p. 112-6.

66. Wathelet, M.G., et al., *Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo.* Mol Cell, 1998. **1**(4): p. 507-18.

67. Doyle, S., et al., *IRF3 mediates a TLR3/TLR4-specific antiviral gene program.* Immunity, 2002. **17**(3): p. 251-63.

68. Shimada, T., et al., *IKK-i, a novel lipopolysaccharide-inducible kinase that is related to IkappaB kinases.* Int Immunol, 1999. **11**(8): p. 1357-62.

69. Fitzgerald, K.A., et al., *IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway.* Nat Immunol, 2003. **4**(5): p. 491-6.

70. Sharma, S., et al., *Triggering the interferon antiviral response through an IKK-related pathway.* Science, 2003. **300**(5622): p. 1148-51.

71. von Bernuth, H., et al., *Pyogenic bacterial infections in humans with MyD88 deficiency.* Science, 2008. **321**(5889): p. 691-696.

72. Picard, C., et al., *Clinical features and outcome of patients with IRAK-4 and MyD88 deficiency.* Medicine (Baltimore), 2010. **89**(6): p. 403-25.

73. Casrouge, A., et al., *Herpes simplex virus encephalitis in human UNC-93B deficiency.* Science, 2006. **314**(5797): p. 308-12.

74. Herman, M., et al., *Heterozygous TBK1 mutations impair TLR3 immunity and underlie herpes simplex encephalitis of childhood.* J Exp Med, 2012. **209**(9): p. 1567-82.

75. Zhang, S.Y., et al., *TLR3 deficiency in patients with herpes simplex encephalitis.* Science, 2007. **317**(5844): p. 1522-7.

76. Guo, Y., et al., *Herpes simplex virus encephalitis in a patient with complete TLR3 deficiency: TLR3 is otherwise redundant in protective immunity.* J Exp Med, 2011. **208**(10): p. 2083-98.

77. Lafaille, F.G., et al., *Impaired intrinsic immunity to HSV-1 in human iPSC-derived TLR3-deficient CNS cells.* Nature, 2012. **491**(7426): p. 769-73.

78. Yamazaki, T., et al., *Expression of programmed death 1 ligands by murine T cells and APC.* J Immunol, 2002. **169**(10): p. 5538-45.

79. Weichhart, T., et al., *The TSC-mTOR signaling pathway regulates the innate inflammatory response.* Immunity, 2008. **29**(4): p. 565-77.

80. Chau, T.A., et al., *Toll-like receptor 2 ligands on the staphylococcal cell wall downregulate superantigen-induced T cell activation and prevent toxic shock syndrome.* Nat Med, 2009. **15**(6): p. 641-8.

81. Wang, J., G. Roderiquez, and M.A. Norcross, *Control of adaptive immune responses by Staphylococcus aureus through IL-10, PD-L1, and TLR2.* Sci Rep, 2012. **2**: p. 606.

82. Mele, T. and J. Madrenas, *TLR2 signalling: At the crossroads of commensalism, invasive infections and toxic shock syndrome by Staphylococcus aureus.* Int J Biochem Cell Biol, 2010. **42**(7): p. 1066-71.

83. Freitas, E.A., et al., *Prevalence of USA300 strain type of methicillin-resistant Staphylococcus aureus among patients with nasal colonization identified with active surveillance.* Infect Control Hosp Epidemiol, 2010. **31**(5): p. 469-75.

84. Kumar, N., et al., *High Staphylococcus aureus colonization prevalence among patients with skin and soft tissue infections and controls in an urban emergency department.* J Clin Microbiol, 2015. **53**(3): p. 810-5.

85. Styers, D., et al., *Laboratory-based surveillance of current antimicrobial resistance patterns and trends among Staphylococcus aureus: 2005 status in the United States.* Ann Clin Microbiol Antimicrob, 2006. **5**: p. 2.

86. Lawrence, J.C., *Burn bacteriology during the last 50 years.* Burns, 1992. **18 Suppl 2**: p. S23-9.

87. Bang, R.L., et al., *Burn septicaemia: an analysis of 79 patients.* Burns, 1998. **24**(4): p. 354-61.

88. Bang, R.L., et al., *Septicaemia after burn injury: a comparative study.* Burns, 2002. **28**(8): p. 746-51.

89. Wang, W.K., et al., *[Analysis of the molecular epidemiology and distribution of pathogenic bacteria in burn wards of Ruijin Hospital from 2004 to 2006].* Zhonghua Shao Shang Za Zhi, 2009. **25**(2): p. 94-7.

90. Kuehnert, M.J., et al., *Methicillin-resistant-Staphylococcus aureus hospitalizations, United States.* Emerg Infect Dis, 2005. **11**(6): p. 868-72.

91. Klein, E., D.L. Smith, and R. Laxminarayan, *Hospitalizations and deaths caused by methicillin-resistant Staphylococcus aureus, United States, 1999-2005.* Emerg Infect Dis, 2007. **13**(12): p. 1840-6.

92. Wertheim, H.F., et al., *Risk and outcome of nosocomial Staphylococcus aureus bacteraemia in nasal carriers versus non-carriers.* Lancet, 2004. **364**(9435): p. 703-5.

93. Shinefield, H.R. and N.L. Ruff, *Staphylococcal infections: a historical perspective.* Infect Dis Clin North Am, 2009. **23**(1): p. 1-15.

94. Nouwen, J.L., et al., *Predicting the Staphylococcus aureus nasal carrier state: derivation and validation of a "culture rule".* Clin Infect Dis, 2004. **39**(6): p. 806-11.

95. van Belkum, A., *Staphylococcal colonization and infection: homeostasis versus disbalance of human (innate) immunity and bacterial virulence.* Curr Opin Infect Dis, 2006. **19**(4): p. 339-44.

96. Ruimy, R., et al., *Are host genetics the predominant determinant of persistent nasal Staphylococcus aureus carriage in humans?* J Infect Dis, 2010. **202**(6): p. 924-34.

97. Andersen, P.S., et al., *Influence of host genetics and environment on nasal carriage of staphylococcus aureus in danish middle-aged and elderly twins.* J Infect Dis, 2012. **206**(8): p. 1178-84.

98. Regev-Yochay, G., et al., *Association between carriage of Streptococcus pneumoniae and Staphylococcus aureus in Children.* JAMA, 2004. **292**(6): p. 716-20.

99. Lebon, A., et al., *The inverse correlation between Staphylococcus aureus and Streptococcus pneumoniae colonization in infants is not explained by differences in serum antibody levels in the Generation R Study.* Clin Vaccine Immunol, 2011. **18**(1): p. 180-3.

100. Nouwen, J.L., et al., *Persistent (not intermittent) nasal carriage of Staphylococcus aureus is the determinant of CPD-related infections.* Kidney Int, 2005. **67**(3): p. 1084-92.

101. van Belkum, A., et al., *Reclassification of Staphylococcus aureus nasal carriage types.* J Infect Dis, 2009. **199**(12): p. 1820-6.

102. Johannessen, M., J.E. Sollid, and A.M. Hanssen, *Host- and microbe determinants that may influence the success of S. aureus colonization.* Front Cell Infect Microbiol, 2012. **2**: p. 56.

103. Hoeksma, A. and K.C. Winkler, *The normal flora of the nose in twins.* Acta Leiden, 1963. **32**: p. 123-33.

104. Aly, R., et al., *Staphylococcus aureus carriage in twins.* Am J Dis Child, 1974. **127**(4): p. 486-8.

105. Weidenmaier, C., et al., *Differential roles of sortase-anchored surface proteins and wall teichoic acid in Staphylococcus aureus nasal colonization.* Int J Med Microbiol, 2008. **298**(5-6): p. 505-13.

106. Burian, M., et al., *Temporal expression of adhesion factors and activity of global regulators during establishment of Staphylococcus aureus nasal colonization.* J Infect Dis, 2010. **201**(9): p. 1414-21.

107. Uehara, Y., et al., *Bacterial interference among nasal inhabitants: eradication of Staphylococcus aureus from nasal cavities by artificial implantation of Corynebacterium sp.* J Hosp Infect, 2000. **44**(2): p. 127-33.

108. Frank, D.N., et al., *The human nasal microbiota and Staphylococcus aureus carriage.* PLoS One, 2010. **5**(5): p. e10598.

109. Olsen, K., et al., *Staphylococcus aureus nasal carriage is associated with serum 25-hydroxyvitamin D levels, gender and smoking status. The Tromso Staph and Skin Study.* Eur J Clin Microbiol Infect Dis, 2012. **31**(4): p. 465-73.

110. van Rijen, M.M., et al., *Intranasal mupirocin for reduction of Staphylococcus aureus infections in surgical patients with nasal carriage: a systematic review.* J Antimicrob Chemother, 2008. **61**(2): p. 254-61.

111. Todd, J., et al., *Toxic-shock syndrome associated with phage-group-I Staphylococci.* Lancet, 1978. **2**(8100): p. 1116-8.

112. CDC, *Follow-up on toxic-shock syndrome.* MMWR, 1980. **29**: p. 441-5.

113. Davis, J.P., et al., *Toxic-shock syndrome: epidemiologic features, recurrence, risk factors, and prevention.* N Engl J Med, 1980. **303**(25): p. 1429-35.

114. CDC, *Historical perspectives - reduced incidence of menstrual toxic-shock syndrome - United States, 1980-1990.* MMWR Morb Mortal Wkly Rep, 1990. **39**(421-3).

115. DeVries, A.S., et al., *Staphylococcal toxic shock syndrome 2000-2006: epidemiology, clinical features, and molecular characteristics.* PLoS One, 2011. **6**(8): p. e22997.

116. Descloux, E., et al., *One in five mortality in non-menstrual toxic shock syndrome versus no mortality in menstrual cases in a balanced French series of 55 cases.* Eur J Clin Microbiol Infect Dis, 2008. **27**(1): p. 37-43.

117. Childs, C., et al., *Patterns of Staphylococcus aureus colonization, toxin production, immunity and illness in burned children.* Burns, 1994. **20**(6): p. 514-21.

118. Edwards-Jones, V., M.M. Dawson, and C. Childs, *A survey into toxic shock syndrome (TSS) in UK burns units.* Burns, 2000. **26**(4): p. 323-33.

119. McCormick, J.K., J.M. Yarwood, and P.M. Schlievert, *Toxic shock syndrome and bacterial superantigens: an update.* Annu Rev Microbiol, 2001. **55**: p. 77-104.

120. Becker, K., et al., *Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of Staphylococcus aureus isolated from blood and nasal specimens.* J Clin Microbiol, 2003. **41**(4): p. 1434-9.

121. Mempel, M., et al., *High prevalence of superantigens associated with the egc locus in Staphylococcus aureus isolates from patients with atopic eczema.* Eur J Clin Microbiol Infect Dis, 2003. **22**(5): p. 306-9.

122. Nashev, D., et al., *Distribution of virulence genes of Staphylococcus aureus isolated from stable nasal carriers.* FEMS Microbiol Lett, 2004. **233**(1): p. 45-52.

123. Parsonnet, J., et al., *Prevalence of toxic shock syndrome toxin 1-producing Staphylococcus aureus and the presence of antibodies to this superantigen in menstruating women.* J Clin Microbiol, 2005. **43**(9): p. 4628-34.

124. Edwards-Jones, V., C. Childs, and H.A. Foster, *A comparison of phenotypic properties of Staphylococcus aureus isolated from burned children and other patient groups.* Burns, 1996. **22**(5): p. 384-9.

125. Fraser, J.D. and T. Proft, *The bacterial superantigen and superantigen-like proteins.* Immunol Rev, 2008. **225**: p. 226-43.

126. Mitchell, D.T., et al., *Structural evidence for the evolution of pyrogenic toxin superantigens.* J Mol Evol, 2000. **51**(6): p. 520-31.

127. Bueno, C., et al., *T cell signalling induced by bacterial superantigens.* Chem Immunol Allergy, 2007. **93**: p. 161-80.

128. Smith-Garvin, J.E., G.A. Koretzky, and M.S. Jordan, *T cell activation.* Annu Rev Immunol, 2009. **27**: p. 591-619.

129. Bueno, C., et al., *Bacterial superantigens bypass Lck-dependent T cell receptor signaling by activating a Galpha11-dependent, PLC-beta-mediated pathway.* Immunity, 2006. **25**(1): p. 67-78.

130. Trede, N.S., R.S. Geha, and T. Chatila, *Transcriptional activation of IL-1 beta and tumor necrosis factor-alpha genes by MHC class II ligands.* J Immunol, 1991. **146**(7): p. 2310-5.

131. Palkama, T. and M. Hurme, *Signal transduction mechanisms of HLA-DR-mediated interleukin-1 beta production in human monocytes. Role of protein kinase C and tyrosine kinase activation.* Hum Immunol, 1993. **36**(4): p. 259-67.

132. Kissner, T.L., et al., *Activation of MyD88 signaling upon staphylococcal enterotoxin binding to MHC class II molecules.* PLoS One, 2011. **6**(1): p. e15985.

133. Lappin, E. and A.J. Ferguson, *Gram-positive toxic shock syndromes.* Lancet Infect Dis, 2009. **9**(5): p. 281-90.

134. Murray, R.J., *Recognition and management of Staphylococcus aureus toxin-mediated disease.* Intern Med J, 2005. **35 Suppl 2**: p. S106-19.

135. Schlievert, P.M., et al., *Alpha and beta chains of hemoglobin inhibit production of Staphylococcus aureus exotoxins.* Biochemistry, 2007. **46**(50): p. 14349-58.

136. Holtfreter, S., et al., *Staphylococcus aureus carriers neutralize superantigens by antibodies specific for their colonizing strain: a potential explanation for their improved prognosis in severe sepsis.* J Infect Dis, 2006. **193**(9): p. 1275-8.

137. Adhikari, R.P., et al., *Lower antibody levels to Staphylococcus aureus exotoxins are associated with sepsis in hospitalized adults with invasive S. aureus infections.* J Infect Dis, 2012. **206**(6): p. 915-23.

138. Verkaik, N.J., et al., *Anti-staphylococcal humoral immune response in persistent nasal carriers and noncarriers of Staphylococcus aureus.* J Infect Dis, 2009. **199**(5): p. 625-32.

139. Verkaik, N.J., et al., *Induction of antibodies by Staphylococcus aureus nasal colonization in young children.* Clin Microbiol Infect, 2010. **16**(8): p. 1312-7.

140. Broker, B.M. and A. van Belkum, *Immune proteomics of Staphylococcus aureus.* Proteomics, 2011. **11**(15): p. 3221-31.

141. Burian, M., et al., *Expression of staphylococcal superantigens during nasal colonization is not sufficient to induce a systemic neutralizing antibody response in humans.* Eur J Clin Microbiol Infect Dis, 2012. **31**(3): p. 251-6.

142. Kasper, K.J., et al., *Bacterial superantigens promote acute nasopharyngeal infection by Streptococcus pyogenes in a human MHC Class II-dependent manner.* PLoS Pathog, 2014. **10**(5): p. e1004155.

143. Xu, S.X., et al., *Superantigens Modulate Bacterial Density during Staphylococcus aureus Nasal Colonization.* Toxins (Basel), 2015. **7**(5): p. 1821-36.

144. Brown, L., et al., *Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi.* Nat Rev Microbiol, 2015. **13**(10): p. 620-30.

145. Lee, C.C., A.M. Avalos, and H.L. Ploegh, *Accessory molecules for Toll-like receptors and their function.* Nat Rev Immunol, 2012. **12**(3): p. 168-79.

146. Wolf, A.J., et al., *Phagosomal degradation increases TLR access to bacterial ligands and enhances macrophage sensitivity to bacteria.* J Immunol, 2011. **187**(11): p. 6002-10.

147. McCully, M.L., et al., *Receptor-interacting protein-2 deficiency delays macrophage migration and increases intracellular infection during peritoneal dialysis-associated peritonitis.* Am J Nephrol, 2008. **28**(6): p. 879-89.

148. Lai, Y., et al., *Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury.* Nat Med, 2009. **15**(12): p. 1377-82.

149. Willems, F., et al., *Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes.* Eur J Immunol, 1994. **24**(4): p. 1007-9.

150. Buelens, C., et al., *Interleukin-10 differentially regulates B7-1 (CD80) and B7-2 (CD86) expression on human peripheral blood dendritic cells.* Eur J Immunol, 1995. **25**(9): p. 2668-72.

151. Murphey, E.D., G. Fang, and E.R. Sherwood, *Pretreatment with the Gram-positive bacterial cell wall molecule peptidoglycan improves bacterial clearance and decreases inflammation and mortality in mice challenged with Staphylococcus aureus.* Crit Care Med, 2008. **36**(11): p. 3067-73.

152. Sing, A., et al., *Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression.* J Exp Med, 2002. **196**(8): p. 1017-24.

153. Jang, S., et al., *IL-6 and IL-10 induction from dendritic cells in response to Mycobacterium tuberculosis is predominantly dependent on TLR2-mediated recognition.* J Immunol, 2004. **173**(5): p. 3392-7.

154. Guo, H., et al., *Toll-like receptor 2 mediates the induction of IL-10 in corneal fibroblasts in response to Fusarium solu.* Immunol Cell Biol, 2008. **86**(3): p. 271-6.

155. Netea, M.G., et al., *Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells.* J Immunol, 2004. **172**(6): p. 3712-8.

156. Round, J.L., et al., *The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota.* Science, 2011. **332**(6032): p. 974-7.

157. Kabelitz, D., *Expression and function of Toll-like receptors in T lymphocytes.* Curr Opin Immunol, 2007. **19**(1): p. 39-45.

158. Taylor, A.L. and M.J. Llewelyn, *Superantigen-induced proliferation of human CD4+CD25- T cells is followed by a switch to a functional regulatory phenotype.* J Immunol, 2010. **185**(11): p. 6591-8.

159. Zielinski, C.E., et al., *Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta.* Nature, 2012. **484**(7395): p. 514-8.

160. Frodermann, V., et al., *A modulatory interleukin-10 response to staphylococcal peptidoglycan prevents Th1/Th17 adaptive immunity to Staphylococcus aureus.* J Infect Dis, 2011. **204**(2): p. 253-62.

161. Chu, C.C., et al., *Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation.* J Exp Med, 2012. **209**(5): p. 935-45.

162. Goodridge, H.S., et al., *Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'.* Nature, 2011. **472**(7344): p. 471-5.

163. Kolar, S.L., et al., *Group B Streptococcus Evades Host Immunity by Degrading Hyaluronan.* Cell Host Microbe, 2015. **18**(6): p. 694-704.

164. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.

165. Santos-Sierra, S., et al., *Mal connects TLR2 to PI3Kinase activation and phagocyte polarization.* EMBO J, 2009. **28**(14): p. 2018-27.

166. Mellett, M., et al., *Mal mediates TLR-induced activation of CREB and expression of IL-10.* J Immunol, 2011. **186**(8): p. 4925-35.

167. Hontelez, S., et al., *Dendritic cell-specific transcript: dendritic cell marker and regulator of TLR-induced cytokine production.* J Immunol, 2012. **189**(1): p. 138-45.

168. Minejima, E., et al., *A Dysregulated Balance of Proinflammatory and Anti-Inflammatory Host Cytokine Response Early During Therapy Predicts Persistence and Mortality in Staphylococcus aureus Bacteremia.* Crit Care Med, 2016. **44**(4): p. 671-9.

169. Rose, W.E., et al., *Increased Endovascular Staphylococcus aureus Inoculum Is the Link Between Elevated Serum Interleukin 10 Concentrations and Mortality in Patients With Bacteremia.* Clin Infect Dis, 2017. **64**(10): p. 1406-1412.

170. Stevens, D.L., et al., *The Eagle effect revisited: efficacy of clindamycin, erythromycin, and penicillin in the treatment of streptococcal myositis.* J Infect Dis, 1988. **158**(1): p. 23-8.

171. Annane, D., B. Clair, and J. Salomon, *Managing toxic shock syndrome with antibiotics.* Expert Opin Pharmacother, 2004. **5**(8): p. 1701-10.

172. Young, M.H., et al., *Therapies for necrotising fasciitis.* Expert Opin Biol Ther, 2006. **6**(2): p. 155-65.

173. Schlievert, P.M. and J.A. Kelly, *Clindamycin-induced suppression of toxic-shock syndrome--associated exotoxin production.* J Infect Dis, 1984. **149**(3): p. 471.

174. Vallespi, M.G., et al., *A Limulus anti-LPS factor-derived peptide modulates cytokine gene expression and promotes resolution of bacterial acute infection in mice.* Int Immunopharmacol, 2003. **3**(2): p. 247-56.

175. Hotchkiss, R.S., et al., *The sepsis seesaw: tilting toward immunosuppression.* Nat Med, 2009. **15**(5): p. 496-7.

176. Funk, D.J., J.E. Parrillo, and A. Kumar, *Sepsis and septic shock: a history.* Crit Care Clin, 2009. **25**(1): p. 83-101, viii.

177. Majno, G., *The ancient riddle of sigma eta psi iota sigma (sepsis).* J Infect Dis, 1991. **163**(5): p. 937-45.

178. Bone, R.C., et al., *Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine.* Chest, 1992. **101**(6): p. 1644-55.

179. Singer, M., et al., *The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3).* JAMA, 2016. **315**(8): p. 801-10.

180. Seymour, C.W., et al., *Assessment of Clinical Criteria for Sepsis: For the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3).* JAMA, 2016. **315**(8): p. 762-74.

181. Shankar-Hari, M., et al., *Developing a New Definition and Assessing New Clinical Criteria for Septic Shock: For the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3).* JAMA, 2016. **315**(8): p. 775-87.

182. Levy, M.M., et al., *2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference.* Crit Care Med, 2003. **31**(4): p. 1250-6.

183. Fleischmann, C., et al., *Assessment of Global Incidence and Mortality of Hospital-treated Sepsis. Current Estimates and Limitations.* Am J Respir Crit Care Med, 2016. **193**(3): p. 259-72.

184. Rhee, C., et al., *Incidence and Trends of Sepsis in US Hospitals Using Clinical vs Claims Data, 2009-2014.* JAMA, 2017. **318**(13): p. 1241-1249.

185. Torio, C.M. and B.J. Moore, *National Inpatient Hospital Costs: The Most Expensive Conditions by Payer, 2013: Statistical Brief #204*, in *Healthcare Cost and Utilization Project (HCUP) Statistical Briefs*. 2016: Rockville (MD).

186. Husak, L., et al., *National analysis of sepsis hospitalizations and factors contributing to sepsis in-hospital mortality in Canada.* Healthc Q, 2010. **13 Spec No**: p. 35-41.

187. Martin, G.S., et al., *The epidemiology of sepsis in the United States from 1979 through 2000.* N Engl J Med, 2003. **348**(16): p. 1546-54.

188. Iwashyna, T.J., et al., *Population burden of long-term survivorship after severe sepsis in older Americans.* J Am Geriatr Soc, 2012. **60**(6): p. 1070-7.

189. Gaieski, D.F., et al., *Benchmarking the incidence and mortality of severe sepsis in the United States.* Crit Care Med, 2013. **41**(5): p. 1167-74.

190. Fleischmann, C., et al., *Hospital Incidence and Mortality Rates of Sepsis.* Dtsch Arztebl Int, 2016. **113**(10): p. 159-66.

191. Vincent, J.L., et al., *International study of the prevalence and outcomes of infection in intensive care units.* JAMA, 2009. **302**(21): p. 2323-9.

192. Cohen, J., et al., *New method of classifying infections in critically ill patients.* Crit Care Med, 2004. **32**(7): p. 1510-26.

193. Mayr, F.B., S. Yende, and D.C. Angus, *Epidemiology of severe sepsis.* Virulence, 2014. **5**(1): p. 4-11.

194. van der Poll, T., et al., *The immunopathology of sepsis and potential therapeutic targets.* Nat Rev Immunol, 2017. **17**(7): p. 407-420.

195. Weber, G.F., et al., *Interleukin-3 amplifies acute inflammation and is a potential therapeutic target in sepsis.* Science, 2015. **347**(6227): p. 1260-5.

196. Munford, R.S. and J. Pugin, *Normal responses to injury prevent systemic inflammation and can be immunosuppressive.* Am J Respir Crit Care Med, 2001. **163**(2): p. 316-21.

197. Hotchkiss, R.S., G. Monneret, and D. Payen, *Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy.* Nat Rev Immunol, 2013. **13**(12): p. 862-74.

198. Shalova, I.N., et al., *Human monocytes undergo functional re-programming during sepsis mediated by hypoxia-inducible factor-1alpha.* Immunity, 2015. **42**(3): p. 484-98.

199. Biswas, S.K. and E. Lopez-Collazo, *Endotoxin tolerance: new mechanisms, molecules and clinical significance.* Trends Immunol, 2009. **30**(10): p. 475-87.

200. Monneret, G., et al., *The anti-inflammatory response dominates after septic shock: association of low monocyte HLA-DR expression and high interleukin-10 concentration.* Immunol Lett, 2004. **95**(2): p. 193-8.

201. Leentjens, J., et al., *Immunotherapy for the adjunctive treatment of sepsis: from immunosuppression to immunostimulation. Time for a paradigm change?* Am J Respir Crit Care Med, 2013. **187**(12): p. 1287-93.

202. Hotchkiss, R.S., et al., *Apoptotic cell death in patients with sepsis, shock, and multiple organ dysfunction.* Crit Care Med, 1999. **27**(7): p. 1230-51.

203. Hotchkiss, R.S., et al., *Sepsis-induced apoptosis causes progressive profound depletion of B and CD4+ T lymphocytes in humans.* J Immunol, 2001. **166**(11): p. 6952-63.

204. Hotchkiss, R.S., et al., *Depletion of dendritic cells, but not macrophages, in patients with sepsis.* J Immunol, 2002. **168**(5): p. 2493-500.

205. Felmet, K.A., et al., *Prolonged lymphopenia, lymphoid depletion, and hypoprolactinemia in children with nosocomial sepsis and multiple organ failure.* J Immunol, 2005. **174**(6): p. 3765-72.

206. Grimaldi, D., et al., *Profound and persistent decrease of circulating dendritic cells is associated with ICU-acquired infection in patients with septic shock.* Intensive Care Med, 2011. **37**(9): p. 1438-46.

207. Carson, W.F.t., et al., *Impaired CD4+ T-cell proliferation and effector function correlates with repressive histone methylation events in a mouse model of severe sepsis.* Eur J Immunol, 2010. **40**(4): p. 998-1010.

208. Venet, F., et al., *Early assessment of leukocyte alterations at diagnosis of septic shock.* Shock, 2010. **34**(4): p. 358-63.

209. Venet, F., et al., *Increased percentage of CD4+CD25+ regulatory T cells during septic shock is due to the decrease of CD4+CD25- lymphocytes.* Crit Care Med, 2004. **32**(11): p. 2329-31.

210. Venet, F., et al., *Regulatory T cell populations in sepsis and trauma.* J Leukoc Biol, 2008. **83**(3): p. 523-35.

211. Tiemessen, M.M., et al., *CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages.* Proc Natl Acad Sci U S A, 2007. **104**(49): p. 19446-51.

212. Merlino, J.I., C.J. Yowler, and M.A. Malangoni, *Nosocomial infections adversely affect the outcomes of patients with serious intraabdominal infections.* Surg Infect (Larchmt), 2004. **5**(1): p. 21-7.

213. Luyt, C.E., et al., *Herpes simplex virus lung infection in patients undergoing prolonged mechanical ventilation.* Am J Respir Crit Care Med, 2007. **175**(9): p. 935-42.

214. Limaye, A.P., et al., *Cytomegalovirus reactivation in critically ill immunocompetent patients.* JAMA, 2008. **300**(4): p. 413-22.

215. Monneret, G., et al., *ICU-acquired immunosuppression and the risk for secondary fungal infections.* Med Mycol, 2011. **49 Suppl 1**: p. S17-23.

216. van Vught, L.A., et al., *Incidence, Risk Factors, and Attributable Mortality of Secondary Infections in the Intensive Care Unit After Admission for Sepsis.* JAMA, 2016. **315**(14): p. 1469-79.

217. Yan, J., S. Li, and S. Li, *The role of the liver in sepsis.* Int Rev Immunol, 2014. **33**(6): p. 498-510.

218. Kramer, L., et al., *Incidence and prognosis of early hepatic dysfunction in critically ill patients--a prospective multicenter study.* Crit Care Med, 2007. **35**(4): p. 1099-104.

219. Mayr, F.B., et al., *Infection rate and acute organ dysfunction risk as explanations for racial differences in severe sepsis.* JAMA, 2010. **303**(24): p. 2495-503.

220. Cray, C., J. Zaias, and N.H. Altman, *Acute phase response in animals: a review.* Comp Med, 2009. **59**(6): p. 517-26.

221. Morgan, E.T., *Regulation of cytochrome p450 by inflammatory mediators: why and how?* Drug Metab Dispos, 2001. **29**(3): p. 207-12.

222. Sakamori, R., et al., *Signal transducer and activator of transcription 3 signaling within hepatocytes attenuates systemic inflammatory response and lethality in septic mice.* Hepatology, 2007. **46**(5): p. 1564-73.

223. Lamacchia, C., et al., *Distinct roles of hepatocyte- and myeloid cell-derived IL-1 receptor antagonist during endotoxemia and sterile inflammation in mice.* J Immunol, 2010. **185**(4): p. 2516-24.

224. Deng, M., et al., *Lipopolysaccharide clearance, bacterial clearance, and systemic inflammatory responses are regulated by cell type-specific functions of TLR4 during sepsis.* J Immunol, 2013. **190**(10): p. 5152-60.

225. Adhikari, A., et al., *Hepatocyte SHP-1 is a Critical Modulator of Inflammation During Endotoxemia.* Sci Rep, 2017. **7**(1): p. 2218.

226. Weis, S., et al., *Metabolic Adaptation Establishes Disease Tolerance to Sepsis.* Cell, 2017. **169**(7): p. 1263-1275 e14.

227. Foster, S.L., D.C. Hargreaves, and R. Medzhitov, *Gene-specific control of inflammation by TLR-induced chromatin modifications.* Nature, 2007. **447**(7147): p. 972-8.

228. Cheng, S.C., et al., *Broad defects in the energy metabolism of leukocytes underlie immunoparalysis in sepsis.* Nat Immunol, 2016. **17**(4): p. 406-13.

229. Randow, F., et al., *Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor beta.* J Exp Med, 1995. **181**(5): p. 1887-92.

230. Liu, Z.J., et al., *Up-regulation of IRAK-M is essential for endotoxin tolerance induced by a low dose of lipopolysaccharide in Kupffer cells.* J Surg Res, 2008. **150**(1): p. 34-9.

231. Lu, M., et al., *Host inactivation of bacterial lipopolysaccharide prevents prolonged tolerance following gram-negative bacterial infection.* Cell Host Microbe, 2008. **4**(3): p. 293-302.

232. Bessede, A., et al., *Aryl hydrocarbon receptor control of a disease tolerance defence pathway.* Nature, 2014. **511**(7508): p. 184-90.

233. Draisma, A., et al., *Development of endotoxin tolerance in humans in vivo.* Crit Care Med, 2009. **37**(4): p. 1261-7.

234. del Fresno, C., et al., *Potent phagocytic activity with impaired antigen presentation identifying lipopolysaccharide-tolerant human monocytes: demonstration in isolated monocytes from cystic fibrosis patients.* J Immunol, 2009. **182**(10): p. 6494-507.

235. Wolk, K., et al., *Impaired antigen presentation by human monocytes during endotoxin tolerance.* Blood, 2000. **96**(1): p. 218-23.

236. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease.* Science, 2016. **352**(6284): p. aaf1098.

237. Cheng, S.C., et al., *mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity.* Science, 2014. **345**(6204): p. 1250684.

238. Saeed, S., et al., *Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity.* Science, 2014. **345**(6204): p. 1251086.

239. Bekkering, S., et al., *Metabolic Induction of Trained Immunity through the Mevalonate Pathway.* Cell, 2018. **172**(1-2): p. 135-146 e9.

240. Novakovic, B., et al., *beta-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance.* Cell, 2016. **167**(5): p. 1354-1368 e14.

241. Okey, A.B., *An aryl hydrocarbon receptor odyssey to the shores of toxicology: the Deichmann Lecture, International Congress of Toxicology-XI.* Toxicol Sci, 2007. **98**(1): p. 5-38.

242. Poland, A., et al., *3,4,3',4'-Tetrachloro azoxybenzene and azobeneze: potent inducers of aryl hydrocarbon hydroxylase.* Science, 1976. **194**(4265): p. 627-30.

243. Conney, A.H., E.C. Miller, and J.A. Miller, *Substrate-induced synthesis and other properties of benzpyrene hydroxylase in rat liver.* J Biol Chem, 1957. **228**(2): p. 753-66.

244. Conney, A.H., *Induction of drug-metabolizing enzymes: a path to the discovery of multiple cytochromes P450.* Annu Rev Pharmacol Toxicol, 2003. **43**: p. 1-30.

245. Hankinson, O., *Single-step selection of clones of a mouse hepatoma line deficient in aryl hydrocarbon hydroxylase.* Proc Natl Acad Sci U S A, 1979. **76**: p. 373-6.

246. Legraverend, C., et al., *Regulatory gene product of the Ah locus. Characterization of receptor mutants among mouse hepatoma clones.* J Biol Chem, 1982. **257**.

247. Abel, J. and T. Haarmann-Stemmann, *An introduction to the molecular basics of aryl hydrocarbon receptor biology.* Biol Chem, 2010. **391**(11): p. 1235-48.

248. Gu, Y.Z., J.B. Hogenesch, and C.A. Bradfield, *The PAS superfamily: sensors of environmental and development signals.* Annu Rev Pharmacol Toxicol, 2000. **40**: p. 519-61.

249. Frericks, M., M. Meissner, and C. Esser, *Microarray analysis of the AHR system: tissue-specific flexibility in signal and target genes.* Toxicol Appl Pharmacol, 2007. **220**(3): p. 320-32.

250. Hahn, M.E., et al., *Molecular evolution of two vertebrate aryl hydrocarbon (dioxin) receptors (AHR1 and AHR2) and the PAS family.* Proc Natl Acad Sci U S A, 1997. **94**: p. 13743-8.

251. Ema, M., et al., *Human arylhydrocarbon receptor: functional expression and chromosomal assignment to 7p21.* J Biochem, 1994. **116**(4): p. 845-51.

252. Le Beau, M.M., et al., *Chromosomal localization of the human AHR locus encoding the structural gene for the Ah receptor to 7p21-->p15.* Cytogenet Cell Genet, 1994. **66**(3): p. 172-6.

253. Schmidt, J.V., L.A. Carver, and C.A. Bradfield, *Molecular characterization of the murine Ahr gene. Organization, promoter analysis, and chromosomal assignment.* J Biol Chem, 1993. **268**(29): p. 22203-9.

254. Eguchi, H., et al., *Molecular cloning of the human AH receptor gene promoter.* Biochem Biophys Res Commun, 1994. **203**(1): p. 615-22.

255. Garrison, P.M. and M.S. Denison, *Analysis of the murine AhR gene promoter.* J Biochem Mol Toxicol, 2000. **14**(1): p. 1-10.

256. Fitzgerald, C.T., D.W. Nebert, and A. Puga, *Regulation of mouse Ah receptor (Ahr) gene basal expression by members of the Sp family of transcription factors.* DNA Cell Biol, 1998. **17**(9): p. 811-22.

257. Wolff, S., et al., *Cell-specific regulation of human aryl hydrocarbon receptor expression by transforming growth factor-beta(1).* Mol Pharmacol, 2001. **59**(4): p. 716-24.

258. Fukunaga, B.N. and O. Hankinson, *Identification of a novel domain in the aryl hydrocarbon receptor required for DNA binding.* J Biol Chem, 1996. **271**(7): p. 3743-9.

259. Dong, L., Q. Ma, and J.P. Whitlock, Jr., *DNA binding by the heterodimeric Ah receptor. Relationship to dioxin-induced CYP1A1 transcription in vivo.* J Biol Chem, 1996. **271**(14): p. 7942-8.

260. Bacsi, S.G. and O. Hankinson, *Functional characterization of DNA-binding domains of the subunits of the heterodimeric aryl hydrocarbon receptor complex imputing novel and canonical basic helix-loop-helix protein-DNA interactions.* J Biol Chem, 1996. **271**(15): p. 8843-50.

261. Minsavage, G.D., S.K. Park, and T.A. Gasiewicz, *The aryl hydrocarbon receptor (AhR) tyrosine 9, a residue that is essential for AhR DNA binding activity, is not a phosphoresidue but augments AhR phosphorylation.* J Biol Chem, 2004. **279**(20): p. 20582-93.

262. Burbach, K.M., A. Poland, and C.A. Bradfield, *Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor.* Proc Natl Acad Sci U S A, 1992. **89**(17): p. 8185-9.

263. Fukunaga, B.N., et al., *Identification of functional domains of the aryl hydrocarbon receptor.* J Biol Chem, 1995. **270**(49): p. 29270-8.

264. Rowlands, J.C., I.J. McEwan, and J.A. Gustafsson, *Trans-activation by the human aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator proteins: direct interactions with basal transcription factors.* Mol Pharmacol, 1996. **50**(3): p. 538-48.

265. Ikuta, T., et al., *Nuclear localization and export signals of the human aryl hydrocarbon receptor.* J Biol Chem, 1998. **273**(5): p. 2895-904.

266. DeGroot, D., et al., *AHR Ligands: Promiscuity in Binding and Diversity in Response*, in *The AH Receptor in Biology and Toxicology*. 2011, John Wiley & Sons, Inc. p. 63-79.

267. Soshilov, A.A. and M.S. Denison, *Ligand promiscuity of aryl hydrocarbon receptor agonists and antagonists revealed by site-directed mutagenesis.* Mol Cell Biol, 2014. **34**(9): p. 1707-19.

268. Mandal, P.K., *Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology.* J Comp Physiol B, 2005. **175**(4): p. 221-30.

269. Denison, M.S. and S.R. Nagy, *Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals.* Annu Rev Pharmacol Toxicol, 2003. **43**: p. 309-34.

270. Shertzer, H.G. and A.P. Senft, *The micronutrient indole-3-carbinol: implications for disease and chemoprevention.* Drug Metabol Drug Interact, 2000. **17**(1-4): p. 159-88.

271. Jin, U.H., et al., *Microbiome-derived tryptophan metabolites and their aryl hydrocarbon receptor-dependent agonist and antagonist activities.* Mol Pharmacol, 2014. **85**(5): p. 777-88.

272. Cervantes-Barragan, L., et al., *Lactobacillus reuteri induces gut intraepithelial CD4(+)CD8alphaalpha(+) T cells.* Science, 2017. **357**(6353): p. 806-810.

273. Zelante, T., et al., *Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22.* Immunity, 2013. **39**(2): p. 372-85.

274. Schiering, C., et al., *Feedback control of AHR signalling regulates intestinal immunity.* Nature, 2017.

275. Moura-Alves, P., et al., *AhR sensing of bacterial pigments regulates antibacterial defence.* Nature, 2014. **512**(7515): p. 387-92.

276. Nguyen, L.P. and C.A. Bradfield, *The search for endogenous activators of the aryl hydrocarbon receptor.* Chem Res Toxicol, 2008. **21**(1): p. 102-16.

277. Nebert, D.W. and C.L. Karp, *Endogenous functions of the aryl hydrocarbon receptor (AHR): intersection of cytochrome P450 1 (CYP1)-metabolized eicosanoids and AHR biology.* J Biol Chem, 2008. **283**(52): p. 36061-5.

278. Fernandez-Salguero, P., et al., *Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor.* Science, 1995. **268**(5211): p. 722-6.

279. Schmidt, J.V., et al., *Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development.* Proc Natl Acad Sci U S A, 1996. **93**(13): p. 6731-6.

280. Mimura, J., et al., *Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor.* Genes Cells, 1997. **2**(10): p. 645-54.

281. Lahvis, G.P. and C.A. Bradfield, *Ahr null alleles: distinctive or different?* Biochem Pharmacol, 1998. **56**(7): p. 781-7.

282. Cervenka, I., L.Z. Agudelo, and J.L. Ruas, *Kynurenines: Tryptophan's metabolites in exercise, inflammation, and mental health.* Science, 2017. **357**(6349): p. eaaf9794.

283. Nguyen, N.T., et al., *Aryl hydrocarbon receptor negatively regulates dendritic cell immunogenicity via a kynurenine-dependent mechanism.* Proc Natl Acad Sci U S A, 2010. **107**(46): p. 19961-6.

284. Mezrich, J.D., et al., *An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells.* J Immunol, 2010. **185**(6): p. 3190-8.

285. Rannug, A., et al., *Certain photooxidized derivatives of tryptophan bind with very high affinity to the Ah receptor and are likely to be endogenous signal substances.* J Biol Chem, 1987. **262**(32): p. 15422-7.

286. Rannug, U., et al., *Structure elucidation of two tryptophan-derived, high affinity Ah receptor ligands.* Chem Biol, 1995. **2**(12): p. 841-5.

287. Oberg, M., et al., *Identification of the tryptophan photoproduct 6-formylindolo[3,2-b]carbazole, in cell culture medium, as a factor that controls the background aryl hydrocarbon receptor activity.* Toxicol Sci, 2005. **85**(2): p. 935-43.

288. Fritsche, E., et al., *Lightening up the UV response by identification of the arylhydrocarbon receptor as a cytoplasmatic target for ultraviolet B radiation.* Proc Natl Acad Sci U S A, 2007. **104**(21): p. 8851-6.

289. Wincent, E., et al., *The suggested physiologic aryl hydrocarbon receptor activator and cytochrome P4501 substrate 6-formylindolo[3,2-b]carbazole is present in humans.* J Biol Chem, 2009. **284**(5): p. 2690-6.

290. Smirnova, A., et al., *Evidence for New Light-Independent Pathways for Generation of the Endogenous Aryl Hydrocarbon Receptor Agonist FICZ.* Chem Res Toxicol, 2016. **29**(1): p. 75-86.

291. Di Meglio, P., et al., *Activation of the aryl hydrocarbon receptor dampens the severity of inflammatory skin conditions.* Immunity, 2014. **40**(6): p. 989-1001.

292. Quintana, F.J., et al., *Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor.* Nature, 2008. **453**(7191): p. 65-71.

293. Veldhoen, M., et al., *The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins.* Nature, 2008. **453**(7191): p. 106-9.

294. Henry, E.C. and T.A. Gasiewicz, *Transformation of the aryl hydrocarbon receptor to a DNA-binding form is accompanied by release of the 90 kDa heat-shock protein and increased affinity for 2,3,7,8-tetrachlorodibenzo-p-dioxin.* Biochem J, 1993. **294 (Pt 1)**: p. 95-101.

295. Carver, L.A., V. Jackiw, and C.A. Bradfield, *The 90-kDa heat shock protein is essential for Ah receptor signaling in a yeast expression system.* J Biol Chem, 1994. **269**(48): p. 30109-12.

296. Kazlauskas, A., L. Poellinger, and I. Pongratz, *Evidence that the co-chaperone p23 regulates ligand responsiveness of the dioxin (Aryl hydrocarbon) receptor.* J Biol Chem, 1999. **274**(19): p. 13519-24.

297. Carver, L.A. and C.A. Bradfield, *Ligand-dependent interaction of the aryl hydrocarbon receptor with a novel immunophilin homolog in vivo.* J Biol Chem, 1997. **272**(17): p. 11452-6.

298. Meyer, B.K., et al., *Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity.* Mol Cell Biol, 1998. **18**(2): p. 978-88.

299. Antonsson, C., et al., *Distinct roles of the molecular chaperone hsp90 in modulating dioxin receptor function via the basic helix-loop-helix and PAS domains.* Mol Cell Biol, 1995. **15**(2): p. 756-65.

300. Perdew, G.H. and C.A. Bradfield, *Mapping the 90 kDa heat shock protein binding region of the Ah receptor.* Biochem Mol Biol Int, 1996. **39**(3): p. 589-93.

301. Meyer, B.K. and G.H. Perdew, *Characterization of the AhR-hsp90-XAP2 core complex and the role of the immunophilin-related protein XAP2 in AhR stabilization.* Biochemistry, 1999. **38**(28): p. 8907-17.

302. Kazlauskas, A., L. Poellinger, and I. Pongratz, *The immunophilin-like protein XAP2 regulates ubiquitination and subcellular localization of the dioxin receptor.* J Biol Chem, 2000. **275**(52): p. 41317-24.

303. Petrulis, J.R., et al., *The hsp90 Co-chaperone XAP2 alters importin beta recognition of the bipartite nuclear localization signal of the Ah receptor and represses transcriptional activity.* J Biol Chem, 2003. **278**(4): p. 2677-85.

304. Probst, M.R., et al., *Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action.* Mol Pharmacol, 1993. **44**(3): p. 511-8.

305. Soshilov, A. and M.S. Denison, *Role of the Per/Arnt/Sim domains in ligand-dependent transformation of the aryl hydrocarbon receptor.* J Biol Chem, 2008. **283**(47): p. 32995-3005.

306. Lusska, A., E. Shen, and J.P. Whitlock, Jr., *Protein-DNA interactions at a dioxin-responsive enhancer. Analysis of six bona fide DNA-binding sites for the liganded Ah receptor.* J Biol Chem, 1993. **268**(9): p. 6575-80.

307. Tijet, N., et al., *Aryl hydrocarbon receptor regulates distinct dioxin-dependent and dioxin-independent gene batteries.* Mol Pharmacol, 2006. **69**(1): p. 140-53.

308. Davarinos, N.A. and R.S. Pollenz, *Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytosplasmic proteasome following nuclear export.* J Biol Chem, 1999. **274**(40): p. 28708-15.

309. Roberts, B.J. and M.L. Whitelaw, *Degradation of the basic helix-loop-helix/Per-ARNT-Sim homology domain dioxin receptor via the ubiquitin/proteasome pathway.* J Biol Chem, 1999. **274**(51): p. 36351-6.

310. Pollenz, R.S. and E.R. Barbour, *Analysis of the complex relationship between nuclear export and aryl hydrocarbon receptor-mediated gene regulation.* Mol Cell Biol, 2000. **20**(16): p. 6095-104.

311. Ikuta, T., Y. Kobayashi, and K. Kawajiri, *Cell density regulates intracellular localization of aryl hydrocarbon receptor.* J Biol Chem, 2004. **279**(18): p. 19209-16.

312. Ikuta, T., Y. Kobayashi, and K. Kawajiri, *Phosphorylation of nuclear localization signal inhibits the ligand-dependent nuclear import of aryl hydrocarbon receptor.* Biochem Biophys Res Commun, 2004. **317**(2): p. 545-50.

313. Ma, Q. and K.T. Baldwin, *2,3,7,8-tetrachlorodibenzo-p-dioxin-induced degradation of aryl hydrocarbon receptor (AhR) by the ubiquitin-proteasome pathway. Role of the transcription activaton and DNA binding of AhR.* J Biol Chem, 2000. **275**(12): p. 8432-8.

314. Chen, H.S., S.S. Singh, and G.H. Perdew, *The Ah receptor is a sensitive target of geldanamycin-induced protein turnover.* Arch Biochem Biophys, 1997. **348**(1): p. 190-8.

315. Mimura, J., et al., *Identification of a novel mechanism of regulation of Ah (dioxin) receptor function.* Genes Dev, 1999. **13**(1): p. 20-5.

316. Ohtake, F., et al., *Dioxin receptor is a ligand-dependent E3 ubiquitin ligase.* Nature, 2007. **446**(7135): p. 562-6.

317. Hart, S.N., et al., *Three patterns of cytochrome P450 gene expression during liver maturation in mice.* Drug Metab Dispos, 2009. **37**(1): p. 116-21.

318. Liang, H.C., et al., *Cyp1a2(-/-) null mutant mice develop normally but show deficient drug metabolism.* Proc Natl Acad Sci U S A, 1996. **93**(4): p. 1671-6.

319. Wincent, E., et al., *Inhibition of cytochrome P4501-dependent clearance of the endogenous agonist FICZ as a mechanism for activation of the aryl hydrocarbon receptor.* Proc Natl Acad Sci U S A, 2012. **109**(12): p. 4479-84.

320. Mitchell, K.A. and C.J. Elferink, *Timing is everything: consequences of transient and sustained AhR activity.* Biochem Pharmacol, 2009. **77**(6): p. 947-56.

321. Stockinger, B., et al., *The aryl hydrocarbon receptor: multitasking in the immune system.* Annu Rev Immunol, 2014. **32**: p. 403-32.

322. Gutierrez-Vazquez, C. and F.J. Quintana, *Regulation of the Immune Response by the Aryl Hydrocarbon Receptor.* Immunity, 2018. **48**(1): p. 19-33.

323. Hinsdill, R.D., D.L. Couch, and R.S. Speirs, *Immunosuppression in mice induced by dioxin (TCDD) in feed.* J Environ Pathol Toxicol, 1980. **4**(2-3): p. 401-25.

324. Holsapple, M.P., et al., *2,3,7,8-tetrachlorodibenzo-p-dioxin-induced changes in immunocompetence: possible mechanisms.* Annu Rev Pharmacol Toxicol, 1991. **31**: p. 73-100.

325. Kerkvliet, N.I., *Recent advances in understanding the mechanisms of TCDD immunotoxicity.* Int Immunopharmacol, 2002. **2**(2-3): p. 277-91.

326. Cella, M. and M. Colonna, *Aryl hydrocarbon receptor: Linking environment to immunity.* Semin Immunol, 2015. **27**(5): p. 310-4.

327. Veldhoen, M., et al., *Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells.* J Exp Med, 2009. **206**(1): p. 43-9.

328. Ishigame, H., et al., *Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses.* Immunity, 2009. **30**(1): p. 108-19.

329. Mohinta, S., et al., *Differential regulation of Th17 and T regulatory cell differentiation by aryl hydrocarbon receptor dependent xenobiotic response element dependent and independent pathways.* Toxicol Sci, 2015. **145**(2): p. 233-43.

330. Apetoh, L., et al., *The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27.* Nat Immunol, 2010. **11**(9): p. 854-61.

331. Gandhi, R., et al., *Activation of the aryl hydrocarbon receptor induces human type 1 regulatory T cell-like and Foxp3(+) regulatory T cells.* Nat Immunol, 2010. **11**(9): p. 846-53.

332. Roncarolo, M.G., et al., *Interleukin-10-secreting type 1 regulatory T cells in rodents and humans.* Immunol Rev, 2006. **212**: p. 28-50.

333. Fitzgerald, D.C., et al., *Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells.* Nat Immunol, 2007. **8**(12): p. 1372-9.

334. Awasthi, A., et al., *A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells.* Nat Immunol, 2007. **8**(12): p. 1380-9.

335. Stumhofer, J.S., et al., *Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10.* Nat Immunol, 2007. **8**(12): p. 1363-71.

336. Gagliani, N., et al., *Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation.* Nature, 2015. **523**(7559): p. 221-5.

337. Kiss, E.A., et al., *Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles.* Science, 2011. **334**(6062): p. 1561-5.

338. Lee, J.S., et al., *AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch.* Nat Immunol, 2011. **13**(2): p. 144-51.

339. Qiu, J., et al., *The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells.* Immunity, 2012. **36**(1): p. 92-104.

340. Li, Y., et al., *Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation.* Cell, 2011. **147**(3): p. 629-40.

341. Garman, R.D., P.J. Doherty, and D.H. Raulet, *Diversity, rearrangement, and expression of murine T cell gamma genes.* Cell, 1986. **45**(5): p. 733-42.

342. Martin, B., et al., *Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals.* Immunity, 2009. **31**(2): p. 321-30.

343. Sekine, H., et al., *Hypersensitivity of aryl hydrocarbon receptor-deficient mice to lipopolysaccharide-induced septic shock.* Mol Cell Biol, 2009. **29**(24): p. 6391-400.

344. Kimura, A., et al., *Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses.* J Exp Med, 2009. **206**(9): p. 2027-35.

345. Yamada, T., et al., *Constitutive aryl hydrocarbon receptor signaling constrains type I interferon-mediated antiviral innate defense.* Nat Immunol, 2016. **17**(6): p. 687-94.

346. Kado, S., et al., *Aryl hydrocarbon receptor signaling modifies Toll-like receptor-regulated responses in human dendritic cells.* Arch Toxicol, 2017. **91**(5): p. 2209-2221.

347. Wang, C., et al., *Activation of the aryl hydrocarbon receptor affects activation and function of human monocyte-derived dendritic cells.* Clin Exp Immunol, 2014. **177**(2): p. 521-30.

348. Quintana, F.J., et al., *An endogenous aryl hydrocarbon receptor ligand acts on dendritic cells and T cells to suppress experimental autoimmune encephalomyelitis.* Proc Natl Acad Sci U S A, 2010. **107**(48): p. 20768-73.

349. Goudot, C., et al., *Aryl Hydrocarbon Receptor Controls Monocyte Differentiation into Dendritic Cells versus Macrophages.* Immunity, 2017. **47**(3): p. 582-596 e6.

350. Volk, H.D., et al., *Monocyte deactivation--rationale for a new therapeutic strategy in sepsis.* Intensive Care Med, 1996. **22 Suppl 4**: p. S474-81.

351. Takeuchi, O., et al., *Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins.* J Immunol, 2002. **169**(1): p. 10-4.

352. Brightbill, H.D., et al., *Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors.* Science, 1999. **285**(5428): p. 732-6.

353. Ozinsky, A., et al., *The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors.* Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13766-71.

354. Takeuchi, O., et al., *Discrimination of bacterial lipoproteins by Toll-like receptor 6.* Int Immunol, 2001. **13**(7): p. 933-40.

355. Hasan, U., et al., *Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88.* J Immunol, 2005. **174**(5): p. 2942-50.

356. Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3.* Nature, 2001. **413**(6857): p. 732-8.

357. Hayashi, F., et al., *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5.* Nature, 2001. **410**(6832): p. 1099-103.

358. Heil, F., et al., *Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8.* Science, 2004. **303**(5663): p. 1526-9.

359. Diebold, S.S., et al., *Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA.* Science, 2004. **303**(5663): p. 1529-31.

360. Hemmi, H., et al., *A Toll-like receptor recognizes bacterial DNA.* Nature, 2000. **408**(6813): p. 740-5.

361. Hess, N.J., et al., *TLR10 Is a B Cell Intrinsic Suppressor of Adaptive Immune Responses.* J Immunol, 2017. **198**(2): p. 699-707.

362. Wharton, M., et al., *Case definitions for public health surveillance.* MMWR Recomm Rep, 1990. **39**(RR-13): p. 1-43.

363. Dantes, R., et al., *National burden of invasive methicillin-resistant Staphylococcus aureus infections, United States, 2011.* JAMA Intern Med, 2013. **173**(21): p. 1970-8.

364. Eriksen, N.H., et al., *Carriage of Staphylococcus aureus among 104 healthy persons during a 19-month period.* Epidemiol Infect, 1995. **115**(1): p. 51-60.

365. Lozano, C., et al., *Staphylococcus aureus nasal carriage, virulence traits, antibiotic resistance mechanisms, and genetic lineages in healthy humans in Spain, with detection of CC398 and CC97 strains.* Int J Med Microbiol, 2011. **301**(6): p. 500-5.

366. Netsvyetayeva, I., et al., *Staphylococcus aureus nasal carriage in Ukraine: antibacterial resistance and virulence factor encoding genes.* BMC Infect Dis, 2014. **14**: p. 128.

367. Marshall, C. and E. McBryde, *The role of Staphylococcus aureus carriage in the pathogenesis of bloodstream infection.* BMC Res Notes, 2014. **7**(1): p. 428.

368. Brown, A.F., et al., *Staphylococcus aureus Colonization: Modulation of Host Immune Response and Impact on Human Vaccine Design.* Front Immunol, 2014. **4**: p. 507.

369. Fournier, B. and D.J. Philpott, *Recognition of Staphylococcus aureus by the innate immune system.* Clin Microbiol Rev, 2005. **18**(3): p. 521-40.

370. Kawai, T. and S. Akira, *Toll-like receptors and their crosstalk with other innate receptors in infection and immunity.* Immunity, 2011. **34**(5): p. 637-50.

371. Ip, W.K., et al., *Phagocytosis and phagosome acidification are required for pathogen processing and MyD88-dependent responses to Staphylococcus aureus.* J Immunol, 2010. **184**(12): p. 7071-81.

372. Enright, M.C., et al., *Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus.* J Clin Microbiol, 2000. **38**(3): p. 1008-15.

373. Baroja, M.L., et al., *Specific CD3 epsilon association of a phosphodiesterase 4B isoform determines its selective tyrosine phosphorylation after CD3 ligation.* J Immunol, 1999. **162**(4): p. 2016-23.

374. Feng, Y., et al., *Evolution and pathogenesis of Staphylococcus aureus: lessons learned from genotyping and comparative genomics.* FEMS Microbiol Rev, 2008. **32**(1): p. 23-37.

375. Saraiva, M. and A. O'Garra, *The regulation of IL-10 production by immune cells.* Nat Rev Immunol, 2010. **10**(3): p. 170-81.

376. Aksoy, E., et al., *The p110delta isoform of the kinase PI(3)K controls the subcellular compartmentalization of TLR4 signaling and protects from endotoxic shock.* Nat Immunol, 2012. **13**(11): p. 1045-54.

377. Wang, P., et al., *IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells.* J Immunol, 1994. **153**(2): p. 811-6.

378. Arthur, J.S. and S.C. Ley, *Mitogen-activated protein kinases in innate immunity.* Nat Rev Immunol, 2013. **13**(9): p. 679-92.

379. Stuart, L.M., et al., *Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain.* J Cell Biol, 2005. **170**(3): p. 477-85.

380. Depaolo, R.W., et al., *Toll-like receptor 6 drives differentiation of tolerogenic dendritic cells and contributes to LcrV-mediated plague pathogenesis.* Cell Host Microbe, 2008. **4**(4): p. 350-61.

381. DePaolo, R.W., et al., *A specific role for TLR1 in protective T(H)17 immunity during mucosal infection.* J Exp Med, 2012. **209**(8): p. 1437-44.

382. Skabytska, Y., et al., *Cutaneous innate immune sensing of Toll-like receptor 2-6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells.* Immunity, 2014. **41**(5): p. 762-75.

383. Kenny, E.F., et al., *MyD88 adaptor-like is not essential for TLR2 signaling and inhibits signaling by TLR3.* J Immunol, 2009. **183**(6): p. 3642-51.

384. Hilmi, D., et al., *Heterogeneity of Host TLR2 Stimulation by Staphylocoocus aureus Isolates.* PLoS One, 2014. **9**(5): p. e96416.

385. Katholnig, K., et al., *p38alpha senses environmental stress to control innate immune responses via mechanistic target of rapamycin.* J Immunol, 2013. **190**(4): p. 1519-27.

386. Over, B., et al., *IRAK4 turns IL-10+ phospho-FOXO+ monocytes into pro-inflammatory cells by suppression of protein kinase B.* Eur J Immunol, 2013. **43**(6): p. 1630-42.

387. Parcina, M., et al., *Pathogen-triggered activation of plasmacytoid dendritic cells induces IL-10-producing B cells in response to Staphylococcus aureus.* J Immunol, 2013. **190**(4): p. 1591-602.

388. Ziegler, S., et al., *Ca(2+) -related signaling events influence TLR9-induced IL-10 secretion in human B cells.* Eur J Immunol, 2014. **44**(5): p. 1285-98.

389. Van Dyken, S.J. and R.M. Locksley, *Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease.* Annu Rev Immunol, 2013. **31**: p. 317-43.

390. Grainger, J.R., et al., *Inflammatory monocytes regulate pathologic responses to commensals during acute gastrointestinal infection.* Nat Med, 2013. **19**(6): p. 713-21.

391. Szkaradkiewicz, A., et al., *Cytokine response in patients with chronic infections caused by Staphylococcus aureus strains and diversification of their Agr system classes.* Eur J Clin Microbiol Infect Dis, 2012. **31**(10): p. 2809-15.

392. Kimura, A., et al., *Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells.* Proc Natl Acad Sci U S A, 2008. **105**(28): p. 9721-6.

393. Perdew, G.H., *Association of the Ah receptor with the 90-kDa heat shock protein.* J Biol Chem, 1988. **263**(27): p. 13802-5.

394. Denis, M., et al., *Association of the dioxin receptor with the Mr 90,000 heat shock protein: a structural kinship with the glucocorticoid receptor.* Biochem Biophys Res Commun, 1988. **155**(2): p. 801-7.

395. Reyes, H., S. Reisz-Porszasz, and O. Hankinson, *Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor.* Science, 1992. **256**(5060): p. 1193-5.

396. Whitelaw, M., et al., *Ligand-dependent recruitment of the Arnt coregulator determines DNA recognition by the dioxin receptor.* Mol Cell Biol, 1993. **13**(4): p. 2504-14.

397. Morales, J.L. and G.H. Perdew, *Carboxyl terminus of hsc70-interacting protein (CHIP) can remodel mature aryl hydrocarbon receptor (AhR) complexes and mediate ubiquitination of both the AhR and the 90 kDa heat-shock protein (hsp90) in vitro.* Biochemistry, 2007. **46**(2): p. 610-21.

398. Gonzalez, F.J., *The molecular biology of cytochrome P450s.* Pharmacol Rev, 1988. **40**(4): p. 243-88.

399. Wincent, E., et al., *Biological effects of 6-formylindolo[3,2-b]carbazole (FICZ) in vivo are enhanced by loss of CYP1A function in an Ahr2-dependent manner.* Biochem Pharmacol, 2016. **110-111**: p. 117-29.

400. Brandstatter, O., et al., *Balancing intestinal and systemic inflammation through cell type-specific expression of the aryl hydrocarbon receptor repressor.* Sci Rep, 2016. **6**: p. 26091.

401. Vogel, C.F., et al., *Transgenic Overexpression of Aryl Hydrocarbon Receptor Repressor (AhRR) and AhR-Mediated Induction of CYP1A1, Cytokines, and Acute Toxicity.* Environ Health Perspect, 2016. **124**(7): p. 1071-83.

402. Lacey, D.C., et al., *Defining GM-CSF- and macrophage-CSF-dependent macrophage responses by in vitro models.* J Immunol, 2012. **188**(11): p. 5752-65.

403. Tholander, J. and J. Bergman, *Syntheses of 6-substituted indolo[3,2-b]carbazoles, including 6-formylindolo[3,2-b]carbazole, an extremely efficient ligand for the TCDD (Ah) receptor.* Tetrahedron, 1999. **55**(19): p. 6243-6260.

404. Radenac, G., et al., *Measurement of EROD activity: Caution on spectral properties of standards used.* Marine Biotechnology, 2004. **6**(4): p. 307-311.

405. Takeuchi, O., K. Hoshino, and S. Akira, *Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection.* J Immunol, 2000. **165**(10): p. 5392-6.

406. Tian, Y., et al., *Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity.* J Biol Chem, 1999. **274**(1): p. 510-5.

407. Ke, S., et al., *Mechanism of suppression of cytochrome P-450 1A1 expression by tumor necrosis factor-alpha and lipopolysaccharide.* J Biol Chem, 2001. **276**(43): p. 39638-44.

408. Joiakim, A., et al., *Superinduction of CYP1A1 in MCF10A cultures by cycloheximide, anisomycin, and puromycin: a process independent of effects on protein translation and unrelated to suppression of aryl hydrocarbon receptor proteolysis by the proteasome.* Mol Pharmacol, 2004. **66**(4): p. 936-47.

409. Bain, J., et al., *The selectivity of protein kinase inhibitors: a further update.* Biochem J, 2007. **408**(3): p. 297-315.

410. Khatsenko, O.G., et al., *Nitric oxide is a mediator of the decrease in cytochrome P450-dependent metabolism caused by immunostimulants.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 11147-51.

411. Delaporte, E. and K.W. Renton, *Cytochrome P4501A1 and cytochrome P4501A2 are downregulated at both transcriptional and post-transcriptional levels by conditions resulting in interferon-alpha/beta induction.* Life Sci, 1997. **60**(10): p. 787-96.

412. Becher, B., S. Tugues, and M. Greter, *GM-CSF: From Growth Factor to Central Mediator of Tissue Inflammation.* Immunity, 2016. **45**(5): p. 963-973.

413. Cheong, C., et al., *Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas.* Cell, 2010. **143**(3): p. 416-29.

414. Vogel, C.F., et al., *Cross-talk between aryl hydrocarbon receptor and the inflammatory response: a role for nuclear factor-kappaB.* J Biol Chem, 2014. **289**(3): p. 1866-75.

415. Memari, B., et al., *Engagement of the Aryl Hydrocarbon Receptor in Mycobacterium tuberculosis-Infected Macrophages Has Pleiotropic Effects on Innate Immune Signaling.* J Immunol, 2015. **195**(9): p. 4479-91.

416. Vogel, C.F., et al., *Aryl hydrocarbon receptor signaling regulates NF-kappaB RelB activation during dendritic-cell differentiation.* Immunol Cell Biol, 2013. **91**(9): p. 568-75.

417. Vogel, C.F., et al., *RelB, a new partner of aryl hydrocarbon receptor-mediated transcription.* Mol Endocrinol, 2007. **21**(12): p. 2941-55.

418. Baba, T., et al., *Structure and expression of the Ah receptor repressor gene.* J Biol Chem, 2001. **276**(35): p. 33101-10.

419. Guo, M., A. Joiakim, and J.J. Reiners, Jr., *Suppression of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-mediated aryl hydrocarbon receptor transformation and CYP1A1 induction by the phosphatidylinositol 3-kinase inhibitor 2-(4-morpholinyl)-8-phenyl-4H-1- benzopyran-4-one (LY294002).* Biochem Pharmacol, 2000. **60**(5): p. 635-42.

420. Vrzal, R., et al., *The effects of drugs with immunosuppressive or immunomodulatory activities on xenobiotics-metabolizing enzymes expression in primary human hepatocytes.* Toxicol In Vitro, 2015. **29**(5): p. 1088-99.

421. Shibazaki, M., et al., *Blockade by SB203580 of Cyp1a1 induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin, and the possible mechanism: possible involvement of the p38 mitogen-activated protein kinase pathway in shuttling of Ah receptor overexpressed in COS-7 cells.* Ann N Y Acad Sci, 2004. **1030**: p. 275-81.

422. Crawford, J.H., et al., *Down-regulation of hepatic CYP1A2 plays an important role in inflammatory responses in sepsis.* Crit Care Med, 2004. **32**(2): p. 502-8.

423. Aitken, A.E., T.A. Richardson, and E.T. Morgan, *Regulation of drug-metabolizing enzymes and transporters in inflammation.* Annu Rev Pharmacol Toxicol, 2006. **46**: p. 123-49.

424. Pineau, T., et al., *Neonatal lethality associated with respiratory distress in mice lacking cytochrome P450 1A2.* Proc Natl Acad Sci U S A, 1995. **92**(11): p. 5134-8.

425. Burke, M.D. and R.T. Mayer, *Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene.* Drug Metab Dispos, 1974. **2**(6): p. 583-8.

426. Corchero, J., et al., *Organization of the CYP1A cluster on human chromosome 15: implications for gene regulation.* Pharmacogenetics, 2001. **11**(1): p. 1-6.

427. Nukaya, M. and C.A. Bradfield, *Conserved genomic structure of the Cyp1a1 and Cyp1a2 loci and their dioxin responsive elements cluster.* Biochem Pharmacol, 2009. **77**(4): p. 654-9.

428. Nukaya, M., S. Moran, and C.A. Bradfield, *The role of the dioxin-responsive element cluster between the Cyp1a1 and Cyp1a2 loci in aryl hydrocarbon receptor biology.* Proc Natl Acad Sci U S A, 2009. **106**(12): p. 4923-8.

429. Dragin, N., et al., *Phenotype of the Cyp1a1/1a2/1b1-/- triple-knockout mouse.* Mol Pharmacol, 2008. **73**(6): p. 1844-56.

430. Feil, E.J., et al., *How clonal is Staphylococcus aureus?* J Bacteriol, 2003. **185**(11): p. 3307-16.

431. Shurland, S.M., et al., *Colonization sites of USA300 methicillin-resistant Staphylococcus aureus in residents of extended care facilities.* Infect Control Hosp Epidemiol, 2009. **30**(4): p. 313-8.

432. Diep, B.A., et al., *Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus.* Lancet, 2006. **367**(9512): p. 731-9.

433. Li, J., D.S. Lee, and J. Madrenas, *Evolving Bacterial Envelopes and Plasticity of TLR2-Dependent Responses: Basic Research and Translational Opportunities.* Front Immunol, 2013. **4**: p. 347.

434. Gao, J., et al., *Impact of the Gut Microbiota on Intestinal Immunity Mediated by Tryptophan Metabolism.* Front Cell Infect Microbiol, 2018. **8**: p. 13.

435. Haas, K., et al., *Aryl Hydrocarbon Receptor in Keratinocytes Is Essential for Murine Skin Barrier Integrity.* J Invest Dermatol, 2016. **136**(11): p. 2260-2269.

436. Schaaf, B.M., et al., *Pneumococcal septic shock is associated with the interleukin-10-1082 gene promoter polymorphism.* Am J Respir Crit Care Med, 2003. **168**(4): p. 476-80.

437. Tattevin, P., et al., *Enhanced indoleamine 2,3-dioxygenase activity in patients with severe sepsis and septic shock.* J Infect Dis, 2010. **201**(6): p. 956-66.

438. Darcy, C.J., et al., *An observational cohort study of the kynurenine to tryptophan ratio in sepsis: association with impaired immune and microvascular function.* PLoS One, 2011. **6**(6): p. e21185.

439. Marshall, J.C., *Why have clinical trials in sepsis failed?* Trends Mol Med, 2014. **20**(4): p. 195-203.

440. Nemzek, J.A., K.M. Hugunin, and M.R. Opp, *Modeling sepsis in the laboratory: merging sound science with animal well-being.* Comp Med, 2008. **58**(2): p. 120-8.

441. Angus, D.C., *The search for effective therapy for sepsis: back to the drawing board?* JAMA, 2011. **306**(23): p. 2614-5.

442. Docke, W.D., et al., *Monocyte deactivation in septic patients: restoration by IFN-gamma treatment.* Nat Med, 1997. **3**(6): p. 678-81.

443. Flohe, S., et al., *Influence of granulocyte-macrophage colony-stimulating factor (GM-CSF) on whole blood endotoxin responsiveness following trauma, cardiopulmonary bypass, and severe sepsis.* Shock, 1999. **12**(1): p. 17-24.

444. Meisel, C., et al., *Granulocyte-macrophage colony-stimulating factor to reverse sepsis-associated immunosuppression: a double-blind, randomized, placebo-controlled multicenter trial.* Am J Respir Crit Care Med, 2009. **180**(7): p. 640-8.

445. Leentjens, J., et al., *Reversal of immunoparalysis in humans in vivo: a double-blind, placebo-controlled, randomized pilot study.* Am J Respir Crit Care Med, 2012. **186**(9): p. 838-45.

446. Delsing, C.E., et al., *Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series.* BMC Infect Dis, 2014. **14**: p. 166.

447. *GM-CSF to Decrease ICU Acquired Infections*. https://ClinicalTrials.gov/show/NCT02361528.

448. Ito, S., et al., *Interleukin-10 inhibits expression of both interferon alpha- and interferon gamma- induced genes by suppressing tyrosine phosphorylation of STAT1.* Blood, 1999. **93**(5): p. 1456-63.

449. Li, J., S.G. Kim, and J. Blenis, *Rapamycin: one drug, many effects.* Cell Metab, 2014. **19**(3): p. 373-9.

450. Sanchez-Rangel, E. and S.E. Inzucchi, *Metformin: clinical use in type 2 diabetes.* Diabetologia, 2017. **60**(9): p. 1586-1593.

451. Guilliams, M., et al., *Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF.* J Exp Med, 2013. **210**(10): p. 1977-92.

452. Herold, S., K. Mayer, and J. Lohmeyer, *Acute lung injury: how macrophages orchestrate resolution of inflammation and tissue repair.* Front Immunol, 2011. **2**: p. 65.

453. Helft, J., et al., *GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells.* Immunity, 2015. **42**(6): p. 1197-211.

454. Chahin, A., et al., *EROD activity induction in peripheral blood lymphocytes, liver and brain tissues of rats orally exposed to polycyclic aromatic hydrocarbons.* Food Chem Toxicol, 2013. **56**: p. 371-80.

Appendices

Appendix A: Supplemental Tables

Table S1. Sequences of primers used for RT-qPCR of human genes examined in this thesis.

Target	Primer Sequences	Amplicon Size (bp)	Accession Number
Ahrr	F- CTGACCCGCTGCTTCATCTG R- ATCGTCATGAGTGGCTCGGG	119	NM_020731
B2m	F- GGCTATCCAGCGTACTCCAAA R- CGGCAGGCATACTCATCTTTTT	246	NM_004048
Cyp1a1	F- AGCTCTGAAGAACTCTCTGG R- TCTCTTCCCTTCACTCTTGG	149	NM_000499
Cyp1b1	F- CTAGGCAAAGGTCCCAGTTC R- GGATGGACAGCGGGTTTAG	108	NM_000104
Hprt	F- ATTGTAATGACCAGTCAACAGGG R- GCATTGTTTTGCCAGTGTCAA	117	NM_000194
Nqo1	F- CGGACCTCTATGCCATGAAC R- GAACAGACTCGGCAGGATAC	102	NM_000903

Table S2. Sequences of primers used for RT-qPCR of mouse genes examined in this thesis.

Target	Primer Sequences	Amplicon Size (bp)	Accession Number
Ahr	F- GCGCCAACATCACCTATGCC R- TTCAGCCGGTCTCTGTGTCG	118	NM_013464
B2m	F- ACCCGCCTCACATTGAAATCC R- CGATCCCAGTAGACGGTCTTG	199	NM_009735
Cyp1a1	F- AGGTTACTGGCTCTGGATAC R- CAATGAGGCTGTCTGTGATG	183	NM_001136059
Cyp1a2	F- AAACTGTCCAGGAGCACTAC R- CTGTGGTGACTGTGTCAAAG	174	NM_009993
Cyp1b1	F- TGGCTGCTCATCCTCTTTAC R- CATGACATATGGCAGGTTGG	126	NM_009994
Hprt	F- AGTCCCAGCGTCGTGATTAG R- CAGAGGGCCACAATGTGATG	185	NM_013556
Ido1	F- ACTGAGAGGACACAGGTTAC R- CAGGACACAGTCTGCATAAG	187	NM_008324
Tdo2	F- GTGCTGCTCTGCTTGTTTG R- CTGGAAAGGGACCTGGAATC	141	NM_019911