THE MANY FACES OF MACROPHAGES:
Polarization, Endotoxin Priming, and Salmonella Resistance

by

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The Many Faces of Macrophages: Polarization, Endotoxin Priming, and Salmonella Resistance

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Abstract

Different forms of macrophage activation or polarization are relevant in the pathogenesis of a variety of diseases from inflammatory conditions to infections. It has been previously established that classically activated or M1 macrophages such as those produced by IFNγ stimulation are non-permissive for intracellular *Salmonella* infection, while alternatively activated or M2 macrophages such as those produced by IL-4 stimulation are permissive for *Salmonella* growth. It is not known whether endotoxin tolerant macrophages (primed with endotoxin stimulation), such as those observed in sepsis, are permissive for *Salmonella* growth. A gentamicin protection assay was performed for these three types of differently polarized human monocyte-derived macrophages (MDM) *in vitro*, and bacterial load measured through colony counts and microscopy. Endotoxin primed MDM (M^{EP}) had a similar bacterial load to M1 macrophages at the initial and 2-hour time-points, but became more susceptible to *Salmonella* by the 4- and 24-hour time-points. Transcriptomic comparisons using RNA-Seq were performed to generate hypotheses regarding mechanisms for the differences observed between these polarization types, based on differential gene expression. Key immune pathways including JAK-STAT were enriched in uninfected M1 and M^{EP} compared to uninfected M2 macrophages, suggesting a priming effect on these pathways due to polarization. Meanwhile, *Salmonella*-infected M1 showed increased expression of key inflammasome genes and *Salmonella* resistance genes compared to M2 and M^{EP} macrophages. These effects were also observed in similarly treated human induced-pluripotent stem cell derived macrophages (iPSDM), further validating the usefulness of iPSDM as a macrophage model in polarization and infection experiments. In order to investigate the mechanistic relevance of these observations, Ruxolitinib was applied to inhibit JAK1-2 during the polarization phase of the experiment. This increased *Salmonella* permissiveness at the 4-hour time point in resistant M1 macrophages, but not in M2 or M^{EP} macrophages, which are susceptible at this time point. This is consistent with an important role for JAK-STAT priming and resistance to *Salmonella* infection. These observations provide insights into the effects of polarization on *Salmonella* resistance in macrophages, and the suitability of iPSDM for macrophage study.
Lay Summary

Macrophages are white blood cells that come in many varieties with different jobs. One variety is common in the deadly condition of sepsis, in which the immune system fails to respond to infection. *Salmonella* can grow inside repair macrophages, but not inside antimicrobial macrophages. I found that *Salmonella* grows well in sepsis macrophages only during the first two hours of infection. Analysis of active genes in these cells shows that even before they see *Salmonella*, antimicrobial macrophages and those primed for sepsis have high activity in specific anti-infection genes. Repair macrophages only activate these genes when exposed to *Salmonella*. This may mean that antimicrobial and sepsis-primed macrophages are initially better prepared when they encounter *Salmonella*. Antimicrobial macrophages also express some anti-*Salmonella* genes that sepsis macrophages do not. Better understanding of these genes could help us develop treatments to improve the ability of macrophages to resist infection and treat sepsis.
Preface

Research was primarily conducted at the University of British Columbia (UBC), Vancouver, in accordance with the ethics approval from the UBC Office of Research Services Clinical Research Ethics board for human subject research under ethics certificate H04-70232. The dataset from Chapter 5 involving iPSDM was received from the Wellcome Sanger Institute, and collected in accordance with the approved ethics guidelines of the Wellcome Trust Sanger Institute described at https://www.sanger.ac.uk/about/who-we-are/research-policies/working-human-material.

Dr. R.E.W. (Bob) Hancock was the thesis supervisor and provided guidance on the thesis concepts, research and writing. Lipopolysaccharide used in the experiments was isolated by Ms. Manjeet Bains.

In Chapter 3, I designed, performed, analyzed, and wrote up the experiments with the following acknowledgements: Dr. Evan Haney and Ms. Beverlie Baquir collected the blood samples from which primary cells were isolated. Dr. Mike Trimble and Dr. Heidi Wolfmeier provided technical assistance with the confocal microscope.

In Chapter 4, I designed, performed, analyzed, and wrote up the experiments with the following acknowledgements: Dr. Evan Haney and Ms. Beverlie Baquir collected the blood samples. Mr. Reza Falsafi provided assistance with isolation of RNA samples, and prepared the RNA samples for sequencing. Mr. Travis Blimkie aligned the RNA-Seq results and generated count tables, and he and Dr. Amy Lee provided guidance on the analysis of this data.

In Chapter 5, I performed the analysis and wrote up the experiments. Dr. Christine Hale at the Gordon Dougan Lab, Wellcome Sanger Institute, collected the iPSDM RNA-Seq data. Dr. Amy Lee aligned the RNA-Seq output files and generated count tables, and provided guidance on the analysis of this data.

In Chapter 6, I designed, performed, analyzed, and wrote up the experiments with the following acknowledgement: blood samples were collected by Dr. Evan Haney and Ms. Beverlie Baquir.
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List of Abbreviations

AIM2 – absent in melanoma 2
Amp – ampicillin
ASC – apoptosis-associated speck-like protein containing a CARD (also called PYCARD)
ATP – adenosine triphosphate
BMDM – bone-marrow-derived macrophages (murine)
CASP1 – caspase-1
CFU – colony-forming unit
COX - cyclooxygenase
DE – differentially expressed
ESDM – embryonic stem cell derived macrophages (murine)
ET – endotoxin tolerant
FBS – fetal bovine serum
FcγRs – Fcγ receptor
GAGs – glycosaminoglycans
GBP – guanylate binding proteins
GC – glucocorticoids
GTPase – guanosine triphosphatase
HDP – host defense peptide
HOPS – Homotypic fusion and Protein Sorting
IC – immune complex
INFγ – interferon gamma
IL – interleukin
ILC3 – group 3 innate lymphoid cells
iPSC – induced pluripotent stem cells
iPSDM – induced pluripotent stem cell derived macrophages (human)
JAK – Janus kinase
LB – Luria-Bertani medium
LDH – lactate dehydrogenase
LPS – lipopolysaccharide
M1 – macrophage polarized by 24-hour treatment with IFNγ
M2 – macrophage polarized by 24-hour treatment with IL4
M\textsuperscript{EP} – macrophage polarized by 24-hour treatment with LPS (endotoxin priming to create endotoxin tolerant macrophage after Salmonella treatment)
MDM – monocyte-derived macrophage (human)
MHC – major histocompatibility complex
MOI – multiplicity of infection
NADPH – nicotinamide adenine dinucleotide phosphate, reduced
NAIP – NLR Family Apoptosis Inhibitory Protein
NEK7 – NIMA related kinase 7
NK – natural killer (cells)
NKT – natural killer T (cells)
NLR – nod-like receptor
NLRP3 – NLR family pyrin domain containing 3
NLRP4 – NLR family pyrin domain containing 4
NO – nitric oxide
OD\textsubscript{600} – optical density at 600nm
P2RX7 - purinergic receptor P2X 7
PANX1 – pannexin 1
PG – prostaglandin
PL – phospholipase
PBMC – peripheral blood mononuclear cell
PBS – phosphate-buffered saline
PCA – principal component analysis
PRR – (pathogen/danger) pattern recognition receptors
Rlog – regularized log2 expression (of gene counts)
SCV – Salmonella-containing vacuole
SPI – Salmonella pathogenicity islands
STAT – signal transducers and activators of transcription
SYK – spleen associated tyrosine kinase
T3SS – type-III secretion system
TLR – toll-like receptor
TRAM (Trif-related adaptor molecule)
TNF – tumour necrosis factor

In addition, official gene symbols are used.
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Dedication

To my mother and my husband.
Chapter 1: Introduction

1.1 Salmonella infection: pathogen versus host

*Salmonella* is a common foodborne pathogen that causes significant morbidity and mortality worldwide\(^1\). Infections can be acute or chronic, and consequences range from localized gastroenteritis to systemic infection and sepsis\(^2\). The ability to evade and manipulate the immune response renders *Salmonella* of great interest to microbiologists and immunologists alike\(^2\). Indeed, *Salmonella* is even capable of surviving and replicating inside immune cells such as macrophages. However, its ability to do so depends on the polarization state of the macrophage\(^3\)–\(^5\). Understanding the dynamics of *Salmonella* infection of differently polarized macrophages could lead to improved treatments for this pathogen.

1.1.1 General characteristics of *Salmonella*

*Salmonella* are Gram-negative aerobic enteric bacteria from the family *Enterobacteriaceae*\(^2\). The genus is diverse, containing species specialized for different hosts, and possessing different levels of virulence from commensal to seriously pathogenic\(^2\). Most infections in humans are caused by a single species, *Salmonella enterica*, which is divided into six subspecies and over 2500 serovars defined by the flagellar, carbohydrate, and LPS antigens found on their surfaces\(^2,6\). This thesis concerns itself from here on with *Salmonella enterica*.

Most varieties of *Salmonella* typically cause localized gastroenteritis. However, systemic infection, as seen in typhoid fever, can result from infection with particular serovars such as *S. Typhi*\(^2\). Some non-typhoidal varieties can also cause systemic infection, particularly in immunocompromised patients such as those with HIV\(^2\). According to 2010 estimates by Majowicz et al\(^1\), there are globally 93.8 million cases of non-typhoidal gastroenteritis, causing 155,000 deaths. Between 55% and 95% of these cases are believed to be foodborne\(^1\), with alternative routes being contaminated water or contact with *Salmonella* carriers\(^2\). Antibiotic use in agriculture has led to a significant increase in antibiotic resistance in *Salmonella* isolates within the food supply\(^7\). These resistant *Salmonella* are particularly dangerous in the event that they enter the blood and cause septicemia or sepsis\(^8,9\).

*S. enterica* serovar Typhimurium is a common cause of gastroenteritis and thus a significant human pathogen. Moreover, Typhimurium is commonly used as a model for systemic infection with *S. Typhi*. Serovar Typhi is a specifically human pathogen, and few animal models exist to study it directly\(^10\), but mice are susceptible to systemic salmonellosis caused by *S.
Typhimurium. Thus, much of the research into mechanisms of Salmonella infection has been performed with Salmonella Typhimurium.

1.1.2 Dynamics of Salmonella infection

Following ingestion, Salmonella reaches the stomach. Acid resistance allows some portion of the Salmonella to survive, colonize the intestine, and adhere to the apical membrane of epithelial cells. Salmonella then invades intestinal epithelial cells, through mechanisms that are not entirely clear and may vary between different Salmonella isolates. Upon invasion, epithelial cells secrete cytokines and chemokines, such as IL-8, MCP-1, and MIP-3α, which attract neutrophils, monocytes, and dendritic cells (DCs) respectively. After entering a cell, Salmonella promotes the formation of a modified acidified phagosome, the Salmonella-containing vacuole or SCV. Salmonella virulence largely depends on the ability of the bacteria to survive inside of host cells, including macrophages. This is achieved through mechanisms such as suppressing immune defenses and formation of a variety of tubular networks within the cell.

Epithelial cells have few mechanisms to limit growth of intracellular Salmonella, in contrast to phagocytes, which contain high levels of defensins and other antimicrobial molecules. Thus in epithelial cells, Salmonella which leave the SCV and enter the cytosol can hyper-replicate. This process can activate host cell surveillance through the inflammasome, discussed in more detail in 1.3.3.2. Replication in epithelial cytosol also allows invasion-primed Salmonella to escape through the intestinal lining to the lamina propria where it can be phagocytosed by macrophages or other polymorphic cells. In these cells, Salmonella blocks the SCV from fusing with the bactericidal phagocyte NADPH oxidase complex, which would lead to oxidative killing of the bacteria. During systemic infection, Salmonella then spreads from these phagocytes to tissues such as the mesenteric lymph nodes, spleen, and liver, where they form granulomatous foci. In particular, Salmonella spreads to sites that have not yet activated local inflammation in response to the infection. Salmonella can penetrate the intestinal lining without invading epithelial cells, but cannot progress further without intracellular replication in phagocytes.

During the early stage of infection, macrophages and neutrophilic granulocytes typically kill large numbers of bacteria through reactive oxygen species. Immune protection depends on cytokines such as IFNγ, which is produced by many types of immune cells during Salmonella infection, with various reports indicating that the main producers early in infection are natural killer (NK) cells, neutrophils, and Group 3 Innate Lymphoid Cells (ILC3s). Natural killer
T (NKT) cells also produce IFNγ during early infection (starting at 2-3 days)\textsuperscript{35}. IFNγ-activated macrophages produce cytokines including IL-12, IL-15, IL-18, and IL-23, which activate IFNγ-producing cells, creating a positive feedback loop\textsuperscript{34,36–39}. Specifically, IL-12 and IL-23 induce IFNγ production from T cells, NK cells, and NKT cells\textsuperscript{39}; IL-12 stimulates IFNγ production from ILC3s\textsuperscript{34}; and IL-15\textsuperscript{36} and IL-18\textsuperscript{37,38} activate NK cells. TNFα produced by macrophages and neutrophils also enhances bacterial killing\textsuperscript{40}.

Once \textit{Salmonella} have transitioned to intracellular infection, after several days of infection, the rate of killing slows\textsuperscript{2,29}. IFNγ production shifts to NK cells and T cells, which are also the primary sources of IFNγ in secondary infection\textsuperscript{33}. Persistent inflammation can result in fibrosis, mediated by production of IL-17 and IL-22 by ILC3s\textsuperscript{41}. The balance between growth and killing at this point determines the ultimate resolution: bacterial clearance, or failure to control bacterial growth, endotoxin shock, and death\textsuperscript{2}. Even hosts able to recover from systemic infection can carry bacteria, in the absence of symptoms, for months or years\textsuperscript{6}.

\textbf{1.1.3 \textit{Salmonella} detection and evasion}

The success of \textit{Salmonella} infection depends on the interplay between host detection and killing mechanisms, and bacterial evasion and survival. \textit{Salmonella}‘s infective toolbox is largely located in different pathogenicity islands, or clusters of genes that are not found in similar non-pathogenic species\textsuperscript{42}. \textit{Salmonella} expresses these sets of genes at different times during infection. Two \textit{Salmonella} Pathogenicity Islands (SPI) encode separate versions of a Type-III Secretion System (T3SS), which represent a complex assembly of proteins capable of injecting bacterial effector proteins into a target host cell. These proteins can manipulate the host immune system in order to benefit the bacteria\textsuperscript{2}.

\textit{Salmonella} populations can be highly heterogeneous, with different sub-populations of \textit{Salmonella} activating different gene programs, even during the same phases of infection. This diversity improves the ability of the population as a whole to adapt to differing conditions and to variations in the immune response or host cell activation\textsuperscript{27}. The following describes typical events in infection at the population level.

\textbf{1.1.3.1 Phagocytosis of \textit{Salmonella}}

\textit{Salmonella} uptake into macrophages occurs either through invasion mediated by SPI-1 effectors (discussed later in section 1.1.3.3) or via host-mediated phagocytosis. This phagocytosis can be assisted by opsonizing the bacteria with complement or immunoglobulin\textsuperscript{43}. Phagocytosis is
initiated by the recognition of a pathogen by one or more cell surface receptors such as the CD14 receptor or Fcγ Receptors (FcγRs), which detect pathogen signatures or antibody-marked particles. Receptor activation results in actin polymerization, causing the cell membrane to deform around the pathogen in a phagocytic cup, bringing additional receptors to contact and engage with the pathogen. When the plasma membrane has completely surrounded the pathogen, it fuses and internalizes the pathogen in a phagosome\textsuperscript{44,45}.

CD11b, also called Complement Receptor 3 or CD18, is expressed on a variety of phagocytes. It can recognize complement-opsonized particles, as well as a variety of pathogen signatures, and is involved in phagocytosis of \textit{Salmonella} Typhimurium by human macrophages\textsuperscript{46}. Complement-opsonized bacteria sink into the phagocyte, remaining closely associated with the membrane in a tight phagosome, in a process mediated by the GTPase Rho\textsuperscript{47}. Immunoglobulin-opsonized bacteria are phagocytosed through a different process. Binding of the FcγR results in membrane ruffling and the extension of pseudopods over the bacteria, creating a larger compartment. This process involves GTPases Cdc42 and Rac\textsuperscript{47}. The SPI-1 mediated invasion of \textit{Salmonella} more closely resembles the latter FcγR-dependent process\textsuperscript{48}.

Non-opsonic phagocytosis of \textit{Salmonella} occurs via receptors such as MARCO and CD36\textsuperscript{49,50} or the mannose receptor\textsuperscript{43}. Toll-like Receptor 4 (TLR4) can promote phagocytosis of \textit{Salmonella} by upregulating scavenger receptors and promoting actin polymerization\textsuperscript{51}. A recent study by Skejsol \textit{et al} additionally found that TLR4 adaptor TRAM (Trif-related adaptor molecule) and Rab11 family interacting protein 2 form a complex at the phagocytic cups of \textit{E. coli}, resulting in actin remodelling mediated by Rac1 and Cdc42\textsuperscript{52}; a similar process presumably occurs in phagocytosis of other Gram-negative pathogens such as \textit{Salmonella}.

The ability of \textit{Salmonella} to avoid killing by phagocytes depends on its mode of internalization. When \textit{Salmonella} are phagocytosed after being opsonized by immunoglobulin or by complement, the acidification of the SCV is delayed compared to \textit{Salmonella} phagocytosed by a non-opsonic route, or that mediate their own entry into the macrophage. This coincides with reduced survival of the opsonized \textit{Salmonella}\textsuperscript{43}. Internalization of \textit{Salmonella} by FcγRIII-mediated phagocytosis also prevents \textit{Salmonella}’s from interfering with antigen presentation by DCs\textsuperscript{53}.

\textbf{1.1.3.2 Inflammasomes and caspases}

If the SCV is disrupted, bacteria can be more readily detected\textsuperscript{54}. This can result in activation
of multi-protein complexes known as inflammasomes. In macrophages, inflammasome activation can lead to pyroptosis, a rapid pro-inflammatory cell death characterized by the activation of caspase-1 (or in humans, caspase-4 and-555), which leads to cell rupture and release of IL-1β, IL-18, and danger-associated molecular patterns55–57 (Figure 1.1).

**Figure 1.1: Inflammasome signalling pathway diagram.** *Salmonella* flagellin or needle protein [with NLR Family Apoptosis Inhibitory Protein (NAIP)] leads to activation of the NLRP4 inflammasome, in combination with ASC and pro-caspase 1 (Pro-CASP1)58,59. A variety of endogenous danger signals, including K+ efflux (requiring PANX1 and P2RX7), ATP (via SYK60), and reactive oxygen species, lead to NLRP3 activation and formation of an inflammasome along with NEK761,62. Similarly, dsDNA and other pathogen signatures lead to the activation of AIM263 and Pyrin64 inflammasome receptors, respectively. Inflammasome formation causes the cleavage of pro-caspase 1 to caspase-1. Caspase-1 in turn cleaves Gasdermin D (GSDMD), pro-IL-1β, and pro-IL1859, leading to the secretion of IL-1α, IL-1β, IL-18, along with other alarmins, through mechanisms including Gasdermin D pores55,61,65. Figure created for this thesis based on synthesis of cited articles55,58–65.

The molecules released during pyroptosis promote inflammation and can activate various cells in the innate or adaptive immune system55. The release of inflammasome adapter protein ASC encourages further pyroptosis in nearby cells66. Although pyroptosis is often considered to
be an antimicrobial defence mechanism, *Salmonella* are also released following pyroptosis. These bacteria can spread and contribute to systemic infection, but can also be phagocytosed and killed by neutrophils via reactive oxygen species independently of cytokine production. While neutrophils activate the inflammasome and produce IL-1β, they do not experience pyroptotic death, thus allowing them to maintain defenses against *Salmonella*.

Pyroptosis is important to host defense against *Salmonella*; mice lacking caspase-1, IL-1β, or IL-18 are more vulnerable to *Salmonella* infection. IL-1β is necessary during the intestinal phase of infection. In contrast, IL-18 provides defense against systemic spread of *Salmonella* infection by recruiting natural killer cells, and inducing these natural killer cells to produce IFN-γ. At least one effector of pyroptosis, Gasdermin D, can also kill bacteria directly. Further, in addition to inducing pyroptosis, caspase-1 regulates the formation of phagosomes and modulates cell rigidity in order to control bacterial uptake, and ultimately bacterial load.

Pyroptosis can easily be mistaken for the similar, but non-inflammatory, apoptotic programmed cell death. Pyroptotic cells are swollen and Annexin-V positive, similar to cells undergoing apoptosis. Chromatin condensation is also observed in pyroptosis, although with an intact nucleus in pyroptotic cells, and TUNEL staining is present at lower levels than in apoptosis, resulting in minimal DNA laddering. Thus pyroptosis can be identified by the combination of Annexin-V positivity combined with the absence of DNA laddering.

The pattern recognition receptors NLRC4 and NLRP3 are responsible for detection of cytosolic *Salmonella* by inflammasomes. *Salmonella* flagellin activates NLRC4, which leads to the activation of caspase-1 and pyroptosis (Figure 1.1). This activation is generally dependent on the *Salmonella* T3SS, which can deliver flagellin to the cytosol. Recent studies show that, in humans, NLRC4 may also be activated by the *Salmonella* SPI-1 T3SS needle protein via interaction with NAIP. *Salmonella* suppresses these needle proteins as well as flagellin upon entering the host cell, in order to escape detection by the NLRC4 inflammasome. NLRP3 senses a more diverse array of signals (Figure 1.1). While it is unclear exactly how this inflammasome is activated in macrophages, the process is independent both of flagellin and of SPI-2, and may involve oxidative stress and/or other host-cell-derived stress and danger signals. The extent and significance of NLRP3 activation in *Salmonella* infection is currently the subject of debate. Other bacterial sensors may be involved in *Salmonella* infection (Figure 1.1). AIM2 is also an NLR and recognizes double-stranded DNA in the cytosol. While AIM2 is important in...
maintaining epithelial integrity within the intestine during *Salmonella* infection\(^{81}\), it does not appear to be involved in inflammasome activation in macrophages in response to *Salmonella*\(^ {82}\). In addition, bacterial toxins can modify Rho-GTPases, which, in the presence of microtubules, results in the activation of pyrin and caspase-1\(^ {55}\). *Salmonella* is known to activate and deactivate at least one Rho-GTPase, Cdc42\(^ {18,19}\).

### 1.1.3.3 *Salmonella* Pathogenicity Islands: SPI-1 and SPI-2

*Salmonella* infection relies, in sequence, on SPI-1 for entry and SPI-2 for intracellular survival\(^ {42,83}\). Replication of *Salmonella* in MDM depends on whether the bacteria are in log phase (in which case they are SPI-1 induced) or stationary phase (not SPI-1 induced)\(^ 4\). Log-phase, SPI-1 induced *Salmonella* show higher levels of replication, but also trigger pyroptosis, unlike slower-replicating stationary phase bacteria that have not induced SPI-1\(^ 4\). Much previous work with macrophages has used stationary-phase bacteria under the assumption that this best represents bacteria encountered by macrophages *in vivo*\(^ 4\). However, SPI-1 induced, invasion-primed *Salmonella* can be released from epithelial cells and thus also encountered by monocytes or macrophages\(^ {84,85}\).

SPI-1 is necessary for invasion of epithelial cells\(^ {42}\). SPI-1 effectors delivered by the T3SS-1, including SopE, activate host cell signaling and cytoskeletal machinery, including Cdc42, in order to induce bacterial uptake\(^ 6\). At the end of this process, a different effector SptP deactivates Cdc42 and Rac1, leading to vacuole formation\(^ {11}\).

SPI-1 effectors also activate parts of the immune response. SPI-1 effector SipA induces pathogen elicited epithelial chemoattractant (PEEC) production, causing recruitment and activation of neutrophils\(^ 6\). The activation of Cdc42 by SopE also activates NF-κB\(^ {18}\), resulting in an early inflammatory response that lasts until Cdc42 is deactivated by SptP\(^ {19}\). The observed tendency of SPI-1 induced *Salmonella* to cause pyroptosis in host macrophages\(^ 4\) is likely due to effector SipB binding to caspase-1 in the cytosol\(^ {86}\). SPI-1 can also induce apoptosis in macrophages\(^ {42}\). Despite having an important role in early inflammation, SPI-1 is not the sole vehicle eliciting *Salmonella*-induced inflammation; *Salmonella* LPS induces a strong inflammatory response in macrophages through TLR4\(^ {87}\).

Once *Salmonella* enters a cell, SPI-1 is downregulated. At this time SPI-2, the T3SS-2, and the two-component PhoP/Q system (the latter mediating resistance to non-oxidative killing) are upregulated and the SCV acidifies\(^ {85,88,89}\). SPI-2 is induced both within epithelial cells and in
phagocytes including macrophages, and includes genes necessary for survival within both types of cells. Expression of SPI-2 genes may be triggered by low concentrations of Mg\(^{2+}\), Ca\(^{2+}\), or phosphate starvation, and secretion of SPI-2 proteins may be linked to low pH. These triggers, low nutrient levels and acidity, are characteristic of the phagosome. SPI-2 and PhoP/Q are responsible for shielding *Salmonella* from the immune system by means of masking pathogen signatures. Activation of PhoP-PhoQ system results in modifications to the immunostimulatory lipid A portion of *Salmonella* LPS, decreasing TLR4 activation. Further, SPI-2 induction is associated with suppression of flagellin, allowing *Salmonella* to evade detection by NLRC4. This does not entirely prevent an inflammatory response, since *Salmonella* containing SPI-2 alone activates MyD88-dependent innate immune responses, while those containing SPI-1 alone do not. Finally, the SPI-2 T3SS needle protein does not appear to activate NLRC4 as the SPI-1 needle protein does.

In phagocytes, SPI-2 serves to protect the SCV by manipulating vesicle trafficking. Bacterial effectors such as SifA interact with host Pleckstrin homology domain containing, family M member 1 (PLEKHM1), Rab7, and Homotypic fusion and protein sorting protein (HOPS), or with SifA kinesin interacting protein (SKIP) to recruit microtubules and promote their association with the SCV. *Salmonella* prevents fusion of the SCV with the lysosome through the retention of early endosome markers: Rab5, transferrin receptor (TfnR) and early endosome associated antigen 1 (EEA1). The SPI-2 virulence protein SpiC also interferes with phagosome-lysosome maturation, at least in part through interaction with macrophage TassC. The SPI-2 is responsible for blocking fusion of the SCV with the phagocyte oxidase complex (NADPH oxidase), though this may not result from endosomal trafficking.

During early infection, *Salmonella* can replicate in an SPI-2 independent fashion, but later infection is SPI-2 dependent. In contrast to the pyroptosis observed in infection with SPI-1-induced *Salmonella*, subsequent SPI-2 dependent replication leads to apoptosis and the spread of the *Salmonella*. Overall, SPI-2 is necessary for systemic disease, and manipulations of the immune system effected by SPI-2 are important to the success of the pathogen.

### 1.1.4 Immune modulation as a strategy for treating *Salmonella*

Because the ability of *Salmonella* to replicate within a macrophage depends on the macrophage activation state, and the spread of *Salmonella* depends on its ability to replicate within phagocytes such as macrophages, manipulating macrophage activation or functions could
lead to new treatments for this infection. This strategy could be particularly important in the case of drug-resistant *Salmonella*, which are increasingly common and particularly dangerous when progressing to sepsis.

As a response to increasing numbers of drug-resistant pathogens observed in hospitals, and slowed development of new antibiotics, immune stimulation and modulation have been proposed as an alternative to antibiotic treatment. These approaches include the use of TLR agonists and immunomodulatory host defense peptides. Notably, host defense peptides have shown protective ability in infections including drug-resistant *Mycobacterium tuberculosis* which is attributed not to direct antimicrobial action, but to modulation of inflammation and enhanced macrophage activation.

Developing immunomodulatory treatments requires effectively targeting the complex responses involved in host-pathogen interactions. Yeung *et al.* recently used a genome-wide knockout screen to identify genes that are involved in *Salmonella* uptake in THP-1 cells. Inhibitors for selected candidate genes reduced intracellular *Salmonella* load, suggesting possible application of these inhibitors in *Salmonella* treatment. Immunomodulatory treatments are more likely to be successful given a strong understanding of the underlying biology of *Salmonella*-host interactions, which requires a specialized research approach.

### 1.2 A systems approach to innate immunity

The systems regulating innate immunity and inflammation are highly complex. Over 2,000 genes have been identified as up- and downregulated in response to immune stimuli. Moreover, the pathways involved contain feedback and feed-forward regulatory mechanisms and there is crosstalk between pathways. This degree of complexity makes it challenging to understand these systems in efforts to develop appropriate therapeutics. Failure to understand innate immunity has resulted in difficulties in bringing anti-inflammatory and immune modulatory agents through clinical trials. In particular, immune modulatory treatments for sepsis have been ineffective to date due to incomplete understanding of the immune dysfunction involved.

The innate immune system contains a wide variety of receptors that recognize conserved pathogen signatures. These so-called “pattern recognition receptors” (PRRs) are classified by family, including the Toll-like receptor (TLR), Nod-like receptor (NLR), and C-type lectin receptor (CLR) families. A selection of PRRs are described in Table 1. In total, there are at least 10 TLR and 23 NLR genes in humans, 12 TLR and 34 NLR genes in mice, and others including...
TLRs can form heterodimeric complexes with other receptors, and PRRs use a variety of adaptors to transmit signals. The signaling pathways of different PRRs overlap in some ways. For example, several TLRs signal through MyD88, and TLRs and some NLRs activate the transcription factor NF-κB. This results in synergy and redundancy in the response to certain signatures. At the same time, differences between pathways result in an immune response tailored to the type of pathogen marker recognized. For example, TLR3, which recognizes retroviruses, signals through TRIF, which activates IRF3 and antiviral Type 1 interferon in addition to NF-κB. Meanwhile, TLR4 signals both through MyD88 and TRIF, and NLRs are known for the formation of inflammasomes.

Table 1.1: Selected pathogen recognition receptors, their localization, recognized pathogens, and signatures. Abbreviations: P. fal = Plasmodium falciparum. Gram (+) = Gram positive. Gram (-) = Gram-negative. Sources

<table>
<thead>
<tr>
<th>PRR</th>
<th>Cellular location</th>
<th>Pathogen detected</th>
<th>Signatures</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TLRs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR1/2</td>
<td>Plasma membrane</td>
<td>Gram (+) bacteria</td>
<td>Peptidoglycan, triacyl lipoprotein, lipoteichoic acid</td>
</tr>
<tr>
<td>TLR3</td>
<td>Endolysosome</td>
<td>Retrovirus</td>
<td>dsRNA, Poly(I:C) (synthetic dsRNA analogue)</td>
</tr>
<tr>
<td>TLR4</td>
<td>Plasma membrane</td>
<td>Gram (-) bacteria</td>
<td>LPS, mannans, glycoinositol phospholipids</td>
</tr>
<tr>
<td>TLR5</td>
<td>Plasma membrane</td>
<td>Flagellated bacteria</td>
<td>Flagellin</td>
</tr>
<tr>
<td>TLR6/2</td>
<td>Plasma membrane</td>
<td>Mycobacteria</td>
<td>Diacyl lipoprotein</td>
</tr>
<tr>
<td>TLR8</td>
<td>Endolysosome</td>
<td>Viruses</td>
<td>ssRNA</td>
</tr>
<tr>
<td>TLR9</td>
<td>Endolysosome</td>
<td>Bacteria, DNA viruses, P. fal.</td>
<td>Unmethylated CpG-DNA, hemozoin</td>
</tr>
<tr>
<td><strong>NLRs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAIP</td>
<td>Cytoplasm</td>
<td>Flagellated bacteria</td>
<td>Flagellin</td>
</tr>
<tr>
<td>NLRC4</td>
<td>Cytoplasm</td>
<td>Flagellated bacteria</td>
<td>Flagellin</td>
</tr>
<tr>
<td>NOD2</td>
<td>Cytoplasm</td>
<td>Bacteria, Mycobacterium</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td><strong>RLRs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA5</td>
<td>Cytoplasm</td>
<td>Picornaviruses, retroviruses</td>
<td>Long dsRNA, Poly(I:C) (synthetic dsRNA analogue)</td>
</tr>
<tr>
<td>RIG-1</td>
<td>Cytoplasm</td>
<td>Viruses</td>
<td>dsRNA, Poly(dA:dT) (synthetic dsDNA)</td>
</tr>
<tr>
<td><strong>CLRs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dectin-1</td>
<td>Plasma membrane</td>
<td>Fungi</td>
<td>β-glucan</td>
</tr>
<tr>
<td>Dectin-2</td>
<td>Plasma membrane</td>
<td>Fungi</td>
<td>α-mannans</td>
</tr>
<tr>
<td><strong>DNA Sensors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIM2</td>
<td>Cytoplasm</td>
<td>Intracellular pathogens, DNA damage</td>
<td>dsDNA, Poly(dA:dT) (synthetic dsDNA)</td>
</tr>
</tbody>
</table>
Salmonella contains a variety of these pathogen signatures, including LPS, flagellin, lipoproteins, CpG-DNA, and the SPI-1 needle complex rod protein (activating NLRP4)\textsuperscript{116–118}. These molecules activate 8 different PRRs: TLR1, TLR2, TLR4, TLR5, TLR6, TLR9, NAIP, and NLRC4 (Table 1.1). Salmonella effectors can also activate NOD1/2 signalling in addition to their effects on actin\textsuperscript{119}. All these signals are integrated in the host response to Salmonella.

In addition to microbial signatures, one must consider the immunomodulatory effects of inflammatory cytokines, host defense peptides, and endogenous danger signals such as heat shock proteins. Cytokines are generally involved in stimulating or suppressing inflammation and in regulating wound repair\textsuperscript{120}, and are expressed by a wide variety of cell types, as detailed in Table 1.2. Chemokines, a subset of cytokines that have the ability to promote chemotaxis\textsuperscript{105}, are involved in recruiting immune cells to the site of infection as well as homeostasis\textsuperscript{121}. Cytokines signal in part through a set of four Janus kinases (JAKs): JAK1, JAK2, JAK3, and TYK2, which phosphorylate transcription factors known as signal transducers and activators of transcription (STATs)\textsuperscript{121}. Despite a large amount of overlap in the JAKs and STATs activated by different cytokines, cytokines have a variety of effects. This variety may partly result from the activation of other signaling pathways. For example, IL-4 activates insulin response substrate -1 and -2, resulting in resistance to apoptosis\textsuperscript{121}. Cytokines can act synergistically, or suppress the function of other cytokines\textsuperscript{122}.

Not only do the signaling pathways used by different cytokines partially overlap, but the pathways activated by endogenous signals such as cytokines and danger molecules can also overlap with those used by microbial signals. For example, both LPS and the cytokine IFNγ can induce transcription of members of the Interferon Regulatory Factor (IRF) family\textsuperscript{126}, heat shock protein HSP60 activates TLR4\textsuperscript{127}, and NLRs can respond to cellular stress\textsuperscript{115}. The mechanisms by which the integration of these different signals occur are not well understood, and some signaling molecules appear to have different roles depending on the extracellular environment and activity of other signals\textsuperscript{112,128,129}. For example, individual cytokines sometimes have contradictory functions that depend on circumstances including adhesion to the extracellular matrix or to other cells\textsuperscript{120}.

To untangle this complexity and investigate innate immunity on a systems level, special methods and tools have been developed. Cytometry allows the assessment of mixed populations
of cells, while “omics” technologies such as transcriptomics, proteomics, and metabolomics offer high-level detailed insights into the activities of those cells\textsuperscript{136,137}. In the future, tools such as single-cell transcriptomics or epigenomics may become more common in assessing the activation of innate immune cells, especially the diverse population that represents macrophages\textsuperscript{138}.

Table 1.2: Selected cytokines and chemokines and their innate immune functions, and cellular sources including both innate and adaptive immune cells. Sources\textsuperscript{105,120,121,128,130–135}.

<table>
<thead>
<tr>
<th>Cytokine / Chemokine</th>
<th>Function</th>
<th>Primary Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNα/β</td>
<td>Antiviral, gut homeostasis</td>
<td>B cells, T cells, NK cells</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Antiviral, proinflammatory (increasing TNF, NO, adhesion molecules), macrophage activation, gut homeostasis</td>
<td>Th1, NK cells</td>
</tr>
<tr>
<td>IL-1α/β</td>
<td>Proinflammatory (increase prostaglandin and NO production, adhesion molecules), T lymphocyte activation, proliferation, apoptosis, differentiation</td>
<td>Macrophages, monocytes, dendritic cells</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Anti-inflammatory (competitive inhibitor or IL-1α/β)</td>
<td>Macrophages, monocytes, dendritic cells</td>
</tr>
<tr>
<td>IL-4</td>
<td>Anti-inflammatory, stimulates IL-1ra, cell proliferation (T cells, B cell, structural cells), B-cell and macrophage activation (humoral response)</td>
<td>Th2 cells, mast cells, basophils</td>
</tr>
<tr>
<td>IL-6</td>
<td>Pro- and anti-inflammatory (downregulates TNF, IL-1, and IFNγ), activates T and B lymphocytes, differentiation</td>
<td>Macrophages, Th2 cells, adipocytes</td>
</tr>
<tr>
<td>IL-8 / CXCL8</td>
<td>Pro-inflammatory, recruits and activates neutrophils, angiogenesis (produced 3-24 hours after infection)</td>
<td>Macrophages, epithelial and endothelial cells</td>
</tr>
<tr>
<td>IL-10</td>
<td>Potently anti-inflammatory, inhibition of LPS and TNF signaling and pro-inflammatory cytokines, macrophage activation, humoral response</td>
<td>Macrophages, monocytes, Th1 and Th2 cells, B cells</td>
</tr>
<tr>
<td>IL-12</td>
<td>Proinflammatory, cell differentiation, activates NK cells</td>
<td>Macrophages, dendritic cells, neutrophils</td>
</tr>
<tr>
<td>IL-13</td>
<td>Anti-inflammatory</td>
<td>Th2 cells</td>
</tr>
<tr>
<td>MCP-1 / CCL2</td>
<td>Attracts monocytes, enhances IL-4 production from T cells, angiogenesis</td>
<td>Monocytes, macrophages, T cells, NK cells, immature dendritic cells, B cells, basophils, epithelial cells</td>
</tr>
<tr>
<td>TNFα/β</td>
<td>Proinflammatory (increase prostaglandin and NO, adhesion molecules), cytokine production, cell proliferation, apoptosis, neutrophil activation, anti-infection</td>
<td>Macrophages, NK cells, CD4+ lymphocytes, adipocytes, Th1 cells, mast cells</td>
</tr>
</tbody>
</table>
1.3 Macrophages

Macrophages can be found in a variety of tissues, and patrol the body to detect infection\(^{44}\). As a result, resident tissue macrophages are often the first cells to encounter pathogens\(^ {44}\). Macrophages are highly heterogeneous cells that can serve a variety of functions depending on their tissue location and environmental signals, including phagocytosis of pathogens and debris, bacterial killing, regulation of inflammation through production of pro- and anti-inflammatory cytokines, and wound repair\(^ {139}\). The plasticity of macrophage functions\(^ {140}\) makes them a target for immunomodulatory therapies.

1.3.1 Macrophage origins and development

Macrophages are recruited during an infection, due to the production of chemokines at the site of infection. They enter tissues in the form of immature monocytes which then differentiate into mature macrophages, known as monocyte-derived-macrophages (MDM)\(^ {141}\). Monocytes are typically described as classical monocytes, expressing LPS co-receptor CD14, and patrolling monocytes, which express low levels of CD14 but higher levels of CD16 (phagocytic receptor FcγRIII)\(^ {141}\). Classical monocytes are found in the blood and some tissues such as the spleen\(^ {142}\).

During inflammation, they migrate to tissues and leave the blood vessels in response to MCP-1 (also called CCL2), and differentiate into macrophages in response to M-CSF (also called CSF-1)\(^ {143,144}\).

Monocytes are not the only source of macrophages, however. Macrophages found in tissues can be derived from embryo yolk sac or fetal liver progenitors rather than from blood monocytes\(^ {141,145}\). Many varieties of embryonic yolk-sac derived tissue macrophages, such as microglia in the brain, are capable of renewing their populations without assistance from blood monocytes\(^ {145}\). In contrast, some tissue macrophages such as those in the intestine are exclusively regenerated through blood monocytes rather than yolk-sac derived cells\(^ {146,147}\). Inflammation can cause proliferation of local resident macrophages\(^ {148}\), or cause monocytes to differentiate into varieties of tissue macrophages, such as bone osteoclasts, that would not be replenished in this way under homeostatic conditions\(^ {145}\). Tissue macrophages have diverse transcriptomic profiles, reflecting differences in their functions and origins\(^ {149}\); for example, monocytes differentiating into tissue macrophages will have somewhat different profiles from neighbors derived from the yolk-sac\(^ {138}\).
1.3.2 Macrophage functions in immunity and homeostasis

Macrophages participate in all phases of the defense against infection. Early in infection, inflammatory macrophages are phagocytic and antimicrobial; they produce chemokines, pro-inflammatory cytokines, and reactive oxygen/nitrogen species, and present antigen to effector CD4+ T cells. During the resolution of inflammation, macrophages are immunosuppressive. They also perform the key task of removing debris, including dead or dying neutrophils from the site of infection. Neutrophils are rapidly recruited during infection and are important in killing and cytokine/chemokine release, but are short-lived (circulating half-life 6-8 hours) thus requiring their removal. Macrophages are also major contributors to wound healing and regeneration, producing an array of growth factors and soluble mediators that stimulate the differentiation of fibroblasts and synthesis of extracellular matrix.

In addition to their immune functions, macrophages are important in development and homeostasis. They are responsible for clearing apoptotic cells from a variety of tissues. They are involved at both ends of the life cycle of red blood cells, consuming the nuclei extruded from maturing red blood cells, and then phagocytosing senescent red blood cells, which is critical for recycling iron and preventing its toxic accumulation. Phagocytosis by microglia is important in synaptic pruning and remodeling. In the gut, subepithelial macrophages sense cell damage through TLR4 and MyD88 signaling, and respond to promote epithelial proliferation and survival.

The various functions of macrophages are regulated in response to a variety of signals. These include not only by microbial signatures such as LPS, flagellin, and so forth, but also endogenous signals from cytokines such as IFNγ or IL4, or host defense peptides. These signals can cause changes involving epigenetic as well as transcriptional modification. Ultimately, macrophage phenotype is dependent not only on the type of pathogen recognized, but also on the extracellular environment. The diversity in phenotype of activated macrophages that results from these environmental signals is sometimes referred to as macrophage polarization.

1.3.3 The macrophage polarization spectrum

The activation states known as polarization have a profound impact on macrophage function. Polarization affects how the cells react to immune stimuli including LPS and host defense peptides. Transcriptionally, macrophage polarization is associated with significant changes in gene expression, including differing sets of lipid mediators, G-protein-coupled receptors, and...
chemokines\textsuperscript{167}. In addition to recognized immune pathways, macrophage activation involves changes in metabolic responses, and some of these metabolic activities produce further immunomodulatory effects\textsuperscript{168–170}.

Macrophage polarization states are often characterized as the M1 inflammatory type induced by LPS and Th1 cytokines such as IFN\(\gamma\), and the M2 wound healing and/or anti-inflammatory type induced by Th2 cytokines such as IL-4, IL-13, and IL-10\textsuperscript{139}. However, activation is heterogeneous within the M1 and M2 categories, with different polarizing stimuli producing different subsets of markers and functional responses\textsuperscript{171}, and stimuli often producing a spectrum of intermediate states\textsuperscript{165,172}. Thus, a more nuanced characterization has been proposed based on the specific signals used to induce activation\textsuperscript{171}. Macrophage activation is also plastic; after an initial polarization, macrophages may still be shifted from one state to another, e.g. by IFN\(\gamma\) treatment of M2 macrophages\textsuperscript{173}.

\textit{In vivo}, macrophages may take on states with markers and functions resembling different points on the polarization spectrum\textsuperscript{171}. Reflecting plasticity, tissue macrophages generally exist in an M2-like state, executing repair functions, but can switch to an M1-like state in response to infection\textsuperscript{145}. There is however variation between tissues. Alveolar macrophages, found in the lung, show a hybrid type characterized by expression of both M1 and M2 markers\textsuperscript{141}. They are involved in clearance of \textit{Klebsiella pneumonia}\textsuperscript{174} and \textit{Pseudomonas aeruginosa}\textsuperscript{175} (M1-like), recruiting neutrophils in response to \textit{P. aeruginosa} infection\textsuperscript{176} (M1-like) and phagocytosing apoptotic neutrophils during the resolution of \textit{Streptococcus pneumoniae} infection (M2-like)\textsuperscript{177}. Intestinal tissue macrophages also show a hybrid activation, highly phagocytic and microbicidal but not inflammatory, in order to non-destructively clear commensal bacteria that escape from the gut\textsuperscript{146}. In addition to M1 or M2-like states, microglia can adopt a unique activation state characterized by specific neural development genes\textsuperscript{145}.

A holistic, systems-based approach to investigating the role of macrophage activation is advantageous because of both the variety and plasticity in macrophage activation and the potential for discovering effects outside of known immune system functions (such as metabolic differences). A recent study investigating 299 macrophage transcriptomes supported the idea that macrophage activation and polarization exists on a spectrum, rather than in discrete categories, identifying 9 different programs triggered by particular microbial molecules, cytokines, and environmental stimuli\textsuperscript{178}. Some of these programs correspond to traditional M1/M2 polarization, but others do
not, and a linear axis of activation was ultimately not observed\textsuperscript{178}.

With these cautions in mind, I here describe some of the characteristics typically observed in particular types of polarized macrophages. Where the literature refers to specific activating factors, these are indicated in parentheses, e.g. M1(IFN\(\gamma\)) or M2(IL-4). Phenotypes observed in response to multiple stimuli are indicated with a slash, e.g. M2(IL-4/IL-13), while phenotypes requiring combinations of stimuli are indicated additively, e.g. M1(IFN\(\gamma\)+LPS).

1.3.3.1 Characteristics of M1 and M2 activation

Macrophages in the M1 state primarily clear initial infections and contribute to host inflammation (Figure 1.2)\textsuperscript{139}. IFN\(\gamma\) promotes phagocytosis and phagosomes in macrophages\textsuperscript{11}, and M1(IFN\(\gamma\)+LPS) produce reactive oxygen species\textsuperscript{173} and inflammatory cytokines such as TNF, IL-1\(\beta\), and IL-12 as well as IL-6\textsuperscript{139,171}. As a result of this activation, M1 macrophages can cause cell death and tissue damage\textsuperscript{144}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{m1_m2_activation.png}
\caption{M1 and M2 macrophage activation.} \textsuperscript{139,143,144,168,171,182–184}
\end{figure}

M2 macrophages generally perform some combination of the following functions: defense against extracellular parasites, wound healing and tissue repair, and the modulation of immune
responses including inflammation (Figure 1.2)\textsuperscript{139,172,173,179}. Allergy is generally associated with M2 polarization, though it may also involve M1 responses such as inflammasomes\textsuperscript{173}.

In order to avoid excessive damage, the immune system must control inflammation. This occurs in a resolution phase in which pro-inflammatory M1 cells are replaced with wound healing and anti-inflammatory M2 cells\textsuperscript{144}. Interestingly, this can occur even in the apparent absence of M2-inducing cytokines\textsuperscript{144,171}. It is unclear whether this represents some timed switch by which M1 cells convert to M2, or results from a different population of M2 cells such as tissue macrophages becoming predominant\textsuperscript{144}.

M2 macrophages have been characterized in more variations than have M1. Noted variants include activation by IL-4 or IL-13, IL-10, TGFβ, immune complexes (IC) with or without TLR agonists, or glucocorticoids (GC)\textsuperscript{139,171}. These are sometimes referred to via sub-classifications such as M2a, M2b, and so forth, although that terminology is not currently recommended\textsuperscript{171}. A wide variety of receptors, cytokines, chemokines, and other markers have been proposed in characterizing these variants\textsuperscript{171}. Gaps exist in this characterization, however, and there is disagreement regarding whether arginase is expressed in M2b macrophages\textsuperscript{139,180}. Markers can also vary between humans and mice. For example, while arginase is a key marker of the M2 state in mice, human macrophages do not express arginase\textsuperscript{179}, and it is unclear whether IL-10 is an M2 marker in mice\textsuperscript{139,181}.

Just as the immune response requires coordination between M1-driven inflammation and M2-driven resolution, resolution of inflammation involves its own balance between the wound healing and immunosuppressive functions of M2 cells. In executing their wound healing functions, M2(IL4) macrophages may promote fibrosis, in part via production of pro-fibrotic cytokine TGF-β1 and specific extracellular matrix degrading matrix metalloproteinases (MMPs)\textsuperscript{143,144}. In contrast, immune suppressive M2(IL-10) macrophages are antifibrotic, partly due to the expression of a different set of MMPs\textsuperscript{143,144}, and inhibition of myofibroblasts that are involved in the repair response.

Among the various classifications of M2 macrophages, this thesis will focus on those activated by IL-4. M2(IL-4) express a variety of receptors including the mannose receptor 1 (MRC-1/CD206), the macrophage scavenger receptor 1 (MSR-1), the C-type lectin-like receptor Dectin-1, and DC-Sign (CD209)\textsuperscript{167}. M2(IL-4) produce IL-1ra, and inhibit IL-1 activity\textsuperscript{139}. Interestingly, IL-4 stimulation can also induce the proliferation of tissue macrophages, through
either self-renewal of resident tissue macrophages\textsuperscript{185} or conversion of IL-4 activated MDM to peritoneal tissue macrophages\textsuperscript{186}.

Consistent with their different roles, M1 and M2 macrophages are specialized for different types of phagocytosis. M1 cells express FcγRs for phagocytosis of pathogens marked with antibody, while M2 cells generally express efferocytic receptors for apoptotic cells, used in clearing neutrophils in the resolution phase\textsuperscript{141}. Phagocytic ability may vary, however, according to the specific form of polarization. One experiment showed that M2(IL-10) have increased phagocytic activity towards an antibody-opsonized target, while M2(IL-4) have reduced phagocytosis of that target\textsuperscript{187}. In contrast, M2(IL-4) increase sampling of soluble antigens, which may be produced by parasites, through upregulation of pinocytosis and endosome recycling\textsuperscript{179}.

Similarly, M1 and M2 macrophages produce different chemokines suited to their roles. M1(IFNγ/LPS) macrophages produce chemokines that support defenses against intracellular pathogens and viruses by recruiting cells such as Th1 cells and natural killer cells. M2 varieties, including M2(IC+LPS), inhibit these Th1 chemokines and instead produce chemokines that recruit cells such as Th2 cells, involved in tissue remodeling, allergic responses, and anti-helminth defenses\textsuperscript{139}.

Metabolic activity also reflects specialized roles. M1 macrophages derive quick energy from glycolysis, whereas M2(IL-4) macrophages generate sustained energy efficiently through fatty acid oxidation and oxidative glucose metabolism\textsuperscript{168,182}. M1(IFNγ+LPS) engage in iron import, presumably to withhold this valuable resource from pathogens, and M2(IL-4) participate in iron efflux\textsuperscript{183}.

As noted previously, tissue macrophages may take on intermediate states in order to better execute necessary functions in a particular tissue. For example, M1/M2 hybrid macrophages in the gut express some M1 markers such as TNFα constitutively along with some M2 markers such as IL-10, CD163, and CD206; their TNF likely fills non-inflammatory roles\textsuperscript{146}. Intestinal tissue macrophages do not produce additional inflammatory M1 cytokines or NO in response to TLR stimulation, likely as a result of regulation of TLR adaptors\textsuperscript{146}. During intestinal inflammation, macophages with M1-like pro-inflammatory activity are temporarily recruited\textsuperscript{146}.

Distinct signaling pathways are involved in establishing polarization. Polarization results from NF-κB activation (for M1) and inhibition (for M2), and activation of different STATs: STAT1 for M1(IFNγ), and STAT6 for M2(IL4) activation\textsuperscript{173}. STAT6 acts in part by upregulating
additional transcription factors such as PPARδ and PPARγ. These polarization pathways may be relevant in creating immunomodulatory treatments.

1.3.4 Targeting macrophages for immunomodulatory therapies

As a result of increased understanding of the roles of macrophages in human health and of the mechanisms of macrophage recruitment and function, researchers have begun to investigate immunomodulatory treatments targeting macrophages. In diseases where pathology is attributed to specific macrophage populations such as infiltrating pro-inflammatory MDMs, targeting these populations such as through blocking M-CSF may be a treatment option. Similarly, MCP-1 RNA silencing can reduce inflammatory macrophage accumulation in mice, showing promise for treating atherosclerosis and diabetes, and Amyotrophic Lateral Sclerosis (ALS or Lou Gehrig’s disease).

However, in some diseases it is unclear whether the pathology results from infiltrating MDMs or tissue macrophage dysfunction. For example, Crohn’s disease is characterized by accumulation of pro-inflammatory macrophages in the gut, and these are not confirmed to represent either MDM or incorrectly polarized tissue macrophages. In these cases, directing polarization may be the most effective intervention.

Given the importance of macrophages in both infection and homeostasis, and the striking effects of polarization, it is unsurprising that inappropriate M1/M2 polarization is implicated in a variety of conditions. An increase in the number and proportion of M1 macrophages is associated with chronic venous leg ulcers, and M1 pro-inflammatory activity in adipose and liver macrophages has been implicated in obesity-related diseases such as fatty liver disease. Increased M1 activation in microglia may even be involved in aging within the brain. Thus, modulation of M1 activation may be an effective treatment for these inflammatory conditions.

Inappropriate activation can also involve M2-like responses. M2-like tumour-associated macrophages have been implicated in cancer through promotion of angiogenesis. Tumours can recruit and activate tumour-associated macrophages by production of M-CSF, IL-10, and CCL2. In contrast, M1 macrophages are tumoricidal. As previously noted, excessive M2(IL-4) activation has been implicated in fibrosis. Finally, inappropriate M2 activation can lead to susceptibility to intracellular infections such as tuberculosis, dengue fever, or Salmonella.

Treatments targeting macrophages could involve transfer of beneficial macrophage populations. For example, transfer of M2(IL-4) macrophages to mice protected them during a
sterile gut inflammation model, and infusion of renal transplant patients with immunosuppressive “regulatory” macrophages appears to improve graft acceptance.

Alternatively, pathways involved in M1 and M2 activation could be therapeutic targets. Given that NLRP3, TNFα, and IL-1β all encourage an inflammatory macrophage phenotype, their inhibition may be useful in promoting wound healing. Interestingly, while NO is considered an M1 marker in macrophages, endothelial NO overexpression increases M2-like activation in liver tissue macrophages, resulting in improved insulin responses. JNK signaling appears to promote M1 activation in adipose tissue, and thus may serve as an inhibition target for treatment of obesity-related diseases. PPARγ agonists, which promote M2 states, are already in use for treating diabetes, and may be useful in treating other metabolic disorders. Akt1 and Akt2 signaling are associated with M2 and M1 polarization, respectively, and Akt2 knockout mice (which are M2 skewed) exhibited macrophage-dependent resistance to endotoxin shock and colitis. Since Notch signaling is implicated in the difference in anti-tumour activity between M1 macrophages and M2-like tumour-associated macrophages, Notch might be a target for anticancer therapies.

Finally, as a wide variety of cytokines, including those associated with polarization, signal through JAK/STAT pathways, modulation of specific JAKs, STATs, or of their suppressors of cytokine signaling (SOCS) inhibitors may be effective in treating conditions associated with inappropriate polarization or maladaptive cytokine responses more generally.

1.3.5 Endotoxin tolerance and its role in sepsis

Endotoxin tolerance is defined as the state in which, following exposure to a bacterial molecule such as LPS, macrophages display a reduced responsiveness to subsequent stimulation. The initial exposure to LPS produces a “reprogramming” effect which alters the macrophage response to later stimulation. This reprogramming is similar to polarization: recently, endotoxin tolerant macrophages have been identified as a new form of alternatively activated (M2) macrophage. The desensitized state of tolerance likely serves as a means of controlling hyperinflammation, as endotoxin tolerance is protective against tissue injury and mortality in various models of sterile injury and stress. However, this immunosuppression may have detrimental effects during bacterial infection. Endotoxin tolerance has been observed in patients recovering from some bacterial illnesses, including typhoid fever, and is implicated in sepsis.

Sepsis is a common and highly destructive immune dysfunction characterized by a
combination of hyperinflammation leading to organ failure, and immune suppression leading to a failure to control pathogens. These two responses appear to occur in phases. Patients dying in the first few days of sepsis typically show inflammatory organ failure, while patients who succumb to later stages of sepsis die as a result of ongoing infection with reduced signs of organ damage. The balance of these two phases depends on many factors involving the patient immune system as well as the pathogen. Current therapies are relatively effective in addressing the inflammatory phase, resulting in many patients surviving the first days of infection only to die as a result of immune suppression. In fact, sepsis survivors are at risk for early mortality even years after recovery from the initial condition. This suggests that treatments enhancing the immune system will be highly beneficial in reducing sepsis mortality. It is worth mentioning that sepsis is considered to be a very heterogeneous disease and current thinking is that there are several endotypes (phenotypic subsets of patients with different underlying mechanisms), this limits any generalizations about this syndrome. Notably, a gene signature derived from endotoxin tolerant peripheral blood mononuclear cells (PMBCs) can predict the development of severe sepsis and organ failure.

Protocols for inducing endotoxin tolerance in the lab vary somewhat. Typically, a 16-48 hour initial LPS stimulation is necessary to induce tolerance in vitro. While one such dose of LPS is sufficient to reduce responsiveness to a second dose, repeated doses increase the level of immunosuppression. The extent of suppression is also partly dependent on the concentration of the first LPS dose, with higher initial doses of LPS leading to a greater suppression of responses to the second LPS dose. Once induced, the state of tolerance can last for days or weeks after the initial exposure to LPS. Commonly, a 24 hour initial LPS stimulation is used to induce tolerance, with the second stimulation immediately following. For example, the endotoxin signature was derived from cells stimulated with LPS for 24 hours, followed by a 4-hour second stimulation, after which gene reprogramming was measured.

Experimentally, tolerance is assessed primarily via reduced expression of TNFα in response to pro-inflammatory stimulation, relative to macrophages that have not received a tolerizing prior stimulation. Endotoxin tolerant macrophages also demonstrate suppressed expression of other pro-inflammatory cytokines such as IL-6, though they may still produce IL-10. Endotoxin tolerant macrophages express CD163, similarly to M2(IL-10), but do not express CD206. They express genes associated with phagocytosis and wound healing such as...
proteoglycans (including versican), growth factors, and metalloproteinases, as well as metallothioneins, which are involved in detoxification, modulation of inflammation, and cell proliferation\textsuperscript{162}. Metallothionein expression may be useful in distinguishing endotoxin tolerance since only one metallothionein, MT-2A, has been associated with other M2 types. Endotoxin tolerant monocytes show phagocytic activity\textsuperscript{213}.

While endotoxin tolerance is often studied in the context of two or more stimulations with LPS, a similar reprogramming has been demonstrated with many, though not all, inflammatory stimuli\textsuperscript{206}. Tolerance can be induced using various TLR2 or TLR4 ligands as well as live bacteria\textsuperscript{198,206,214}. Similarly, tolerance affects macrophage responses not only to LPS but to stimuli including killed gram-positive bacteria\textsuperscript{198,215}. However, these forms of “cross-tolerance” often result in a weaker reduction in TNFα production than that seen when an initial LPS stimulation is used to induce tolerance to further LPS stimulation\textsuperscript{206}.

Interestingly, variation in LPS structure leads to variation in immune responses including tolerance. Rough LPS (defined by the absence of an O-antigen) signals through a different, CD14 independent mechanism from smooth LPS, though the ultimate immune responses are similar \textit{in vivo}\textsuperscript{216}. Some forms of LPS are TLR2-dependent rather than TLR4-dependent\textsuperscript{91}, and these TLR2-dependent forms of LPS produce a different immune response\textsuperscript{217}. \textit{Bacteroides dorei} LPS, unlike \textit{E. coli} LPS, does not induce tolerance to fungal zymosan; this may be attributed to differences in the lipid A portion of the LPS molecule\textsuperscript{218,219}.

A variety of mechanisms have been implicated in the establishment of endotoxin tolerance, including epigenetic changes and chromatin remodelling\textsuperscript{206}. The effects of endotoxin tolerance are dependent on Src homology 2-containing inositol phosphatase (SHIP), which suppresses M1 responses\textsuperscript{209}, and may be partially mediated by IL-10 in an organ-specific manner\textsuperscript{198,211}. Glucocorticoids, prostaglandins, and TGF-β may also play a role in endotoxin tolerance\textsuperscript{198}. While the plasma from patients with sepsis-related Systemic Inflammatory Response Syndrome (SIRS) contains high levels of LPS-neutralizing sCD14 and LPS-binding protein, exposing tolerant cells to plasma from healthy patients is not sufficient to reverse tolerance, consistent with persistent changes in the macrophages\textsuperscript{198}. However, IFNγ can prevent or reverse endotoxin tolerance \textit{in vitro}\textsuperscript{198}.

Currently, sepsis is treated with broad-spectrum antibiotics meant to control bacterial infection, or with supportive treatment to prevent organ failure or correct circulatory system
imbalances. The discovery that TNFα was necessary and sufficient for inducing acute septic shock\textsuperscript{220} led researchers to develop sepsis therapies targeting TNFα. However, septic shock represents a small portion of the sepsis syndrome\textsuperscript{104}, and septic patients do not necessarily produce high levels of inflammatory cytokines\textsuperscript{201}. As a result, cytokine blocking and anti-inflammatory treatments have been ineffective in treating sepsis\textsuperscript{130,205}. However, treatment of patients in the immunosuppressive phase of sepsis with IFNγ\textsuperscript{221,222}, or G-MCSF\textsuperscript{223,224} improved outcomes.

Because such IFNγ treatment could reverse endotoxin tolerance\textsuperscript{198}, these clinical trials would support the idea that endotoxin tolerance is part of the immunosuppressive pathology associated with sepsis. Indeed, many studies do link endotoxin tolerance with immunosuppression, such as reduced production of interferon in response to viral challenge\textsuperscript{225}, and reduced ability to kill intracellular \textit{Leishmania major} (of macrophages \textit{in vitro})\textsuperscript{226}.

Other studies, however, indicate that endotoxin tolerance may preserve certain host defenses. Despite suppression of inflammatory responses, endotoxin primed murine bone-marrow-derived macrophages (BMDM) show antimicrobial functions and are protective against \textit{P. aeruginosa}\textsuperscript{214}, and mice primed with endotoxin show an enhanced ability to clear \textit{P. aeruginosa} infection\textsuperscript{211,214}. This protection may be associated with M1-like glycolysis metabolism, which was observed in cells tolerized by priming with the TLR4 ligand monophosphoryl lipid A\textsuperscript{214}. Furthermore, endotoxin priming for 16 hours protects macrophages from cell death induced by \textit{Yersinia enterocolitica}, a pathogen that inhibits anti-apoptotic NF-κB\textsuperscript{227}. The endotoxin tolerance gene signature includes the alarmins S100A8 and S100A9\textsuperscript{205}, which are protective in neonatal sepsis\textsuperscript{228}. In neonates, these alarmins modulate the immune system via MyD88, NF-κB, and IRF5 in order to limit hyperinflammation, while preserving antimicrobial defenses\textsuperscript{228}.

Similarly, studies investigating a possible link between endotoxin tolerance and the susceptibility of sepsis patients to secondary viral infections have shown differing results\textsuperscript{229–231}. This is likely the result of differing experimental designs, particularly factors such as duration and timing of endotoxin priming and infection, and the characteristics of the infectious pathogen. These studies raise questions regarding whether endotoxin tolerance is ultimately pathogenically immunosuppressive, or protectively anti-inflammatory, or both.

1.3.6 Effect of polarization on susceptibility to \textit{Salmonella}

While \textit{Salmonella} Typhimurium is able to replicate within M0 and M2 cells, the M1 state is associated with resistance to intracellular \textit{S. Typhimurium}\textsuperscript{3–5}. Several mechanisms have been
proposed for this based on investigation of specific hypotheses, though no investigation of this resistance has been employed using a broad, systems-based approach. The induction of IFNγ-stimulated production of reactive oxygen species can eventually activate the inflammasome and lead to pro-inflammatory cell death and bacterial clearance\textsuperscript{11,232}. Specific to \textit{Salmonella} infection, IFNγ induces the expression of Guanylate Binding Proteins (GBPs), which rupture the SCV leading to pyroptosis. This process is dependent on JAK/STAT signalling\textsuperscript{11,233}. GBPs can also participate in inflammasome activation, either directly or recruiting IRGB10\textsuperscript{234–236}. Secretion of IL-12 and IL-18 by IFNγ-activated macrophages induces secretion of further IFNγ from T cells and natural killer cells, creating a positive feedback loop\textsuperscript{37,38}.

Mutation studies reinforce this connection between M1 activation and \textit{Salmonella} resistance. Deletion of Akt2, which renders mice deficient in M1 macrophages, also renders them susceptible to \textit{Salmonella}\textsuperscript{237}, possibly as a result of the M1 deficiency. In humans, susceptibility to salmonellosis can result from deficiencies in IFNγR, STAT-1, and the IL-12p30 subunit of the M1 marker IL-12\textsuperscript{2}. This suggests that these pathways have a key role in defense against \textit{Salmonella}. Interestingly, intracellular \textit{Salmonella} is able to interfere with secretion of IL-12p70\textsuperscript{238}, emphasizing the interplay between host defense and bacterial evasion mechanisms.

The effect of endotoxin tolerance or priming on macrophage resistance to \textit{Salmonella} is not well established. Macrophages that have been tolerized to TLR ligands such as LPS can still produce IL-1β in response to NLRP3\textsuperscript{210} or NLRC4\textsuperscript{239} activation; this allows cells that have been exposed to commensal bacteria in the gut to still react, at least some extent, to pathogenic bacteria such as \textit{Salmonella}.

Human endotoxin tolerant monocytes have phagocytic activity\textsuperscript{213}. Despite their lack of an inflammatory response, endotoxin tolerant mice have increased survival and reduced bacterial load after challenge with \textit{Salmonella enterica} serovar Typhimurium\textsuperscript{207}. This effect has been attributed to early accumulation of leukocytes, neutrophil recruitment and Kupffer cell activity, and it is uncertain as to whether this could have reflected resistance of tolerant macrophages to intracellular \textit{Salmonella} replication. A 16-hour tolerizing pretreatment with LPS increases programmed cell death in mouse macrophages subsequently infected with \textit{Salmonella}\textsuperscript{227}, however, because cell death was measured in the form of Annexin-V positive cells, these could represent either apoptotic or pyroptotic macrophages. To my knowledge, no investigation into resistance of endotoxin tolerant cells to intracellular growth of \textit{Salmonella} has been performed in mature human
monocytes.

1.4 Stem cell-derived macrophages

Investigation of human macrophage function has largely relied on murine bone marrow derived macrophages (BMDM), or human blood monocyte-derived macrophages (MDM). These cell sources suffer from some limitations. These cells can be difficult to obtain in large amounts. Human MDM are characterized by high variability between donors, and even between different collection times from the same donor\(^\text{240}\). Macrophages are also difficult to manipulate genetically\(^\text{241}\). Critically viral vectors for gene transfer can activate macrophage immune programs, which represents a confounding factor in experiments that would employ these macrophages. Even if this was not a concern, macrophages degrade nucleic acids, making gene transfer inefficient. Some methods, such as the introduction of genes through lipids (termed lipofection), have to some extent overcome these two challenges\(^\text{241}\). However, as terminally differentiated cells, MDM do not readily divide, preventing the establishment of a self-renewing line of mutated cells\(^\text{242}\).

Recently, techniques have been developed to create murine embryonic stem cell derived macrophages (ESDM) or human induced pluripotent stem cell-derived macrophages (iPSDM). Induced pluripotent stem cells (iPSC) are derived from differentiated cells taken from an adult donor and reverted, through the introduction of specific transcription factors, to a pluripotent state\(^\text{243}\). While iPSDM are still affected by the genetic individuality of the donor, once an iPSC line has been established it can be considered consistent, and as long as it is maintained in the pluripotent state, can be induced to yield theoretically unlimited numbers of differentiated macrophages\(^\text{240}\). Stem cells can also be easily genetically manipulated by tools such as CRISPR-Cas9\(^\text{243}\), yielding mutant knock-out or knock-in iPSDM. If safe and consistent differentiation protocols were developed, iPSDM could be used therapeutically in order to boost immunity or repair functions\(^\text{138,244}\).

iPSDM have already proven useful in several research applications. iPSDM models have been used to study the role of \(ABCA1\) in reverse cholesterol transport, which is significant in atherosclerosis\(^\text{245}\), and screen for phagocytosis of kidney stone-forming calcium oxalate crystals\(^\text{246}\). iPSDM can provide opportunities to investigate the genotype of the stem cell donor\(^\text{244}\). For example, macrophages derived from stem cells provided by a donor with familial Mediterranean fever show characteristics of the disease, allowing research into its pathology\(^\text{247}\). Through production of knockout stem cell lines, stem cell derived macrophages can be used to
study knockout mutants that would be difficult to obtain in primary lines\textsuperscript{248}. For example, Yeung \textit{et al} have used iPSDM with knockout of the IL-10 receptor subunit A and interferon regulatory (transcription) factor IRF5, to determine that IL-10 and IRF5 respectively are important in limiting \textit{Chlamydia} infection\textsuperscript{248}.

iPSDM may be used to closely model tissue macrophages. Differentiation of iPSDM is independent of MYB, which makes them developmentally similar to yolk-sac derived tissue macrophages, which include those found in the lung, kidney, brain, liver, and pancreas\textsuperscript{249}. Human stem cells can be differentiated into macrophages resembling those present in the erythroblastic islands which are responsible for phagocytosis of red blood cell nuclei in development\textsuperscript{250}, as well as cells that closely resemble microglia, and which can be studied in a 3D organ-like culture\textsuperscript{251}. Differentiation of stem cells into tissue macrophages is most effective when the cells are not only differentiated using developmental cytokines, but also co-cultured with relevant tissue cells or organoids\textsuperscript{243}. Indeed, stem cell derived macrophages that are differentiated in the presence of neurons more closely resemble microglia than blood-derived macrophages\textsuperscript{247}.

The resemblance of iPSDM to tissue macrophages may be therapeutically relevant. In a murine model of hepatic injury and fibrosis, ESDM were produced that resembled resident tissue macrophages, and were able to effectively repopulate the liver, and reduce fibrosis\textsuperscript{252}. In mice, stem cell derived macrophage precursors can differentiate into microglia-like cells that respond to neuronal injury and these precursors can also integrate into the brain and differentiate into macrophages in vivo\textsuperscript{247}. Human iPSDM can differentiate \textit{in situ}, in the lungs of humanized mice, to resemble alveolar macrophages, and this reduced the symptoms of alveolar proteinosis\textsuperscript{253}.

These various research and therapeutic opportunities motivate a deeper understanding of the biology of stem cell derived macrophages. Several recent studies show strong similarities between stem cell-derived macrophages and their primary macrophage counterparts. Murine ESDM and BMDM show similar cytokine responses to various agonists\textsuperscript{254}. ESDM can be infected with \textit{Salmonella} Typhimurium, and show similar formation of the characteristic \textit{Salmonella}-containing vacuole\textsuperscript{254}. iPSDM also closely model MDM, including in phagocytosis and killing of \textit{Salmonella}\textsuperscript{255}, and in their response to tuberculosis through Bacillus Calmette-Guérin/BCG infection\textsuperscript{256}. iPSDM respond to polarization via IFN\(\gamma\) or IFN\(\gamma\)+LPS and IL-4 by altering their expression of cytokines and surface markers\textsuperscript{255,257}. A comparison of the expression of polarization markers and angiogenesis genes in M1(IFN\(\gamma\)+LPS), M2(IL-4+IL-13) and M2(IL-10) from
different cell sources found that iPSDM and MDM were more similar to each other than to macrophages derived from the commonly used THP-1 cell line of immortalized monocytes\textsuperscript{240}. To date, several transcriptomic studies have characterized the extent of similarity between stem cell derived macrophages and their blood macrophage counterparts.

### 1.4.1 Transcriptomic studies of stem cell-derived macrophages

On the transcriptional level, BMDM and ESDM share 3,729 genes that are significantly differentially expressed in response to \textit{Salmonella}, out of 5,579 differentially expressed in BMDMs (67\%)\textsuperscript{254}. Most of the commonly up-regulated genes between ESDMs and BMDMs are part of innate immune pathways such as the TLR, NLR, and cytokine and chemokine signaling pathways, or are known to be specifically involved in \textit{Salmonella} infection. Commonly down-regulated genes are in pathways involved in energy metabolism and cell division. In addition, most of the non-shared transcripts are rare (represented by having low read counts) and might not be active\textsuperscript{254}.

Further, the transcriptomic responses of human iPSDM and MDM to LPS have been compared\textsuperscript{258}, and found to be overall similar, with 2,638 genes differentially expressed in response to LPS in both iPSDM and MDM. Just 365 genes showed a significantly different magnitude of response in the two cell types, and 229 genes were regulated in the opposite direction (e.g. upregulated in iPSDM and downregulated in MDM). These 229 genes had a lower magnitude of change than those that were similarly regulated. Some of these differently expressed genes did have immunological relevance; for example, iPSDM and MDM differed in the expression of 11 chemokines with different target cells. However overall, a very strong correlation was observed between the fold change of genes that were regulated in response to LPS in iPSDM and in MDM.

In addition to LPS, the transcriptomic response of iPSDM to \textit{Chlamydia trachomatis} infection has been studied\textsuperscript{248}. This study found 2,209 genes that were differentially expressed as a result of \textit{Chlamydia} infection in both iPSDM and MDM. Pathways over-represented in this gene set include immune pathways such as interferon and TLR signaling, as well as metabolic pathways. From within the interferon pathways, STAT genes were significantly upregulated. Some pathways were determined to be uniquely upregulated in iPSDM (such as extracellular matrix organization) or in MDM (such as antigen presentation). \textit{Chlamydia}, like \textit{Salmonella}, is an intracellular pathogen with the ability to multiply inside M2, but not M1 macrophages\textsuperscript{180,248}.

Overall, these transcriptomic studies speak to significant similarities between stem cell
derived and host macrophages, but with some notable areas of divergence. These differences should be kept in mind as iPSDM models become more common in research. An investigation of how polarization affects the iPSDM response to *Salmonella* infection would improve our understanding of the utility of stem cell derived macrophages as a model for macrophage infection.

1.5 Overall research objectives

1.5.1 Determine the effect of endotoxin priming on the susceptibility of MDM to *Salmonella* infection

While endotoxin tolerant macrophages are non-inflammatory, and in many ways similar to alternatively activated M2 macrophages, endotoxin tolerance is protective during a murine in vivo model of *Salmonella* infection. These observations raise the question as to whether endotoxin tolerance causes macrophages to become *Salmonella* permissive similarly to other alternatively activated macrophages, or whether their protective functions include resistance to intracellular infection. This question will be addressed in Chapter 3.

Endotoxin tolerance is approximated here with a 24-hour stimulation with *P. aeruginosa* LPS immediately prior to infection. Since this represents a cross-tolerance effect rather than a classic double LPS stimulation, these macrophages are referred to throughout as M\(^{EP}\), or endotoxin primed macrophages. Endotoxin priming, followed by infection as a second stimulation, has been used previously to represent endotoxin tolerance, and in BMDM, a 24-hour prestimulation with *E. coli* LPS was sufficient to induce a tolerant response to live *Salmonella* infection. While *P. aeruginosa* induces a weaker cytokine response than *E. coli* or *Salmonella* LPS, this response is still TLR4 dependent rather than TLR2 dependent, and similar effects as *E. coli* or *Salmonella* LPS can be achieved using higher concentrations of *P. aeruginosa* LPS. Thus, I expect that the 24-hour *P. aeruginosa* LPS stimulation will be sufficient to induce a tolerant response to *Salmonella* infection, and this LPS has the advantage of extensive quality testing within our lab.

1.5.2 Transcriptomic analysis of the effect of activation and endotoxin priming on *Salmonella* infection in MDM

*Salmonella* infection causes significant morbidity and mortality worldwide. Its progression depends upon its ability to survive within macrophages, which depends in part on the polarization of the macrophage. To date, several experiments have used whole genome transcriptome sequencing or RNA-Seq to expand our understanding of the pathways involved in the spectrum of macrophage polarization, including revealing pathways not previously identified.
using microarray analysis of incomplete gene sets. Single-cell RNA-Seq has been used to investigate murine BMDM in the course of Salmonella infection and identify markers of polarized states, with Salmonella-resistant cells showing more M1-like transcriptomes. However, RNA-Seq analysis of polarized human macrophages in response to Salmonella has not been performed.

A full transcriptomic analysis comparing macrophages based on both polarization and infection status will provide new insights into the dynamics of how polarization affects Salmonella infection, and will likely identify additional pathways of relevance to Salmonella infection in humans. Further, a transcriptomic analysis of endotoxin primed MDM in response to Salmonella infection has not been performed. Such analysis of the response of endotoxin tolerant MDM to Salmonella will be particularly relevant in the treatment of Salmonella septicemia. This analysis is discussed in Chapter 4.

1.5.3 Transcriptomic comparison of response to Salmonella infection in M1 and M2 polarized MDM & iPSDM

Given the great potential applications for iPSDM in research and medicine, it is important to understand the extent of their similarity to MDM and identify any significant differences. In response to polarization, iPSDM and MDM display similar surface markers and cytokine expression, and similar expression of a specific set of genes known to be affected by polarization. Previous RNA-Seq analyses have demonstrated a strong overall similarity, with some notable differences, between iPSDM and MDM gene expression in response to LPS and to Chlamydia infection. iPSDM are capable of being infected by Salmonella, and murine ESDM and BMDM have similar gene expression in response to Salmonella. However, to my knowledge there has not been a comprehensive comparison between the entire transcriptome of polarized iPSDM and MDM, either without further stimulation or in response to Salmonella infection. This analysis will provide insights into the suitability of iPSDM as a model to study macrophage polarization, and particularly to study the resistance of M1 macrophages to Salmonella infection. This analysis is discussed in Chapter 5.

1.5.4 Test mechanisms of resistance to Salmonella infection

The transcriptomic analyses in Chapters 4 and 5 serve as hypothesis-generating experiments, revealing a number of pathways that may determine the different resistance of M1, M2, and MEP to Salmonella infection. Inhibition of selected pathways will confirm their mechanistic significance. This question is addressed in Chapter 6.
Chapter 2: Methods and materials

2.1 Isolation and infection of MDM

2.1.1 Reagents, blood samples, and ethics statement

The LPS used in this experiment was made in the Hancock laboratory from *Pseudomonas aeruginosa* PAO1 (strain H103), using the previously described Darveau-Hancock method\(^2\). Briefly, bacteria at an optical density at 600nm (OD\(_{600}\)) of 0.6-0.8 were centrifuged and lyophilized. A series of digestion, precipitation and purification steps\(^2\) were performed to remove contaminants. The sample was incubated for 2 hours with DNAse and RNAse to remove nucleic acids, centrifuged at 50,000 x g for 30 min to remove peptidoglycan, and incubated with pronase and centrifuged to remove protein. Finally, LPS was extracted using a 2:1 chloroform/methanol solution to remove lipids. The concentration of LPS was derived by measuring the concentration of the LPS component sugar 2-keto-3-deoxyoctonate using a thiobarbituric acid assay described by Osborn\(^2\), and converting from weight of 2-keto-3-deoxyoctonate to weight of LPS based on known percentages. LPS obtained through this method is typically 93-96.9% pure as measured by absorbance at 260 and 280 nm to estimate contamination from protein and nucleic acids, and measurement of the C16:1 fatty acid to estimate contamination from non-LPS lipids\(^2\).

Blood samples were obtained from healthy donors with approval from the UBC Clinical Research Ethics Board, ethics certificate H04-70232.

2.1.2 MDM isolation and maturation from PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from human blood using Lymphoprep (StemCell cat. no. 07811) density gradient isolation. Briefly, blood samples were diluted 1:1 in sterile phosphate-buffered saline (PBS, ThermoFisher 10010049), layered on top of Lymphoprep, and centrifuged at 800 x g for 20 minutes without braking. A layer containing PBMCs and basophils was removed and washed three times with PBS, then resuspended in serum-free RPMI media (Fisher cat. no. SH30255FS) supplemented with 2mM L-Glutamine (Fisher cat. no. 25030081) and 1mM sodium pyruvate (Fisher cat. no. 11360070). Cell density was assessed using a hemocytometer and Trypan Blue staining (Sigma cat. no. T8154) to exclude dead cells.

In order to select for monocytes, the PBMCs were plated into 24-well plates at 2 x 10\(^6\) cells/mL in serum-free RPMI media (supplemented as described with L-Glutamine and sodium pyruvate) and incubated at 37°C, 5% CO\(_2\) for 1 hour. Under these conditions, monocytes will
adhere to the treated plastic or glass, but other PBMCs will not. To enable microscopy (see 2.2.9), 12-mm glass coverslips (Fisher cat. no. 12-545-81) were inserted into some wells and cells were plated on the glass at the same 2 x 10^6 cells/mL density as was used for treated plastic. After 1 hour, the media containing non-adherent cells was removed, and replaced with RPMI containing 10% heat-inactivated FBS (Gibco cat. no. 12483-020) in addition to 2mM L-glutamine and 1mM sodium pyruvate (this supplementation will be referred to as cRPMI), plus 10ng/mL M-CSF (R&D cat. no. 216-MC-025). FBS was drawn from the same lot as much as possible to minimize effects of variation in serum. The monocytes were matured for 7 days in this media, changing media on days 3 and 5.

The plated PBMC density of 2 x 10^6 cells/well in 1mL media resulted in a final MDM density of 0.42-2.6 x 10^5 cells/well, varying by donor. This number was determined by seeding, for each biological replicate, additional duplicate wells for the purpose of counting. On day 6 of maturation, these wells were washed vigorously with sterile PBS and cells were detached using 0.25% Trypsin-EDTA (Fisher cat. no. 25200056) applied for 5-10 minutes followed by scraping with a rubber policeman (Fisher cat. no. 50-809-208) and collection in cRPMI. Cell density was determined using a hemocytometer and Trypan Blue (Sigma cat. no. T8154) to exclude dead cells.

It was confirmed using flow cytometry that this method produced monocyte derived macrophages (MDM), expressing CD14, CD16, and CD64 (see Appendix A, Figure A.1). Briefly, approximately 4x10^5 cells were fixed using 4% formaldehyde (Fisher PI28906) for 15-20 minutes at room temperature, and stored in PBS overnight at 4°C. Cells were then washed and resuspended in PBS with 1% bovine serum albumin (Sigma A9418), and incubated with fluorescent antibodies for 30-40 minutes on ice. For a final volume of 50µL, 8µL FITC-CD64 (Biolegend 305005), 8µL PE-CD14 (Biolegend 325605), and 1µL APC-CD16 (Biolegend 302011) were used. Equal volumes of isotype antibodies FITC-IgG1 (Biolegend 406605), PE-IgG1 (Biolegend 400111), and APC-IgG1 (Biolegend 400119) were used for the unstained control as well as single-stained controls used to set compensation. Samples were read using a FACSCalibur instrument (BD).

**2.1.3 MDM polarization**

Having been matured for 7 days in cRPMI containing M-CSF, MDM were polarized by removing the media, washing vigorously in PBS, and replacing with cRPMI containing 50ng/mL IFNγ (Biolegend cat. no. 570202) for M1, 50ng/mL IL-4 (Biolegend cat. no. 574002) for M2, and 10ng/mL LPS (see 2.1.1) for MEP. Samples were then incubated for 24 hours. This duration and
these concentrations were chosen to be as per previous studies\textsuperscript{162} and in line with the procedures of collaborators whose iPSDM dataset is compared with MDMs in Chapter 5.

For JAK-STAT inhibition in Chapter 6, Ruxolitinib 10\(\mu\text{M}\) (MedChemExpress cat. no. HY-50856) or 0.1\% DMSO vehicle control were added along with the polarizing treatments. The timing was selected to inhibit JAK-STAT signalling occurring during macrophage polarization, but not during infection; see Chapter 6.3 for details. Inhibitor concentration was chosen to maximize cytokine suppression while minimizing cytotoxicity. Supernatants were collected after 24 hours for measurement of cytokines using ELISA (see 2.2.2).

2.1.4 Bacterial culture

\textit{Salmonella enterica} serovar Typhimurium strain SL1344 (\textit{ssaG::GFP}) was used for most experiments; this strain expresses GFP when it becomes intracellular within a \textit{Salmonella}-containing vacuole (SCV)\textsuperscript{264}. It was received from Dr. Christine Hale of the Dougan laboratory, Wellcome Trust Sanger Institute, Hinxton UK. For experiments taking place at time points earlier than 4 hours, when the GFP reporter would not be expressed in the (\textit{ssaG::GFP}) strain, a constitutively GFP-expressing \textit{S. Typhimurium} strain derived from SL1344 was used, designated MCS003 (SL1344 \{Tn7 PA1lacO:sgfp2 t0 t1 FRT-cat-FRT t0 t1\}) and received from Andrew Santos of the Finlay Lab, University of British Columbia. \textit{Salmonella} strain SL1344 (\textit{ssaG::GFP}) was cultured in Luria-Bertani (LB) medium, or on LB-agar plates, supplemented with ampicillin 100\(\mu\text{g/mL}\) (Sigma cat. no. A9518), while MCS003 was cultured in LB (Fisher cat. no. BP-1427-500) supplemented with streptomycin 100 \(\mu\text{g/mL}\) (Sigma cat. no. S6501) and chloramphenicol 30 \(\mu\text{g/mL}\) (Sigma cat. no. C0378).

Two days prior to infection of MDM, a fresh overnight plate was streaked from a frozen stock and incubated overnight at 37\(^\circ\text{C}\). A colony was picked from this plate the following afternoon and inoculated into aerated LB media with antibiotics (ampicillin 100\(\mu\text{g/mL}\) for SL1344 or streptomycin 100 \(\mu\text{g/mL}\) + chloramphenicol 30 \(\mu\text{g/mL}\) for MCS003) and cultured for approximately 3 hours at 37\(^\circ\text{C}\). A 100 \(\mu\text{L}\) sample of this culture was added to 10 \(\text{mL}\) of fresh LB containing the appropriate antibiotics and subcultured overnight under static conditions at 37\(^\circ\text{C}\).

2.1.5 MDM infection and sample collection

Polarized MDM were washed once with sterile PBS, and media was replaced with 900 \(\mu\text{L}\) cRPMI. For Parthenolide experiments in Chapter 6, Parthenolide (Sigma cat. no. P0667) was added at a final concentration of 1 \(\mu\text{g/mL}\), and DMSO at final concentration 0.002\% was added to
vehicle control wells. Inhibitor concentration was chosen to maximize cytokine suppression while minimizing cytotoxicity.

Plates were returned to the incubator (37°C, 5% CO₂) for <1 hour while the bacterial culture was prepared for infection. *Salmonella* concentration was measured by taking OD₆₀₀, which was typically in the range of 0.5-0.6 after overnight static culture. Based on previous experiments relating *Salmonella* OD₆₀₀ to CFU (whereby OD x 10⁹ = CFU/mL), and the cell density for each biological replicate as determined in 2.1.2, the bacterial culture was diluted in cRPMI to result in a 10x bacterial concentrate. This bacterial concentrate was plated on LB-agar plates with antibiotic (ampicillin 100 µg/mL for SL1344 or streptomycin 100 µg/mL + chloramphenicol 30 µg/mL for MCS003) to confirm *Salmonella* concentration. 100 µL of this concentrate was added to wells for the infected condition, resulting in a final multiplicity of infection (MOI) averaging 27 for each biological replicate. For the uninfected condition, 100µL cRPMI media was instead added to wells.

Following *Salmonella* addition, plates were swirled to distribute bacteria and centrifuged at 23°C at 411 x g for 5 minutes to sediment bacteria onto cells. The plates were then returned to the incubator for an average of 43 minutes to allow association of the bacteria with MDM. Samples were collected immediately (0 hours), or the medium was removed and replaced with cRPMI + 50µg/mL gentamicin (Alfa Aesar cat. no. J62834) to remove most extracellular bacteria and kill any that were not removed. For Parthenolide treatments in Chapter 6, media was supplemented with Parthenolide (final concentration 1 µg/mL) or DMSO 0.002% for the vehicle control. Intracellular infection then progressed a further 2, 4, or 24 hours. For the 24 hour samples, medium was removed at 2 hours and replaced with medium containing 10µg/mL gentamicin⁴,²⁶₅,²⁶₆, in order to limit pinocytosis of antibiotic that might lead to killing of intracellular *Salmonella*²⁶⁷.

At the end of this period, supernatants were collected for ELISA and lactate dehydrogenase (LDH) assays. Cells were washed twice with PBS, and lysed using 1mL 0.1% Triton-X-100 (Sigma cat. no. T8787) for bacterial quantification by CFU or fixed for 25 minutes using 4% formaldehyde (Fisher PL8906) for microscopy. For RNA isolation, cells were not washed with PBS but rather immediately treated with 300µL RNAProtect (Qiagen cat. no. 76506). After 5-10 minutes, cells were gently dislodged by pipetting and collected into sterile RNase-free tubes. The tubes were centrifuged at 400 x g for 5 minutes using a MicroCL 21R microcentrifuge (Thermo Scientific) to pellet the cells, and RNAProtect was removed and replaced with 350µL of lysis
buffer (RLT) from a RNAeasy Plus Mini kit (Qiagen), with added β-mercaptoethanol (Sigma cat. no. M3148). The lysate was stored at -80°C until isolation.

2.2 Analysis of MDM samples

2.2.1 Lactate dehydrogenase (LDH) assay

Supernatants were tested for LDH release immediately, or refrigerated overnight. LDH reagent was combined with LDH catalyst (both Sigma cat. no. 11644793001) at 1:100 v/v, and added to MDM supernatants at a 1:1 v/v ratio. The mixture was incubated for 25 minutes and read on an Epoch plate reader (BioTek). Absorbance from media blanks was subtracted, and results were expressed relative to an uninfected control sample (0% toxicity) and a positive control in which cells had been lysed with 0.1% Triton-X-100 (Sigma cat. no. T8787). Both these controls were matched according to biological replicates.

2.2.2 ELISAs

Supernatants stored at -20°C were thawed prior to measurement of cytokines and chemokines using ELISA. Antibodies and standards were from eBioscience through Fisher, except as noted: TNFα (Fisher primary antibody cat. no. 14-7348-85, secondary antibody cat. no. 13-7349-85, cytokine standard solution cat. no. 14-8329-63), IL-6 (Fisher primary antibody cat. no. 14-7069-81, secondary antibody cat. no. 13-7068-85, cytokine standard solution cat. no. 14-8069-62), IL-10 (Fisher primary antibody cat. no. 14-7108-81, secondary antibody cat. no. 13-7109-81, cytokine standard solution cat. no. 14-8109-62), MCP-1 (Fisher primary antibody cat. no. 14-7099-85, secondary antibody cat. no. 13-7096-85, cytokine standard solution R&D cat. no. 279-MC), and IL-1β (Fisher primary antibody cat. no. 14-7018-85, secondary antibody cat. no. 13-7016-85, cytokine standard solution cat. no. 29-8108-60).

Manufacturer’s protocols were followed for ELISAs, with optimization of antibody concentrations, sample dilutions, and incubation times performed in the laboratory. Capture antibodies were diluted 1:1000 (IL-6 and IL-10), 1:500 (IL-1β and MCP-1), or 1:250 (TNFα); detection antibodies were diluted 1:1000 (MCP-1), 1:500 (TNFα, IL-6, and IL-10), or 1:250 (IL-1β). Standards were used in range of 15.625-1000pg/mL, except for MCP-1 which was used in range of 32.25-2000pg/mL. Plates were incubated with samples for 1 hour, except for MCP-1 which incubated for 2 hours. Plates were developed using TMB (Fisher cat. no. 00-4201-56), and the reaction was stopped using 2N sulfuric acid (VWR cat. no. BDH7500-1). Absorbance was read on an Epoch plate reader (BioTek) and fitted to a 4-parameter non-linear standard curve using.
Gen5 software (version 2.07 and 3.05, BioTek).

2.2.3 Bacterial quantification by colony-forming unit (CFU) counts

Cells were lysed using 1mL 0.1% Triton-X-100 (Sigma cat. no. T8787), mixing by pipetting and leaving cells in Triton for at least 5 minutes. Cells were checked under the microscope to ensure complete lysis. Lysates were subjected to four 1:10 serial dilutions in sterile PBS (Fisher cat. no. 10010-049), and 3-4 spots of 10 µL volume were applied to LB plates containing the appropriate antibiotics (ampicillin 100µg/mL for SL1344 or streptomycin 100 µg/mL + chloramphenicol 30 µg/mL for MCS003). Plates were incubated at 37°C overnight, and colonies were counted. The numbers of CFUs in the original lysate were determined by multiplying by the dilution factor, taking into account the plated volume.

2.2.4 Microscopy

Cells adhering to glass coverslips (see 2.2.2) were fixed for 25 minutes with 0.5 ml 4% formaldehyde (Fisher PI28906) in PBS. After this time, the fixative was removed, and replaced with PBS to keep coverslips moist during mounting. Slips were dipped in dH2O to rinse, blotted, and mounted on rectangular slides (VWR cat. no. 48404-454) over a small drop of mounting medium containing DAPI (Fisher Invitrogen cat. no. S36964) for staining nuclei. Excess mounting medium was blotted, and the glass coverslips were sealed over the rectangular slides using clear nail polish. Slides were stored at 4°C and imaging was typically performed within a week to prevent fading; all samples from the same biological replicate were imaged in the same sitting. Imaging was performed using a LSM 800 confocal microscope (Zeiss) running Zen software (blue edition version 2.6). In order to account for Salmonella internalized at different heights, 3-dimensional images were collected using z-stacks. Images were analyzed using Zen black edition (version 2.3).

2.2.5 RNA Isolation and RNA-Seq count generation

RNA was isolated from MDM lysate stored at -80°C using an RNAeasy Plus Mini kit (Qiagen) with DNAse treatment (RNAfree DNAsel kit, Qiagen). RNA quality met sequencing standards upon analysis on an RNA 6000 Nano Chip (Agilent Technologies, Santa Clara, California, USA) on an Agilent 2100 Bioanalyzer. From total RNA, mRNA was isolated using polyA enrichment with d(T) beads (New England Biolabs, Ipswich, Massachusetts, USA), and cDNA libraries were created using a KAPA RNA HyperPrep kit (Illumina cat. no. KR1350). The library creation process consisted of (a) synthesizing first strand cDNA, (b) synthesizing second strand DNA, (c) A-tailing and blunt end formation, (d) ligating adapters (Bioo Scientific, Austin,
Texas, USA) for multiplexing, (e) amplifying the library via PCR, and (f) bead purification.

Library quality was assessed with an Agilent 2100 Bioanalyzer using a High Sensitivity DNA chip (Agilent, Santa Clara, California, USA). RNA-Seq was performed on HiSeq2500. The FASTQ files resulting from RNA-Seq were quality controlled using FastQC (version 0.11.7)\(^{268}\) and MultiQC (version 1.0.dev0)\(^{269}\), demultiplexed and aligned to Ensembl\(^{270}\) human reference genome JRCH38 version 91 using STAR aligner (version 2.5.4b)\(^{271}\). HTSeq (version 0.8.0)\(^{272}\) was used to generate Read Counts. Sequencing quality was otherwise considered good as assessed using MultiQC\(^{269}\). Four samples were removed due to having fewer than 1.2 million aligned reads, leaving \(n = 3\) or 4 samples for each condition. Otherwise, all samples passed the FastQC quality (base calls) and per sequence quality assessments, and all samples had sequences of 100bp in length. Most reads were uniquely aligned (85-92%) and assigned (69-80%). Per sequence GC content was largely normal, though 7 of the retained samples indicated a warning, likely resulting from adapter contamination.

### 2.3 iPSDM sample treatment and counts generation

Data representing the infection of polarized iPSDM by \(S.\) Typhimurium was obtained from collaborator Dr. Christine Hale at the Wellcome Trust Sanger Institute. The methods for generating this data are summarized below.

#### 2.3.1 Production of iPSDM

iPSDM were produced as described in Alasoo \textit{et al} (2015)\(^{258}\). Briefly, iPSC line Bob (A1ATD-1) was maintained on mouse embryonic feeder cells in Advanced Dulbecco’s modified Eagles/F12 medium (DMEM/F12) containing 8 ng/ml recombinant human fibroblast growth factor (FGF2) (R&D system 233-FB). iPSC were differentiated into macrophages by withdrawing FGF, causing the formation of embryoid bodies (EBs), followed by the addition of 25 ng/ml IL-3 (R&D 203-IL) and 50 ng/ml M-CSF (R&D 279-MC) to induce production of myeloid precursor cells from EBs, which were terminally differentiated and matured into iPSDM in RPMI containing 10% heat-inactivated FBS and 100ng/ml human M-CSF.

#### 2.3.2 Bacterial culture and preparation

\textit{Salmonella} strain SL1344 (\textit{ssaG::GFP}) was spread from a frozen stock onto LB plates containing ampicillin (100 µg/mL) for overnight growth. Several colonies were picked and cultured for approximately 3 hours at 37°C. A subculture was produced by adding 100 µL to a fresh 10 mL LB+Amp plate and spreading, and this was then grown overnight at 37°C under static
conditions. Optical density at 600nm (OD\textsubscript{600}) was measured immediately prior to infection, and the bacterial culture was diluted according to previously determined calculations relating OD\textsubscript{600} to CFU (whereby OD of 1 = 10\textsuperscript{9} CFU/mL), in order to produce a final MOI of 20.

2.3.3 Polarization and infection of iPSDM

Two days before infection, iPSDM were plated into 24-well plates at a concentration of 2\times10^{5} cells/mL, with a 0.5 mL volume (1\times10^{5} cells/well), and grown overnight. The following day, media was replaced with fresh media containing 50 ng/mL IFN\gamma to produce M1 macrophages or 50 ng/mL IL-4 for M2 macrophages. After 24 hours, cells were washed and infected with \textit{Salmonella}, prepared as described in 2.3.2 above, at MOI 20. Plates were centrifuged to sediment bacteria, and left to incubate for 30 minutes. Medium was replaced with medium containing gentamycin at 50 \textmu g/ml to kill extracellular bacteria, and returned to the incubator for 4 hours.

2.3.4 RNA Isolation and RNA-Seq counts generation for iPSDM

RNA isolation was performed using the RNeasy Mini kit (Qiagen) with DNAse treatment. Unstranded, poly-A enriched libraries were created using Illumina TruSeq RNA Sample Preparation v2 Kit, and sequencing was done in 5-plex on Illumina HiSeq 2500.

The received FASTQ files resulting from RNA-Seq were demultiplexed and aligned to Ensembl\textsuperscript{270} human reference genome (version GRCh37) using STAR aligner (version 2.5.2b)\textsuperscript{271}. HTSeq (version 0.6.0)\textsuperscript{272} was used to generate read counts. Quality Control was performed using FastQC (version 0.11.5)\textsuperscript{268}. Sequencing quality was considered good as assessed using MultiQC\textsuperscript{269}, with n = 4 samples per condition.

2.4 Statistical analysis

Data were analyzed using \texttt{R} scripts (version 3.6.0)\textsuperscript{273} produced in RStudio. In addition to my own functions, these scripts draw on existing packages tidyverse (version 1.2.1)\textsuperscript{274} and ggplot2 (version 3.2.0)\textsuperscript{275}. For each experiment, values were indexed to the indicated samples, typically the M2 macrophage measurement for CFU but the M1 macrophage value for ELISA. Statistical significance was determined using the Mann–Whitney–Wilcoxon test, since the distribution of values did not always follow a normal distribution. Analysis of RNA-Seq counts data for both MDM and iPSDM was performed using specialized tools, as follows.

2.4.1 RNA-Seq Analysis

Counts were normalized, Principal Component Analysis was performed, and differentially expressed genes were determined using DESeq2 (version 1.24.0)\textsuperscript{276} using a modified
Bioconductor workflow. Low read count genes, defined as those for which fewer than 3 samples contain 10 or more counts, were eliminated. Differentially expressed genes were considered to be those with a log2 fold change >1 (representing a 2-fold change), with an adjusted p-value of <0.05. Gene IDs and symbols were determined using org.Hs.eg.db (version 3.8.2). Overlap in the lists of differentially expressed genes from the two cell types was performed using GeneOverlap (version 1.20.0).

Gene set analysis was performed using the roast method, using 99999 rotations, on counts normalized using voom (limma version 3.40.2). Gene sets used were taken from Pena et al (2014), Becker et al (2015), BioCarta (h_salmonellaPathway) and KEGG, located using the Molecular Signatures Database (MSigDB.v6.2). These sets can be found in Appendix B, Table A.1.

Further pathway analysis on differentially expressed genes was performed using InnateDB for the relative polarization effect, or Sigora (version 3.0.1) for other comparisons, based on genes from DESeq2, with pathway data from the Reactome repository. Multiple test correction was performed using the methods suggested for each of InnateDB and Sigora: Benjamani-Hochberg and Bonferroni, respectively. Pathways were visualized using functions that I developed for this project by building on tidyverse (version 1.2.1) and ggplot2 (version 3.2.0). Networks were calculated using NetworkAnalyst with the IMEx Interactome and KEGG, and visualized using Cytoscape.

Heatmaps were created using pheatmap (version 1.0.12). For heatmaps showing gene expression, a regularized log transformation was used. This transformation allows data to be visualized along a similar dynamic range, preventing the visualization from being dominated by high-count genes while also adjusting expression changes of low-count genes towards zero to account for exaggerated changes that may be observed in genes with low counts but high variance. The regularized log transformation performs better than the alternative variance-stabilizing transformation in cases where the dynamic range of the size factors (sequencing depth) is greater than 4, and a dynamic range of 17 was observed for the MDM RNA-Seq data in this experiment.
Chapter 3: Susceptibility of differently polarized macrophages to infection

3.1 Introduction and rationale

Sepsis is a known complication of Salmonella infections\(^294\), particularly in patients with immune dysfunctions such as AIDS\(^295\) or systemic lupus erythematosus\(^296\). However, no study has determined whether endotoxin tolerant human macrophages are resistant to or susceptible to Salmonella. Endotoxin tolerant macrophages resemble alternatively activated or M2 macrophages\(^162\) and these are known to permit the growth of intracellular Salmonella, unlike classically activated M1 macrophages\(^3,4\). Further, endotoxin primed macrophages have a reduced ability to kill intracellular Leishmania major\(^226\). These observations suggest that endotoxin tolerant macrophages might be permissive for the growth of intracellular Salmonella. On the other hand, endotoxin primed murine bone marrow derived macrophages (BMDM) demonstrate antimicrobial function\(^214\), and endotoxin tolerant mice are resistant to Salmonella, which may or may not be entirely attributed to the recruitment of other immune cells such as neutrophils\(^207\). In addition, the endotoxin gene expression signature includes the alarmins S100A8 and S100A9\(^205\), which are known in neonates to preserve antimicrobial defenses while limiting inflammation\(^228\).

Thus, at the start of this thesis it was not possible to state if endotoxin tolerant human monocyte-derived macrophages (MDM) are resistant or susceptible to Salmonella infection, despite their known\(^162,198,211,212\) lack of pro-inflammatory activity. By determining the effect of endotoxin priming on macrophage permissiveness to intracellular Salmonella infection, the research covered in this chapter has the potential to shed light on the dynamics of endotoxin tolerance in Salmonella septicemia, and also establish parameters of infection for the transcriptomic analysis to follow. Furthermore it might shed light on the known general susceptibility of septic patients to certain types of secondary infections\(^297,298\).

For this study, MDM were polarized using a 24-hour treatment with IFN\(\gamma\) (for M1), IL-4 (for M2), and P. aeruginosa LPS (for M\(^{EP}\)). M1 and M2 polarization is commonly induced with 24-hour stimulation\(^172,299,300\), and to be consistent with these treatments, M\(^{EP}\) received only a single 24 hour LPS treatment to induce tolerance prior to Salmonella infection, which acts as the second stimulation to which a tolerant response will be observed. While many studies of endotoxin tolerance use two or more consecutive stimulations of LPS to induce endotoxin tolerance\(^162,301\), in BMDM, a 24-hour prestimulation with E. coli LPS was sufficient to induce a tolerant response to live Salmonella infection\(^210\), and in sufficient doses P. aeruginosa LPS induces similar effects to
*E. coli* or *Salmonella* LPS\(^\text{259}\). However, in addition to endotoxin, *Salmonella* contains other macrophage-activating molecules and its own immunomodulatory effectors\(^6\). This might cause a more complex response in endotoxin-primed, *Salmonella* treated cells than would a second LPS exposure.

Ultimately, the expression of cytokines was consistent with expectations for the three types of macrophages and endotoxin signature genes\(^\text{205}\) were observed in M\(^\text{EP}\) macrophages (see Chapter 4). Furthermore, *Salmonella* was visualized inside the cells, confirming SCV formation. M1 macrophages showed reduced bacterial load and improved bacterial killing compared to M2 at all measured time points. In contrast, M\(^\text{EP}\) showed similar bacterial load and killing to the resistant M1 at the 0 hour and 2 hour time points, but similar bacterial load and killing to the more susceptible M2 macrophages at the 4 hour and 24 hour time points. This indicates that the antimicrobial functions of the endotoxin-primed cells still function after the 24 hour polarization, but that tolerance results in a suppressed immune response to *Salmonella* and therefore loss of *Salmonella* resistance at some point between 2 and 4 hours of infection, with a deepening state of immunosuppression observed after 24 hours of infection. Overall, this study provides insights into the ability of endotoxin primed MDM to kill *Salmonella*, and provides a foundation for the transcriptional analysis performed in the next chapter.

### 3.2 Results

#### 3.2.1 Experimental Workflow

The MDM experiments presented in thesis used a consistent workflow, shown in Figure 3.1. Monocytes isolated from blood were matured for 7 days in M-CSF, causing them to differentiate into macrophages. These macrophages were polarized for 24 hours using 50ng/mL IFN\(\gamma\) (for M1), 50ng/mL IL-4 (for M2), or 10ng/mL LPS (for M\(^\text{EP}\)), based on the procedures of collaborators whose iPSDM dataset is compared with MDMs in Chapter 5, and previous studies on endotoxin tolerance in our lab\(^\text{162}\). Polarization was verified based on cytokine expression as presented in the next section, and by RNA signature expression in Chapter 4.
Salmonella was added at a calculated MOI of 20, and MDM were centrifuged to increase Salmonella contact and then incubated for 30 minutes. This process is necessary to allow sufficient numbers of Salmonella to invade macrophages, since fewer than 1% of instances of Salmonella-macrophage contact result in invasion. Measurements were taken immediately after this invasion period (called the 0 hour time point for intracellular infection), or after a further 2 hours, 4 hours, or 24 hours of intracellular infection, using washing followed by gentamicin treatment to remove and kill extracellular Salmonella. Supernatants were collected for cytokine quantification using ELISA, cells were lysed to quantify extracellular CFU, and at the 4 hour time point lysate was also collected for RNA isolation.

### 3.2.2 Verification of polarization and infection

Polarization was verified by measurement of the expression of cytokines known to be associated with M1, M2, and M\textsuperscript{EP} macrophages. In order to account for variation between donors, and better show the trends for individual donors, values are indexed. For each donor, the expression value for infected M1 macrophages values is set to 100%, with the values for M2 and M\textsuperscript{EP} macrophages expressed as percentages relative to the M1 macrophage value for that donor. For example, if the M2 macrophage IL-10 value were 200 and the TNF\textalpha value were 50 for a particular replicate, it would indicate that, for that donor, the IL-10 expression in M2 macrophages was twice that of the M1 macrophage, and the TNF\textalpha expression was half that of the M1 macrophage. Raw, unindexed data for cytokine expression are included in Appendix B (Figures A.2 and A.3).

Cytokine expression between polarized cells was compared after infection with Salmonella, since cytokines TNF\textalpha, IL-6, and IL-10 were induced by infection; uninfected polarized cells show...
negligible expression of these cytokines (Figure 3.2). After 4 hours of intracellular *Salmonella* infection, M2 and M\textsuperscript{EP} macrophages produced higher levels of IL-10 than M1 macrophages, and M\textsuperscript{EP} showed suppressed expression of TNFα. Interestingly, M\textsuperscript{EP} macrophages also showed higher levels of IL-1β expression (Figure 3.2).

![Figure 3.2: Cytokine production in polarized MDM which were uninfected or infected with *Salmonella*, after 4 hours intracellular infection.](image)

For each donor, production is expressed relative to the level observed in infected M1 macrophages; 100% represents a median 66,900pg/mL TNFα, 24,900pg/mL IL-6, 160pg/mL IL-10, 138pg/mL IL-1β, and 7,600pg/mL MCP-1. Colour and shape correspond to polarization, and are used for emphasis. Lines represent the median, with 4 biological replicates for uninfected cells, and for infected cells 16 biological repeats for TNFα, 13 BR IL-6 and IL-10, 9 BR IL-1β, 6 BR MCP-1. Statistics were calculated by Wilcoxon test, with p-values indicated as * (< 0.05), ** (<0.01), *** (<0.001).

The observed differences in cytokine expression became more distinct after 24 hours of infection (Figure 3.3). At this point, M1 macrophages showed statistically significantly increased expression of TNFα, IL-1β, and IL-6 compared to M2 macrophages, and reduced expression of IL-10. At 24 hours, M\textsuperscript{EP} macrophages expressed low levels of TNFα, and IL-10, but expressed comparable levels of IL-6 to M1 macrophages, while IL-1β expression was between that of M1
and M2 macrophages.

Figure 3.3: Cytokine production in polarized MDM which were infected with *Salmonella*, after 24 hours intracellular infection. Colour and shape correspond to polarization, and are used for emphasis. Lines represent medians. Data are from 8 biological repeats and expressed relative to cytokine expression in M1 macrophages for the same donor; 100% represents a median 31,300pg/mL TNFα, 91,000pg/mL IL-6, 1,780pg/mL IL-10, and 339pg/mL IL-1β. Statistics were calculated by Wilcoxon test, with p-values indicated as * (< 0.05), ** (<0.01), *** (<0.001).

At 4 hours after infection, GFP-expressing bacteria could be visualized inside some macrophages (Figure 3.4a), indicating activation of SPI-2 and thus intracellular *Salmonella*-Containing Vacuole formation due to intracellular invasion. Imaging with transmitted light allowed counting of infected macrophages, while three-dimensional confocal microscopy imaging (Figure 3.4b-e) allowed for counting of bacteria within macrophages. The infection rate (% of macrophages that contain one or more GFP-labelled bacteria) ranged in individual experiments from between 4.28% and 59.6% of macrophages, with 1.2 to 10 bacteria per infected macrophage. The mean infection rate across experiments, for all macrophage types, was 21.1% of macrophages infected/GFP-positive, with 3.4 bacteria per infected macrophage.
Figure 3.4: *Salmonella* visualized within macrophages using confocal microscopy. [a] Appearance of the macrophage in 2D with *Salmonella* shown by green fluorescence (GFP), macrophage nuclei shown in blue (DAPI), and macrophage cell boundaries shown using transmitted-light imaging. [b] 3D visualization of a macrophage, nucleus stained blue, infected by 4 bacteria, green rods. [c]-[e] 3D visualization of fields of differentially polarized macrophages with nuclei stained blue, infected with intracellular bacteria in green: [c] M1, [d] M2, and [e] M<sup>EP</sup>.

### 3.2.3 Susceptibility of MDM to *Salmonella* infection

*Salmonella* resistance was assessed using a gentamicin protection assay to measure intracellular bacterial load. In order to more accurately assess trends without interference from donor variability, CFU data are indexed to the values for M2 cells. Raw data are shown in Appendix B (Figure A.4 and A.5). After 4 hours of intracellular infection, both M2 and M<sup>EP</sup> macrophages were statistically significantly more susceptible to *Salmonella* infection than M1 macrophages, with M1 macrophages showing an average bacterial load 41.6% that of M2 macrophages (range 0.8%-78.6% relative to the paired M2 sample), and M<sup>EP</sup> macrophages showing a comparable average bacterial load to M2 macrophages (Figure 3.5a).

To determine the dynamics of infection, I performed a time course experiment. The 0 hour time point quantifies initially internalized bacteria, whether this occurs by macrophage phagocytosis or initiated by *Salmonella* Pathogenicity Island (SPI)-1 effectors<sup>43,303</sup>. The 2 hour time point captures the early stages of infection, just after the SCV has been established as indicated by acidification of the SCV and induction of PhoPQ and *Salmonella* Pathogenicity Island-2<sup>4,43,88,266</sup>. The 24-hour time point measures the ability of *Salmonella* to replicate within the macrophage; bacterial load at this point reflects a balance between killing and replication of the
Interestingly, M\textsuperscript{EP} macrophages showed bacterial loads similar to those of M1 macrophages at the 0-hour (internalization) and 2-hour (SCV formation) time-points (Figure 3.5b). At both these time points, M1 and M\textsuperscript{EP} macrophages had a lower bacterial load than M2.
macrophages, though for M1 macrophages this difference did not meet the significance threshold at these time points (p=0.08 at 0 hours and p=0.1 at 2 hours). All three types of macrophages were able to eliminate some *Salmonella* between internalization and SCV formation, but at different rates. In M1 macrophages, an average of 31% of bacterial survived 0-hour to 2-hour time points, compared to a 53% survival rate within M2 macrophages (Figure 3.5c). In *M*<sup>EP</sup>, only 33% of *Salmonella* survived from 0 hours to 2 hours, similar to the survival rate in M1 macrophages and lower than that in M2 macrophages.

However, at the 4 hour (Figure 3.5a) and 24 hour (Figure 3.5b) replication time points, the bacterial load in *M*<sup>EP</sup> macrophages had increased and was similar to that of M2 macrophages, with M1 macrophages still showing statistically significantly lower levels of bacteria. Indeed at 24 hours after infection, 11% of the bacteria present in M1 macrophages survived; 33% of bacteria survived in M2 macrophages, and 43% survived in *M*<sup>EP</sup> macrophages (Figure 3.5d). This leads me to speculate that endotoxin primed macrophages still exhibited some M1-like functions after the initial tolerizing 24 hour LPS treatment, but that they became increasingly M2-like over the subsequent 2-22 hours of exposure to *Salmonella*.

Modest cytotoxicity was observed after this 24-hour infection (Figure 3.6). The highest median cytotoxicity was for M1 macrophages at 17.2%. This was significantly higher than the 9.0% cytotoxicity observed for *M*<sup>EP</sup> macrophages (p=0.010), and somewhat higher than the 10.8% cytotoxicity observed for M2 macrophages, though the latter was not statistically significant (p=0.083).

![Figure 3.6: Cytotoxicity after 24 hours infection with *Salmonella*. Color and shape represent polarization and are used for emphasis. Lines indicate the median of 8 biological repeats. Statistics were calculated by Wilcoxon test, with p-values indicated as * (<0.05), ** (<0.01), *** (<0.001).](image-url)
3.3 Discussion

The observed cytokine expression was generally consistent with that expected for successful polarization of the macrophages. Over 24 hours of infection, M1 showed increased expression of the pro-inflammatory cytokines TNFα and IL-1β, as well as IL-6, compared to M2 macrophages (Figure 3.3). This was fully consistent with the expected pro-inflammatory nature of M1 macrophages\textsuperscript{139,171}. In contrast, M2 macrophages expressed higher levels of anti-inflammatory IL-10 than M1 macrophages did at both 4 hours and 24 hours after infection (Figure 3.2 and Figure 3.3), which is consistent with M2 polarization\textsuperscript{304–306}. Thus cytokine expression indicated a correct polarization of these M1 and M2 cells.

A reduction of TNFα was observed in M\textsuperscript{EP} macrophages compared to both M1 and M2 macrophages at 4 hours after infection (Figure 3.2); this low production of TNFα is classically characteristic of endotoxin tolerance\textsuperscript{162,199}. Over 24 hours (Figure 3.3), M\textsuperscript{EP} macrophages continued to show reduced expression of TNFα compared to M1 macrophages, and also showed reduced expression of IL-1β when compared to M1 macrophages, which is also characteristic of endotoxin tolerance\textsuperscript{198}. At the 24-hour time point, M\textsuperscript{EP} also showed a reduction in IL-10 compared to both M1 and M2 macrophages (Figure 3.3). While high IL-10 expression has been reported in endotoxin tolerant mice\textsuperscript{212}, our lab found reduced IL-10 expression in endotoxin tolerant PMBCs\textsuperscript{162}, so this observation is still in line with the cytokine expression expected in endotoxin tolerant macrophages.

The observed increased expression of IL-1β by M\textsuperscript{EP} macrophages at 4 hours (Figure 3.2) is not generally characteristic of endotoxin tolerance\textsuperscript{198}. However, it likely arose from the macrophages being stimulated with Salmonella infection rather than a second dose of LPS, since Salmonella infection involves exposure to multiple pathogen signatures including LPS and flagellin as well as bacterial effectors\textsuperscript{6}. In particular, macrophages that have been tolerized to TLR ligands such as LPS can still produce IL-1β, e.g. in response to Nod-like receptor activation by Salmonella flagellin\textsuperscript{210,239}. Furthermore, reduced expression of IL-1β at 24 hours compared to M1 macrophages (Figure 3.3) still indicated a reduction in inflammatory potential. IL-6 expression levels were similar to M1 (Figure 3.2 and Figure 3.3) but since IL-6 is known to have mixed pro- and anti-inflammatory activity\textsuperscript{133}, this did not necessarily indicate that M\textsuperscript{EP} macrophages had an inflammatory character, especially given the statistically significant reduction in TNFα levels. Overall, endotoxin priming did appear to have created a suppressed inflammatory response upon
Salmonella stimulus as expected, though higher doses may have produced a stronger suppression\textsuperscript{198}.

Infection was also successful, and consistent with previous observations of Salmonella replication in M1 and M2 MDM by Lathrop \textit{et al}\textsuperscript{4}. Bacteria were visualized internalized within the macrophage, and GFP expression indicated activation of SPI-2 genes under the \textit{ssaG} promoter (Figure 3.4). The distribution of numbers of bacteria per infected cell (1-10, mean 3.4) was consistent with the findings of Lathrop \textit{et al} that most cells contained fewer than 5 bacteria at 2 hours post infection, although some did contain 10 or more. Lathrop \textit{et al} also reported that between 2 hours and 18 hours post-infection the number of bacteria in M1 macrophages decreased by half, which is similar to what I observed at the 24 hour time point (Figure 3.5b). The mean infection rate in my experiments of 21.1\% at 4 hours was somewhat lower than that observed by Lathrop \textit{et al}, who reported that 40\% of M1 macrophages and 58\% of M2 macrophages contained \textit{Salmonella} at 2 hours after infection, but this can be attributed to differences in time points, culturing conditions and donors. Net replication of bacteria was not observed in M2 macrophages (Figure 3.5b). This might be due to the absence of histidine supplementation, which Lathrop \textit{et al} determined was necessary for replication of the SL1344 strain, consistent with the observation that SL1344 is a histidine auxotroph\textsuperscript{307,308}. However, I consistently observed a greater bacterial load in M2 than in M1 macrophages, at least in part due to increased killing in the case of the M1 macrophages, which is consistent with the reported resistance of M1 macrophages to \textit{Salmonella}\textsuperscript{3–5}.

Having established that the cytokine expression and infection in M1 and M2 macrophages was consistent with expectations, I also considered the effects of endotoxin priming. M\textsuperscript{EP} macrophages showed a very similar bacterial load to M1 macrophages at the internalization phase and early infection (SCV establishment) phase of the infection, representing similar rates of uptake and early killing of the bacteria (Figure 3.5b). The higher rate of bacterial uptake in M2 macrophages could result from either a higher rate of non-opsonic phagocytosis of \textit{Salmonella}, or from a greater responsiveness to \textit{Salmonella}-initiated uptake driven by SPI-1 effectors\textsuperscript{43,303}. M2 macrophages express higher levels of scavenger receptors such as MARCO\textsuperscript{309,310} and CD36\textsuperscript{311}, which are involved in binding and internalization of pathogens such as \textit{Salmonella typhimurium}\textsuperscript{49,50}, suggesting that non-opsonic phagocytosis likely contributes to the higher rate of \textit{Salmonella} internalization in M2 macrophages. However, further experiments would be required
to differentiate between the two internalization mechanisms.

The higher levels of *Salmonella* internalized in M2 macrophages, however, are not solely responsible for the higher bacterial load at the 2-hour time point. Not only is the bacterial load lower in M1 and M<sup>EP</sup> macrophages at this time, but the percentage of bacteria surviving from 0 hours to 2 hours is lower (31% and 33% compared to 53%) (Figure 3.5c), indicating that M1 and M<sup>EP</sup> macrophages are more successful at killing internalized bacteria as the bacteria shift from the invasion to intracellular program.

However, as the infection progressed the M<sup>EP</sup> demonstrated a longer-term permissiveness to replication. By 4 hours after infection, they showed similar bacterial loads to M2 macrophages (Figure 3.5a), and this persisted through to the 24 hour time point, with both the 24 hour bacterial load and the rate of bacteria surviving from 0 hours to 24 hours being similar in M<sup>EP</sup> and M2 macrophages, representing 3-fold the bacterial load and 3- to 4-fold the survival rate observed in M1 macrophages (Figure 3.5b and d). This might indicate that after 24 hours of LPS stimulation, the major antimicrobial functions remained intact, but that these functions became less efficient at some time between 2 hours and 4 hours after the *Salmonella* invasion. This appeared to coincide with the less inflammatory cytokine production (lower TNFα and higher IL-10) at the 4 hour time point (Figure 3.2) compared to M1 macrophages.

This shift in resistance is not surprising given what is known about the dynamics of endotoxin responses and tolerance. LPS induces M1 responses<sup>139,312</sup>; the reprogramming characteristic of endotoxin tolerance occurs after prolonged stimulation<sup>206</sup>. The data suggest that, in response to the initial 24-hour stimulation with LPS, the M<sup>EP</sup> do induce some M1 antimicrobial functions, leading to initial resistance to *Salmonella* (Figure 3.5b and c). At the same time, the initial stimulation reprogrammed M<sup>EP</sup> macrophages such that they have a tolerant response to the *Salmonella* infection; this response manifests as suppressed TNFα expression, increased IL-10 expression, and reduced *Salmonella* resistance measured at the 4-hour time point (Figure 3.2 and Figure 3.5a). Repeated doses of LPS are also known to increase immune suppression<sup>206,207</sup>. It is thus likely that after 24 hours of *Salmonella* infection, the repeated immune stimulation led to a further deepened state of immunosuppressive tolerance, as indicated by the increased cytokine suppression (Figure 3.3) and continued *Salmonella* survival (Figure 3.5b and d) at this time point.

The infection rates and bacterial load seen in the microscopy data were not entirely consistent with the CFU counts (see Appendix C). This is likely because of culturing these cells
directly on glass coverslips as opposed to treated plastic, as growing macrophages on different types of plastic is known to affect their activation\textsuperscript{171}, but could also relate to differential viability of \textit{Salmonella} in differently polarized macrophages.

The moderately elevated rates of cytotoxicity from 10-12\% in M2 and M\textsuperscript{EP} to 18\% in M1 macrophages after 24 hours of infection (Figure 3.6) may reflect higher rates of pyroptosis in the latter cells, particularly in light of the higher IL-1\beta expression in these cells at the 24 hour time point (Figure 3.3). However, other explanations are possible. For example, \textit{Salmonella} is capable of manipulating host cell death processes, such as by suppressing TNF\alpha-induced cell death\textsuperscript{313,314}. The higher bacteria loads in M2 and M\textsuperscript{EP} macrophages at this time point could thus be responsible for the lower cytotoxicity observed in these samples. This difference in cytotoxicity was not high enough that a decreased number of cells could account for the lower numbers of intracellular bacteria isolated in M1 macrophages.

3.3.1 \textbf{Limitations and future directions}

As these experiments used primary cells isolated from blood, donor variability can result in a range of responses to polarization and infection, as well as modest variation in final macrophage density after maturation. While a method is available for approximating the number of \textit{Salmonella} CFUs within a liquid culture using OD, the calculation was not precise. Thus, while I consistently applied an estimated MOI of 20, based on the final macrophage density, confirmatory overnight cultures indicated that the actual MOI varied somewhat, with 4 hour measurements (Figure 3.5a) having an average MOI of 35 while the time course samples (Figure 3.5b) averaged an MOI of 19. This variation, together with donor variability, could partly explain the observed 4.28\%-59.6\% range in infection rates. In order to minimize the effects of these sources of variation, data were indexed such that cytokine expression and bacterial load were expressed relative to samples that had been collected from the same donor and exposed to the same MOI.

The infection rates reflect a second limitation of the analysis: all measurements were made at the population level. Within the resistant populations, some macrophages were still observed to be infected with multiple \textit{Salmonella} (Figure 3.4c), and within the susceptible population some macrophages were still uninfected (Figure 3.4d). Polarization shifted the overall level of resistance of the population, but the measurements in bacterial load and cytokine expression were still averaged over a large number of cells. This experiment was thus unable to distinguish between,
for example, reductions of bacterial load resulting from decreased replication, and those resulting from increased killing\textsuperscript{315}.

One possible extension of this work would be measuring resistance over longer periods of time. During \textit{Salmonella} infection, the period of intracellular replication lasts several days\textsuperscript{2}, and the ultimate clearance, persistence, or replication of \textit{Salmonella} within the differently polarized macrophages over 2-7 days would be relevant to the outcome of infection. Immunosuppression resulting from sepsis can also be long-lasting\textsuperscript{201}. It would be of interest to determine whether the ultimate susceptibility of M\textsuperscript{EP} to \textit{Salmonella} infection could persist if the infection occurred one or more days after the initial tolerance-inducing endotoxin priming. Such studies are ongoing in our lab.

Another direction of inquiry lies in interrogating the mechanisms by which M1 macrophages were resistant to \textit{Salmonella}, and by which M\textsuperscript{EP} were initially resistant but ultimately susceptible to \textit{Salmonella} infection. In order to thoroughly examine the biological processes involved and identify likely mechanisms, I performed a systems biology analysis of the effect of macrophage polarization on infection.
Chapter 4: Transcriptomics analysis of the effect of activation and tolerance on *Salmonella* infection in MDM

4.1 Introduction and rationale

*Salmonella* is a significant human pathogen. It was estimated that it caused close to 100 million cases of non-typhoidal gastroenteritis in 2010, and 155,000 deaths, largely through foodborne routes\(^1\). Agricultural use of antibiotics has led to increased antibiotic resistance\(^7\), which impedes treatment of sepsis cases involving these pathogens\(^8\). Given that the pathology of *Salmonella* depends substantially on its replication within the macrophage\(^17\), a more in-depth understanding of the responses of resistant and susceptible macrophages would improve our ability to treat *Salmonella* infections.

Transcriptional studies have enabled the derivation of signatures for M1 and M2 macrophages\(^261\), and for endotoxin tolerance\(^205\). Further, RNA-Seq and whole genome sequencing of M1 and M2 human monocyte-derived macrophages (MDM) have to date enabled a deeper understanding of the differences between these cell types and revealed novel markers\(^167,260\). Single-cell RNA-Seq in murine bone marrow-derived macrophages (BMDM) has also provided insights into the transcriptional states of individual macrophages in the course of *Salmonella* infection\(^3\). Some innate immune mechanisms of resistance to *Salmonella* are known, including interleukins such as IL-12 and IL-13\(^39\) and inflammasomes\(^70\). Some aspects of *Salmonella* resistance in the specific context of M1 macrophages have been also identified, including production of reactive oxygen species and rupture of the *Salmonella*-containing vesicle (SCV) through guanylate binding proteins\(^11,232,233\). However, there has not been a detailed transcriptomic analysis of *Salmonella* infection in polarized human macrophages, or in endotoxin primed macrophages.

RNA-Seq analysis was performed on M1, M2, and M\(^{EP}\) macrophages polarized and infected as in Chapter 3. Samples were taken at the 4-hour time point. Because this is the first time point at which M\(^{EP}\) are susceptible to *Salmonella* (Figure 3.5), I expect that differences in gene expression between M\(^{EP}\) and M1 macrophages will provide insight into the loss of *Salmonella* resistance in M\(^{EP}\) macrophages.

The differences in gene expression between polarized macrophages was compared for both uninfected and infected cells in order to determine which pathways were differentially modified by polarization alone, and which were differently expressed between polarized cells in response.
to infection. Indeed, it was determined that some genes, such as JAK-STAT genes, were differentially expressed primarily in uninfected cells, while others, including major inflammasome genes, were differentially expressed primarily in infected cells. M1, M2, and M^EP^ macrophages showed differences in expression in genes known to be important in *Salmonella* infection. Finally, genes from the endotoxin tolerance signature\(^205\) and from “chemokine receptors bind chemokines” pathways were found to be upregulated specifically by M^EP^ macrophages while cholesterol synthesis pathways were downregulated. This analysis provided a more complete picture of the effect of polarization and endotoxin priming on the response of macrophages to *Salmonella* infection.

4.2 Results

4.2.1 RNA-Seq Workflow

To produce the data in this chapter and Chapter 5, an RNA-Seq pipeline was implemented as summarized in Figure 4.1. Samples were produced as described in Figure 3.1, and RNA was isolated from cell lysate after 4 hours of intracellular infection. A cDNA library was generated from these samples, and this cDNA was sequenced, producing hundreds of millions of paired reads covering hundreds of billions of base pairs. The reads were mapped to genes using a reference genome, producing a counts table indicating number of reads aligned to each gene for each sample. This counts table was used in bioinformatics methods such as Principal Component Analysis to visualize similarity between samples, differential gene expression analysis to identify genes that were significantly differently expressed between two conditions, and gene set enrichment analysis to determine whether sets of genes of interest were differently regulated between samples. Lists of differentially expressed genes were further analysed using protein:protein interaction networks in order to identify genes that might act as interaction “hubs,” and using pathway enrichment analysis in order to determine which biological pathways were over-represented in the list of differentially expressed genes. These methods are described in further detail as the relevant results are introduced.
4.2.2 MDM samples clustered according to infection and polarization status

Upon generation of a set of gene expression counts from RNA-Seq data, the first step in analysis was to confirm that samples that were similarly stimulated had broadly similar gene expression patterns. This was done using clustering and heat-map methods.

Principal Component Analysis (PCA) is a technique by which an entire gene expression dataset of potentially thousands of differentially expressed genes is mathematically reduced into a smaller number of dimensions referred to as principal components, such that the first few components (i.e. specific sets of genes changing expression) explain most of the variability within the full gene expression dataset. PCA is used to identify similarity between clusters of samples. Since differences in sample treatment should account for most of the variability between samples, samples within the same treatment condition should have similar values for the first principal components. As a result, they are expected to cluster together on a PCA plot. Similarly, the separation between two conditions on one or more dimensions indicates the extent of difference between those conditions.

MDM clustered as anticipated in the PCA plot (Figure 4.2). *Salmonella* infected and uninfected cells were separated along the first principal component (PC1), which explained 63% of the variance within the dataset. Separation of cells by polarization state was primarily along...
PC2, which explained 12% of the variance. While M1 and M2 cells were well separated, M0 macrophages were located between and slightly overlapped with the two. Interestingly, \( M^{\text{EP}} \) macrophages were located closer to M1 macrophages on this plot than to M2 macrophages.

![PCA plot showing clustering of normalized gene expression data for differently activated MDMs that were uninfected or infected with *Salmonella*](image)

**Figure 4.2:** PCA plot showing clustering of normalized gene expression data for differently activated MDMs that were uninfected or infected with *Salmonella*. The first two principal components, plotted on x and y axes, summarize the greatest sources of variation between samples.

Similarity between samples can also be expressed using a heatmap which displays the distance between each pair of samples, calculated by treating normalized expression levels of each gene as one dimension in a multidimensional space (Figure 4.3). The heatmap showed distinct clustering of uninfected and infected cells. While \( M^{\text{EP}} \) cells separated into two distinct groups of infected \( M^{\text{EP}} \) macrophages and uninfected \( M^{\text{EP}} \) macrophages, the M0, M1, and M2 macrophages were more intermixed on the heatmap but still separated according to infection status.
Figure 4.3: Sample distance heatmap showing clustering of gene expression for differently activated macrophages that were infected or uninfected. The colour of the heatmap indicates the distance between samples. On axis labels, ETP indicates M^{EP} macrophages.

4.2.3 M1 and M2 express polarization signatures, and infection affects these genes.

To verify polarization and observe the effect of infection on polarization markers, the Roast gene set enrichment tool was used to assess enrichment of genes that were part of M1 and M2 gene expression signatures that had been derived using integrated transcriptomics by Becker et al.\textsuperscript{261} (see Appendix D, Table A.1 for the lists of genes). The Roast method\textsuperscript{280} is designed for testing for changes of expression in one or more specific gene sets of focused interest in an experiment. Roast uses simulation to calculate whether a set of genes is overall up- or down-regulated when comparing two cell types. Roast determines both a direction of enrichment, and a p-value for that enrichment. This calculation is based on both the number of genes from the set that are up- and down-regulated, and the significance of change of these genes.

Three types of comparisons between cell types were drawn in order to provide a full picture of the differences associated with macrophage polarization and infection. These comparisons are illustrated in Figure 4.4, and I use these icons to indicate which comparison is considered in figures elsewhere in the thesis. First, comparisons between control samples that were polarized but not infected provided a baseline for differences between the polarization types, in the absence of
*Salmonella*. Second, comparisons between uninfected and infected cells of each polarization type provided insights into how genes changed expression during infection for each type of macrophage. Third, comparisons between infected cells of different polarization types demonstrated the differences in gene expression in differently polarized macrophages infected with *Salmonella*. A measurement was also derived describing the difference in transcriptional response to infection between the differently polarized macrophages. Statistically, this measurement is called the “interaction term,” but to avoid confusion with biological interactions such as protein-protein interactions, I will refer to it as the “relative polarization effect.”

![Diagram showing different comparisons made during RNA-Seq analysis](image)

**Figure 4.4: Illustration of the different comparisons made during RNA-Seq analysis.** The information provided by each regarding the effects of polarization and infection indicated below each comparison.

Roast indicated that a statistically significant number of genes from the M1 (polarized with IFNγ and LPS or TNFα) signature\(^{261}\) (Appendix D, Table A.1) were upregulated in M1 and M\(^{EP}\) macrophages compared to M2 macrophages regardless of infection state (Table 4.1). Similarly, the M2 (polarized with IL-4 or IL-13) signature gene set of Becker *et al*\(^{261}\) (see Appendix D, Table A.1) was found to be upregulated in M2 macrophages when compared to M1 and M\(^{EP}\) macrophages for both uninfected and infected cells. This is reflected in Table 4.2 as a downregulation of these M2 signature genes in M1 and M\(^{EP}\) macrophages when compared to M2 macrophages.
Table 4.1: Roast gene set enrichment test for M1 signature genes. The 100-gene M1 signature was derived by Becker et al\cite{261}. Columns are as follows. “MDM Types Compared”: the cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: to the relative polarization effect. “% Up-Regulated” and “% Down-Regulated”: percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. “Direction”: whether the gene set was found to be overall up-or down-regulated, based on both the number of up- and down-regulated genes, and the significance of change. “p-value” was calculated by Roast taking into account both percentage of up- and down-regulated genes and magnitude of change. Statistically significant (p<0.05) comparisons are indicated in bold.

<table>
<thead>
<tr>
<th>MDM Types Compared</th>
<th>% Up-regulated</th>
<th>% Down-regulated</th>
<th>Direction</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 vs. M2 uninfected</td>
<td>92</td>
<td>1</td>
<td>Up</td>
<td>&lt;0.001</td>
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<tr>
<td>M\textsuperscript{EP} vs. M1 uninfected</td>
<td>39</td>
<td>27</td>
<td>Up</td>
<td>0.103</td>
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<tr>
<td>M\textsuperscript{EP} vs. M2 uninfected</td>
<td>91</td>
<td>0</td>
<td>Up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M1 vs. M2 infected</td>
<td>57</td>
<td>1</td>
<td>Up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M1 infected</td>
<td>27</td>
<td>29</td>
<td>Up</td>
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<tr>
<td>M\textsuperscript{EP} vs. M2 infected</td>
<td>53</td>
<td>12</td>
<td>Up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>45</td>
<td>14</td>
<td>Up</td>
<td>0.0016</td>
</tr>
<tr>
<td>M2 infected vs. uninfected</td>
<td>62</td>
<td>5</td>
<td>Up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M\textsuperscript{EP} infected vs. uninfected</td>
<td>26</td>
<td>5</td>
<td>Up</td>
<td>0.125</td>
</tr>
<tr>
<td>Infected/uninfected M1 vs. M2</td>
<td>4</td>
<td>58</td>
<td>Down</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Infected/uninfected M\textsuperscript{EP} vs. M1</td>
<td>6</td>
<td>25</td>
<td>Down</td>
<td>0.222</td>
</tr>
<tr>
<td>Infected/uninfected M\textsuperscript{EP} vs. M2</td>
<td>3</td>
<td>52</td>
<td>Down</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 4.2: Roast gene set enrichment test for M2 signature genes. The 58-gene M2 signature was derived by Becker et al\cite{261}. Columns are as follows. “MDM Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. “% Up-Regulated” and “% Down-Regulated”: percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. “Direction”: whether the gene set was found to be overall up- or down-regulated. “p-value” was calculated by Roast taking into account both percentage of up- and down-regulated genes and magnitude of change. Statistically significant (p<0.05) comparisons are indicated in bold.

<table>
<thead>
<tr>
<th>MDM Types Compared</th>
<th>% Up-regulated</th>
<th>% Down-regulated</th>
<th>Direction</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 vs. M2 uninfected</td>
<td>4</td>
<td>67</td>
<td>Down</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M1 uninfected</td>
<td>19</td>
<td>20</td>
<td>Up</td>
<td>0.977</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M2 uninfected</td>
<td>6</td>
<td>65</td>
<td>Down</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M1 vs. M2 infected</td>
<td>0</td>
<td>70</td>
<td>Down</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M1 infected</td>
<td>39</td>
<td>19</td>
<td>Up</td>
<td>0.050</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M2 infected</td>
<td>15</td>
<td>57</td>
<td>Down</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>9</td>
<td>41</td>
<td>Down</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M2 infected vs. uninfected</td>
<td>9</td>
<td>39</td>
<td>Down</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M\textsuperscript{EP} infected vs. uninfected</td>
<td>2</td>
<td>26</td>
<td>Down</td>
<td>0.004</td>
</tr>
<tr>
<td>Infected/uninfected M1 vs. M2</td>
<td>2</td>
<td>9</td>
<td>Down</td>
<td>0.403</td>
</tr>
<tr>
<td>Infected/uninfected M\textsuperscript{EP} vs. M1</td>
<td>19</td>
<td>2</td>
<td>Up</td>
<td>0.143</td>
</tr>
<tr>
<td>Infected/uninfected M\textsuperscript{EP} vs. M2</td>
<td>13</td>
<td>4</td>
<td>Up</td>
<td>0.508</td>
</tr>
</tbody>
</table>
In response to infection, the M1 signature was statistically significantly upregulated in both M1 and M2 macrophages, but not M^{EP} macrophages (rows 8-10 of Table 4.1). In contrast, the M2 gene signature was downregulated in response to infection for the M1, M2, and M^{EP} macrophages (rows 8-10 of Table 4.2).

4.2.4 Certain anti-infective pathways showed primed activation in uninfected M1 and M^{EP} macrophages

Bioinformatics statistical methods in the DESeq2^276 package were used to determine differentially expressed genes: that is, genes for which expression was significantly different between two conditions. DESeq2 estimates Log$_2$ fold change in gene expression between two conditions. The estimate is adjusted such that lower count genes, and genes with greater variability, are reported as having a lower estimated fold change. This reduces the chances that low count or high variability genes will be identified as showing a significant magnitude of change in the analysis. The standard error of the Log$_2$ fold change estimates is then used to perform a Wald test, and the resulting p-value is corrected for multiple testing using the Benjamani-Hoffberg adjustment. Genes are considered significantly differentially expressed if both the magnitude of the fold change is greater than 2 (Log$_2$ fold change > 1), and the Benjamani-Hoffberg-adjusted p-value is lower than 0.05. DESeq2 automatically removes from the analysis genes containing outlier samples (defined as samples for which the Cook’s distance is greater than the 0.99 quantile).

For the DESeq2 analysis, the same comparisons between cell types were investigated as for Roast analysis and illustrated in Appendix D, namely polarization effect for uninfected cells, polarization effect for infected cells, response to infection (uninfected vs infected cells) for each polarization type, and the relative polarization effect on response to infection. Sigora^288 and InnateDB^102 pathway overrepresentation analysis was used to identify any particular pathways that were enriched, or found at greater levels than would be expected by chance, in the set of differentially expressed genes. These methods take as their input the lists of significantly differentially expressed genes produced by DESeq2, and compare these lists with pathways contained in some database, in this case REACTOME^289. Where more genes from a pathway are found in the list of differentially expressed genes than would be expected by chance, after multiple test correction, the pathway is said to be enriched. Such a statistically significant enrichment would suggest that those pathways were important in determining the functional differences between cell and treatment types.
A pattern became apparent while analyzing enriched pathways. In M2 macrophages certain pathways were poorly expressed in uninfected cells and more highly expressed after infection. In contrast, uninfected M1, and typically also uninfected M\textsuperscript{EP}, showed a higher expression of these pathways than M2 macrophages. However, these pathways were not as substantially upregulated in M1 and M\textsuperscript{EP} macrophages in response to infection, and as a result the differences between infected M1, M2, and M\textsuperscript{EP} macrophages was less extensive than between uninfected macrophages. Since the most significant differences between polarization types for these pathways were their activation as a result of priming of uninfected macrophages, rather than their response to infection, I will here refer to this expression pattern as “primed activation.” It is conceptually similar to other cases of immune priming, such as neutrophils\textsuperscript{316}, in that it represents a response to an initial immune stimulus (IFN\textgamma{} or LPS), but is unlike neutrophil priming in that a second activating stimuli is not necessarily required for a response. Primed activation represents a subset of the transcriptional changes produced by polarization: those in which differences resulting from polarization were most distinct in uninfected cells. The primed activation pattern is conceptually presented in Figure 4.5. This diagram represents pathway activation in absolute terms as a visual aid, translating patterns in Sigora’s representation of differences between pathways enrichment for two types of cells into a level of activation for one of those types of cells. For example, uninfected M1 and infected M2 macrophages both being enriched compared to uninfected M2 macrophages indicates low activation in uninfected M2 macrophages.

![Figure 4.5: Conceptual diagram of activation levels representing a primed activation pathway.](image)

When represented in a pathway enrichment dot plot, this pattern had a distinctive appearance (Figure 4.6a). First (a.1: example 1), upregulation in the M1 vs M2 uninfected
macrophages, which sometimes coincided with upregulation in $M^{EP}$ uninfected macrophages.

Second (a.2: example 2), upregulation in M2 macrophages in response to infection was equal to if not greater than upregulation in M1 and $M^{EP}$ macrophages in response to infection. Third, the differences between infected macrophages (a.3: example 3) were smaller than those observed in uninfected cells (a.1: example 1). In some cases, the relative polarization effect (a.4: example 4) indicated downregulation in the pathway in M1 and/or $M^{EP}$ macrophages in response to infection, or in other words a lesser response to infection, when compared to M2 macrophages. Such a primed activation pattern was observed for many pathways associated with immunity (Figure 4.6b).

**Figure 4.6: Immune pathways showing primed activation.** The colour of the dots indicate the percentage of the differentially expressed genes found in the pathway which were upregulated: pathways that were largely upregulated are red, while pathways that were largely downregulated are green. The size of the dots indicates the significance level of the pathway enrichment, $-\log_{10}(\text{adjusted p-value})$, such that larger dots have smaller p-values. [a] Diagrams representing examples of the primed activation pattern as it appears on a pathway dot plot. [b] Pathways that showed this pattern in MDM data. Abbreviations: “un” for “uninfected”, “inf” for “infected”, and “inter” or “Interaction” refers to the relative polarization effect (the difference between the response to infection – infected/uninfected – in different polarization types). “p-adj” refers to the p-value adjusted for multiple testing. Pathways with an adjusted p-value <0.05 are shown.

These primed activation pathways included several interferon-related pathways: the “interferon (IFN) signaling”, “IFNγ signaling”, “IFNa/β signaling”, and “RIG-I/MDA5 mediated induction of IFNa/β” pathways, as well as “C-type Lectin Receptors (CLRs)”. In particular, the relative polarization effect revealed that IFN α/β and γ pathways were statistically significantly
less upregulated due to infection in M1 and M\(^{EP}\) macrophages, compared to M2 macrophages. Interferon pathways are known to be important in defense against intracellular pathogens including both viruses and bacteria\(^{317,318}\). C-type Lectin Receptors are pathogen recognition receptors that are important in antiviral defenses\(^{319}\). Furthermore, secreted CLR Reg3b prevents the translocation of *Salmonella* into murine tissue, thus reducing the severity of infection\(^{320}\). Primed genes in the CLRs pathway include CLEC4D, which is known to activate antimicrobial functions such as the respiratory burst in myeloid cells\(^{321}\), while related CLR Dectin-2 recognizes *Salmonella* O-antigen\(^{322}\). It is interesting that these pathways specific to intracellular infection and *Salmonella* were active in uninfected M1 macrophages, while I did not observe such changes in the more general TLR signaling pathways.

Additional primed activation immune pathways were “Class A/1 Rhodopsin-like receptors”, “Chemokine Receptors bind Chemokines”, “IL-10 signaling”, and “IL-15 signaling”. Class A/1 Rhodopsin-like receptors include a variety of receptors involved in inflammatory signaling, such as those for prostaglandins, histamine, and chemokines\(^{323}\) (which represent a primed pathway in their own right). The most upregulated member of the Rhodopsin-like receptors pathway in uninfected M1 cells was RXFP1; activation of this receptor can increase LPS-induced secretion of pro-inflammatory cytokines such as IL-6 and IL-8\(^{324}\). A role for RXFP1 in *Salmonella* infection has not yet been identified and if this gene were confirmed to indeed be significant in resistance it would be a novel function for the receptor. Considering the chemokine and chemokine receptors specifically, both uninfected M1 and M\(^{EP}\) macrophages highly upregulated CXCL11 compared to uninfected M2 macrophages; this chemokine binds the CXCR3 receptor, which is known to be an important component in the host defense against *Salmonella*\(^{325,326}\).

IL-10 inhibits defenses against *Salmonella*\(^{327}\); however, its upregulation in M1 macrophages is not surprising as IL-10 is induced by TNF\(\alpha\) in a negative feedback loop to limit inflammation\(^{121}\). In addition, IL-10 protects macrophages from TNF-induced apoptosis during *Salmonella* infection, so its primed expression in M1 macrophages might have some beneficial effect\(^{328}\). IL-15 has been implicated in *Salmonella* killing via activation of natural killer cells\(^{36}\). While natural killer cell activation would not influence the resistance of isolated MDM *in vitro* to *Salmonella* as observed in this study, primed activation of the IL-15 pathway may contribute to additional *in vivo* defenses against *Salmonella* resulting from M1 macrophage activation.
In addition, three pathways connected to metabolism demonstrated primed activation, namely “Tryptophan catabolism” (including genes such as IDO1), “Nicotinate metabolism” (including genes such as CD38) and “Nicotinamide salvaging” (including genes such as NAMPT). These pathways are linked as they all represent routes for production of NAD⁺, which has a variety of effects on immunity. NAD⁺ is necessary for cell survival and for bacterial killing through autophagy, and high levels of extracellular NAD⁺ mobilize monocytes and neutrophils through the activity of CD38. NAD⁺ is typically depleted from macrophages during infection as part of the process of repairing free radical damage resulting from phagocytosis, while increased NAD⁺ synthesis during infection may be protective. Further, reducing NAD⁺ levels via CD38 activation interferes with Salmonella’s ability to invade macrophages. This could represent a feedback mechanism to limit pathogen uptake in a macrophage that has exhausted its ability to complete autophagy and mitigate oxidative damage. These findings suggest that primed activation of tryptophan catabolism, nicotinate metabolism, and nicotinate salvaging in M1 and MEp macrophages would prepare them for a stronger oxidative response to Salmonella infection, increasing killing capacity.

Additional functions of NAD⁺ include promoting IL-10 production in macrophages, and nicotinate and nicotinamide metabolism can also have anti-inflammatory effect. This could represent an inflammation-limiting negative feedback mechanism similar to the induction of IL-10 by TNFα. Finally, NAD⁺ is important in bacterial function, and Salmonella produces NAD⁺ in part by importing nicotinamide riboside, which it produces from the nicotinate salvaging intermediate nicotinamide mononucleotide. Thus, bacterial access to NAD⁺ could be affected by changes in levels of nicotinamide mononucleotide resulting from regulation of the salvage pathway in macrophages. Notably, however, reducing NAD⁺ levels does not interfere with Salmonella replication in macrophages.

Since Salmonella can also produce NAD⁺ from tryptophan, the bacteria’s access to NAD⁺ presumably depends not just on the nicotinamide salvaging pathway but also tryptophan catabolism. Upregulation of key tryptophan catabolic enzyme IDO is associated with IFNγ stimulation, though IDO1 expression in M1 macrophages does not further increase response to Salmonella infection. This is consistent with what was observed in this study. Inhibiting IDO1 reduces Salmonella killing, presumably through one or both of two mechanisms: nutrient restriction, and immunomodulatory properties of Tryptophan catabolites. Intracellular
*Salmonella* relies on the host cell for access to Tryptophan among other nutrients, and Tryptophan catabolism would reduce the nutrient available to the bacteria\(^{338,339}\). Immunomodulatory tryptophan catabolites kynurenine and quinolate\(^{330}\) are increased in patients with typhoid fever; no significant decrease is observed in serum tryptophan\(^{337}\), but this may not reflect concentrations within host macrophages. The primed activation of the tryptophan catabolism pathway in uninfected M1 macrophages that was observed in this study supports the proposition\(^{339}\) that M1 macrophages are metabolically non-permissive for *Salmonella* replication; i.e. that they are inhospitable to the pathogen even before infection occurs.

Uninfected M\(^{EP}\) macrophages strongly upregulated these primed activation pathways compared to M2 macrophages (Figure 4.6b), and showed a moderate number of upregulated genes in some of these pathways when compared to M1 macrophages. Infected M\(^{EP}\) macrophages still upregulated several of these pathways compared to infected M2 macrophages, including interferon, tryptophan, and nicotinate pathways. However, the only primed activation pathway that infected M\(^{EP}\) macrophages upregulated when compared to infected M1 macrophages, was the “chemokine receptors bind chemokines” pathway.

Protein:protein interaction networks are a useful tool for providing visual structure to a collection of differentially expressed genes, based on the known interactions between the protein products of these genes\(^{290,291}\). Proteins are represented as nodes, while interactions between the proteins are represented as “edges” connecting two nodes. Central or “hub” nodes can be identified by measures such as degree: the number of connections the node has with other nodes, or in other words the number of interactions the protein has with other proteins in the network. Minimum protein:protein interaction networks include as nodes both differentially expressed genes of interest, as well as some genes that are not themselves differentially expressed but whose protein products directly interact with the products of two differentially expressed genes, creating a first-order interaction link between the differentially expressed genes.

Minimum networks (Figure 4.7) were created from the list of genes that were differentially expressed in M1 vs. M2 macrophages, and were part of the primed activation pathways indicated in Figure 4.6. Among the genes from primed activation pathways that were upregulated in uninfected M1 compared to M2 macrophages (Figure 4.7a), STAT1 was particularly prominent with the highest hub degree of 63. STAT2 and JAK2 were also represented with degrees of 25 and 23, respectively. When comparing infected M1 and M2 macrophages (Figure 4.7b), STAT1 still
had the highest hub degree of 38, but STAT2 and JAK2 were no longer differentially expressed.

Figure 4.7: Protein-protein interaction networks of differentially expressed genes from primed activation pathways, comparing M1 and M2 macrophages. Gene expression data were
rendered as a minimum protein-protein interaction network using NetworkAnalyst and visualized using Cytoscape. Genes upregulated in M1 macrophages are shown as red nodes; those with higher activation in M2 macrophages are shown as green nodes with the known interactions between the gene products shown by lines (termed “edges”) and gene products that interconnect differentially expressed nodes but which are not themselves differentially expressed shown as grey nodes. The size of the nodes refers to their hub degree (how well interconnected they are to other nodes in the network). Networks include genes differentially expressed when comparing [a] uninfected M1 and M2, and [b] infected M1 and M2 macrophages.

The primed activation pattern can be visualized using a heatmap of the regularized log2 (rlog) expression counts (Figure 4.8). The log transformation allowed changes in expression to be visualized along a similar dynamic range while regularization adjusted for sequencing depth, and shrinking the difference in expression for low-count genes towards zero such that low-count genes did not exhibit deceptively high fold changes, as described by Love et al in their paper on DESeq2. The shown primed activation genes had their lowest expression in uninfected M2 macrophages, with uninfected M1 and MÉP showing higher expression of these genes. For some genes such as CXCL11 and IDO1, I observed an additional upregulation in infected M1 macrophages, but for most genes the differences between infected M1 and M2 macrophages were modest. Infected MÉP macrophages clustered with uninfected MÉP macrophages; thus infection resulted in no change in expression of these primed activation genes. Because of the centrality of JAK2, STAT1, and STAT2 in the protein-protein interaction network, JAK-STAT signaling genes were further investigated.

![Figure 4.8: Heatmap of selected primed activation genes. Colour indicates the difference in regularized log (rlog) gene expression from the mean. Rlog expression represents the log2 of counts, adjusted by sequencing depth for each sample, with the values for genes with low counts adjusted towards zero. X-axis labels: “Un” is uninfected, “Inf” is infected, and “ETP” is MÉP.](image-url)
4.2.4.1 Primed activation of genes involved in JAK-STAT signaling

JAK-STAT signaling pathways are the main mechanism through which cytokine signaling is translated into immune responses\textsuperscript{121,341}, including during macrophage activation\textsuperscript{196}. The pathway is implicated in the progression of \textit{Salmonella} infection in chickens in that suppression of JAK-STAT signaling mRNA leads to \textit{Salmonella} susceptibility\textsuperscript{342}, while \textit{Salmonella enterica} serovar \textit{enteritidis} inhibits JAK-STAT signaling in chickens to subvert host defenses\textsuperscript{25}. Notably, the IFN\textgamma-induced expression of Guanylate Binding Proteins, which rupture the \textit{Salmonella}-containing vacuole, is dependent on JAK/STAT signalling\textsuperscript{11,233}. Three JAK-STAT genes were observed to be hubs in the network of primed activation pathway genes (Figure 4.7a).

Since the JAK-STAT signaling pathway was not included in the Reactome database that was used for pathway analysis, another method was employed to further investigate this signaling pathway. A set of JAK-STAT pathway genes was obtained from KEGG\textsuperscript{283} (see Appendix D, Table A.1). Roast gene set analysis identified in the JAK-STAT pathway genes for all comparisons a mixed differential gene expression, namely a statistically significant change in gene expression, without taking into account whether that change represents up or downregulation (Table 4.3). This suggested extensive modulation of this pathway during polarization and infection.

Directional enrichment, namely consistent upregulation or downregulation in gene expression, was less clear (Table 4.3). A general upregulation occurred in JAK-STAT signaling genes in uninfected M\textsuperscript{EP} macrophages when compared to M2 macrophages. A similar trend for M1 macrophages was not statistically significant (p=0.061). However, uninfected M1 macrophages did show greater upregulation (31 genes) in this gene set when compared to M2 macrophages than did infected M1 macrophages (16 genes), with similar numbers of downregulated genes (13 and 14 respectively). Only M2 macrophages showed a statistically significant overall upregulation of these pathways in response to infection. This is consistent with the primed activation pattern.

Interestingly, while infected M\textsuperscript{EP} macrophages, when compared to M1 macrophages, showed upregulation in only one of the four primed activation pathways that were upregulated in uninfected M\textsuperscript{EP} vs. uninfected M1 macrophages (Figure 4.6b), infected M\textsuperscript{EP} macrophages showed statistically significant upregulation in the JAK-STAT gene set compared to both M1 and M2 macrophages (Table 4.3).
Table 4.3: Roast gene set enrichment test for set of 155 genes in the KEGG\textsuperscript{283} JAK-STAT pathway. Columns are as follows. “MDM Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. “% Up-Regulated” and “% Down-Regulated”: percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. “Direction”: whether the gene set was found to be overall up-or down-regulated. “p-value” was calculated by Roast taking into account both percentage of up- and down-regulated genes and magnitude of change. Statistically significant (p<0.05) comparisons are indicated in bold.

<table>
<thead>
<tr>
<th>MDM Types Compared</th>
<th>% Up-regulated</th>
<th>% Down-regulated</th>
<th>Direction</th>
<th>p-value (direction)</th>
<th>p-value (mixed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 vs. M2 uninfected</td>
<td>31</td>
<td>13</td>
<td>Up</td>
<td>0.061</td>
<td>0.002</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M1 uninfected</td>
<td>23</td>
<td>9</td>
<td>Up</td>
<td>0.084</td>
<td>0.014</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M2 uninfected</td>
<td>44</td>
<td>6</td>
<td>Up</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M1 vs. M2 infected</td>
<td>16</td>
<td>14</td>
<td>Down</td>
<td>0.878</td>
<td>0.03</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M1 infected</td>
<td>42</td>
<td>13</td>
<td>Up</td>
<td>0.010</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M2 infected</td>
<td>39</td>
<td>17</td>
<td>Up</td>
<td>0.029</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>33</td>
<td>45</td>
<td>Up</td>
<td>0.996</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M\textsuperscript{EP} infected vs. uninfected</td>
<td>28</td>
<td>25</td>
<td>Up</td>
<td>0.230</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Infected/uninfected M1 vs. M2</td>
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<td>21</td>
<td>Down</td>
<td>0.113</td>
<td>0.037</td>
</tr>
<tr>
<td>Infected/uninfected M\textsuperscript{EP} vs. M1</td>
<td>22</td>
<td>17</td>
<td>Up</td>
<td>0.417</td>
<td>0.016</td>
</tr>
<tr>
<td>Infected/uninfected M\textsuperscript{EP} vs. M2</td>
<td>14</td>
<td>22</td>
<td>Down</td>
<td>0.418</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Although upregulation of the signature in uninfected M1 macrophages was not quite statistically significant, it was very close (p=0.061). This was likely because uninfected M1 macrophages expressed high levels of several genes involved in the JAK-STAT signaling process, as previously noted (Figure 4.7a). Expression of JAK-STAT genes of interest is shown in Figure 4.9. For many of these genes, higher expression was observed in infected cells of all polarization types, indicating upregulation of the genes in response to infection (Figure 4.9a). For a subset, expression was increased in uninfected M1 cells, with little increase between uninfected and infected M1 cells (Figure 4.9a). The activation of these JAK-STAT genes in uninfected M1 macrophages was particularly apparent when viewing expression in uninfected macrophages (Figure 4.9b) separately from infected macrophages (Figure 4.9c), allowing us to focus on differences resulting from polarization. With this view, a large cluster of primed JAK-STAT genes could be observed that were more expressed in uninfected M1 macrophages, and in some cases M\textsuperscript{EP} macrophages (Figure 4.9b), than in uninfected M2 macrophages. For the most part, the differences in expression resulting from polarization were less distinct in infected cells (Figure 4.9c).
Figure 4.9: Heatmaps of selected JAK-STAT genes differentially expressed in polarized macrophages. The colour indicates the difference in regularized log (rlog) gene expression from the mean. Rlog expression represents the log₂ of counts, adjusted according to sequencing depth for each sample and with the values for genes with low counts adjusted towards zero. For X-axis labels, “Un” indicates uninfected, “Inf: indicates infected, and “ETP” indicates MEP. [a] Both infected and uninfected macrophages are shown on the same scale. [b] Only uninfected macrophages are shown. [c] Only infected macrophages are shown.
Uninfected M1 macrophages upregulated JAK2, STAT1, and SOCS1, as well as the anti-apoptotic effector PIM-1, when compared to both M2 and M\(_{\text{EP}}\) macrophages; compared to M2 macrophages they additionally upregulated STAT2, IL-15, and cytokine receptors IL-15RA, CSF2RB, IL-10RA (Figure 4.9b). These specific JAK-STAT genes are of mechanistic significance with their roles in signaling depicted in Figure 4.10.

**Figure 4.10: Diagram of JAK-STAT signaling pathway showing genes differentially expressed as a result of polarization.** Genes upregulated in M1 macrophages are indicated in red. Abbreviations: EPO – Erythropoietin, TPO – Thrombopoietin, GH – growth hormone. STAT indicates STATs other than STAT1 and STAT2. J/T* indicates some combination of JAKs (other than JAK2) and Tyk2. References\(^{283,343-347}\).

JAK2, STAT1, and STAT2 are known to be associated with M1 polarization. IFN\(\gamma\) signals through JAK1 and JAK2\(^{343,348}\). Persistent *Salmonella* infection is associated with dephosphorylation/deactivation of JAK2\(^{25}\). JAK2 is also involved in IL-12 signaling\(^{349}\) and IL-12 is known to be involved in *Salmonella* resistance\(^{39}\), while its deficiency results in susceptibility to salmonellosis\(^2\). Furthermore, STAT1 deficient mice are defective in immunity to intracellular bacteria\(^{350}\) and STAT1 deficiency or mutation in humans is associated with susceptibility to *Salmonella* infection\(^{351,352}\). STAT2 is known to be expressed in response to *Salmonella* infection\(^3\). Interestingly, STAT2-dependent inflammation in the gut produces a competitive advantage for *Salmonella* over the gut microbiota, leading to *Salmonella* growth\(^{353}\). This would not affect resistance of macrophages to *Salmonella in vitro*, but may suggest a possible complication of inflammatory macrophage activation *in vivo*. SOCS1 is a feedback inhibitor of STAT1\(^{173}\), preventing excessive inflammation and septic shock\(^{354,355}\). As previously noted, both IL10RA and
IL15RA are parts of pathways previously observed to exhibit primed activation.

Infected M1 still expressed higher levels of STAT1 than infected M2 macrophages, and more JAK2 than M\textsuperscript{EP} macrophages (Figure 4.9c), but otherwise their differentially expressed JAK-STAT pathway genes largely represented effectors such as IFNA8, IFNW1, IL-12A, and IL-6, which are also regulated by many other pathways. Type 1 interferons such as IFN\textalpha and IFN\textomega are involved in defenses against intracellular pathogens\textsuperscript{356}. As noted, IL-12 is important in defense against \textit{Salmonella}\textsuperscript{2,39} and it is known to activate natural killer cells and a Th1 response\textsuperscript{30}. IL-6 has a generally pro-inflammatory character, but is not currently known to have a specific role in \textit{Salmonella} infection\textsuperscript{327}.

All three cell types upregulated SOCS2 and SOCS3 in response to infection (Figure 4.9a), although M\textsuperscript{EP} macrophages did so to a lesser extent. This was consistent with the finding that \textit{Salmonella} SPI-2 upregulates SOCS3 via ERK1/2, inhibiting STAT1 and STAT3 and suppressing host defenses\textsuperscript{24}. Interestingly, IL-22 was upregulated in all three cell types in response to infection, but its receptor IL22RA\textsuperscript{2} was only upregulated in M2 macrophages, both uninfected and infected, when compared to the other cell types. IL-22 is involved in epithelial homeostasis in the gut\textsuperscript{357}. The upregulation of its receptor in M2 macrophages, observed here, might reflect the role of M2-like macrophages in homeostatic functions.

\textbf{4.2.5 Interleukin and Inflammasome pathways did not show primed activation, but central inflammasome genes were enriched in infected M1 compared to infected M2 macrophages}

Given their central role in antimicrobial immunity it was notable that Interleukin and Inflammasome pathways showed a pattern that did not reflect primed activation (Figure 4.11). Interleukins are involved in regulating inflammation and the immune response (see Table 1.2). In particular, IL-4, IL-13, and IL-10 promote M2 macrophage polarization\textsuperscript{139}, IL-10 protects macrophages from apoptosis during \textit{Salmonella} infection\textsuperscript{328}, and IL-12, IL-15, and IL-23 are known to be specifically important in defense against \textit{Salmonella}\textsuperscript{36,39}. Inflammasomes are multi-protein complexes that trigger inflammatory cell death (pyroptosis) and the processing and release of IL-1\textbeta and IL-18\textsuperscript{55–57}, activating immune responses\textsuperscript{55} and exposing \textit{Salmonella} to killing by neutrophils\textsuperscript{68}. This process is believed to be important in detecting and defending against intracellular \textit{Salmonella} infection, since mice lacking the pyroptotic caspase-1, IL-1\textbeta, or IL-18 are susceptible to \textit{Salmonella}\textsuperscript{70}. 
Figure 4.11: Differential enrichment in signaling in interleukin and inflammasome pathways. The colour of the dots indicate the percentage of the differentially expressed genes found in the pathway which were upregulated: pathways that were largely upregulated are red, while pathways that were largely downregulated are green. The size of the dots indicates the significance level of the pathway enrichment, \(-\log_{10}(\text{adjusted } p\text{-value})\), such that larger dots have smaller \(p\)-values. Abbreviations: “un” means “uninfected”, “inf” means “infected”, and “inter” or “Interaction” refers to the relative polarization effect (the difference between the response to infection in different polarization types). “\(p\text{-adj}\)” refers to the \(p\)-value adjusted for multiple testing. Only pathways for which the adjusted \(p\)-value was \(\leq 0.05\) are shown.

Aside from the IL-10 and IL-15 pathways, which were discussed in section 4.2.4, interleukin pathways were not differently regulated between polarization types in uninfected cells. All three polarization types showed statistically significant upregulation in interleukin pathways when comparing uninfected and infected cells. Furthermore, based on the relative polarization effect, IL-1 signaling and IL-1 processing showed statistically significantly greater upregulation in M1 than in M2 macrophages in response to infection, and there was greater upregulation in IL-1 processing in M1 macrophages than in M\(^E\) macrophages (graphically represented as a downregulation in M\(^E\) macrophages).

M\(^E\) macrophages showed little enrichment in interleukin and inflammasome pathways relative to other macrophage types. Both uninfected and infected M\(^E\) macrophages showed only downregulation in these pathways compared to M1 macrophages. Compared to uninfected M2 macrophages, uninfected M\(^E\) macrophages showed modest upregulation of IL-10, IL-15, and IL-17 signaling and the NLRP3 inflammasome pathways. In response to infection, M\(^E\) macrophages upregulated five pathways including IL-10 signaling and IL-4 and IL-13 signaling, but overall
upregulated fewer pathways than did M1 or M2 macrophages (10 and 11 pathways respectively).

Because inflammasomes are believed to be important in *Salmonella* infection\(^{68,70}\), the genes involved in this process were examined more closely. The number of genes differentially expressed in M1 macrophages was modest: there were 9 inflammasome genes that were upregulated in uninfected M1 macrophages compared to uninfected M2 macrophages, and 10 upregulated in infected M1 macrophages. However, these genes, particularly those that were M1-associated for infected cells, included key genes at multiple stages of the inflammasome pathway (Figure 4.12).

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**Figure 4.12: Diagram of the inflammasome pathway showing genes differentially expressed as a result of polarization and infection.** Genes upregulated in infected M1 macrophages compared to infected M2 macrophages are shown in red. Genes downregulated in infected M1 macrophages are in green. Genes similarly regulated in MEP compared to M2 macrophages are indicated with (*). Genes downregulated in MEP compared to M1 macrophages are indicated with (-). The NLRP3 inflammasome can be activated through a variety of cell stress signals including reactive oxygen species production, and K+ efflux\(^{61}\), which involves Pannexin-1 (PANX1) and
Purinergic Receptor P2X7 activation. Formation of the NLRP3 inflammasome requires NEK7 as well as ASC/PYCARD and caspase-1 (CASP1), and is facilitated by Pellino E3 Ubiquitin Protein Ligase Family Member 2 (PELI2). NLRC4, AIM2, Pyrin/MEFV, and NLRP1 can also form inflammasomes with caspase-1 and ASC; the NLRC4 inflammasome also includes NAIP receptors while the NLRP1 inflammasome includes caspase-5 (CASP5). On being activated by the inflammasome, caspase-1 cleaves IL1β and IL18 as well as Gasdermin D (GSDMD). IL-18, IL-1α, and IL-1β are secreted through mechanisms that include, but are not limited to, pores formed by the Gasdermin D N-terminus. TPL2 (MAP3K8) increases caspase-1 activation through a mechanism that appears to be initiated by NOD2 and may require ERK; differing findings regarding ERK dependence were reported in studies using different PAMPs. Caspase-8 (CASP8) can promote activation of caspase-1 through the NLRP3 inflammasome, or directly cleave pro-IL1β. Syk is mostly associated with inflammasome activation in response to fungal pathogens, which involves formation of a scaffold with CARD9, BLC10, and MALT1 and activation of caspase-8. Syk may also be involved in inflammasome activation in response to other pathogens as it responds to ligands such as ATP, but the involvement of the CARD9-BCL10-MALT1 scaffold has not been established in this interaction, and CARD9 overall inhibits the NLRP3 inflammasome in the context of Salmonella infection. BCL-2 and BCL-XL bind and inhibit NLRP1, and IL-18 binding protein (IL18BP) binds and inhibits IL-18.

Inflammasome initiating receptor AIM2, as well as caspase-5 and pyroptosis mediator Gasdermin-D, were upregulated in both infected and uninfected M1 macrophages. Infected M1 macrophages additionally upregulated genes encoding the initiating receptor NLRP3, facilitator PELI2, central pyroptosis coordinator CASP1 (caspase-1), and effector cytokines IL-1A and IL18, which are released by pyroptotic cells. As noted, caspase-1, IL-1β, and IL-18 are specifically linked to Salmonella resistance. Also upregulated was MAP3K8 (TPL2), which is required for IL-1β secretion in response to various PRR agonists as well as Salmonella. In addition, CARD9, which inhibits the NLRP3 inflammasome in Salmonella infection, was downregulated in infected M1 macrophages. Genes upregulated in uninfected M1 macrophages but not infected M1 macrophages included receptors Pyrin and NOD2, and caspase-8, but also included the negative regulator IL18BP and K+ efflux genes PANX1 and P2RX7, which are not involved in inflammasome activation in response to Salmonella infection. The inflammasome genes that were differently regulated in M1 macrophages in response to infection based on relative polarization effect were IL-1α and IL-1β (more expressed in M1 than M2 macrophages) and PANX1 and P2RX7 (less expressed in M1 vs. M2 macrophages in response to infection). This was consistent with the observation that PANX1 and P2RX7 were only upregulated in uninfected M1 macrophages, while IL-1α was only upregulated in infected M1 macrophages. IL-1 β was also modestly but not significantly upregulated in M1 macrophages (FC 1.68, p= 0.18).
While uninfected M<sup>EP</sup> macrophages overall upregulated inflammasome genes, infected M<sup>EP</sup> macrophages mostly downregulated inflammasome genes when compared to M1 macrophages, and only upregulated 6 genes compared to infected M2 macrophages, with 4 genes downregulated (Table 4.4).

**Table 4.4: Numbers of differentially expressed inflammasome and IL-1 genes in M<sup>EP</sup> macrophages.** The number includes genes considered significantly differentially expressed (p<0.05, fold change>2).

<table>
<thead>
<tr>
<th></th>
<th>Uninfected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M&lt;sup&gt;EP&lt;/sup&gt; vs M1</td>
<td>M&lt;sup&gt;EP&lt;/sup&gt; vs M2</td>
</tr>
<tr>
<td>Upregulated</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Downregulated</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

Compared to M2 macrophages, infected M<sup>EP</sup> macrophages had higher expression of the inflammasome receptor NLRP3, and the effectors IL1A and IL18, which would contribute to inflammasome activation and *Salmonella* resistance (Figure 4.12). However, M<sup>EP</sup> macrophages also downregulated IL-1 receptor 1 (IL1R1) and IL-1 receptor accessory protein (IL1RAP) which would reduce their responsiveness to any IL-1 released by pyroptotic neighbors and thus limit the effectiveness of inflammasome activation. Based on the relative polarization effect, M<sup>EP</sup> macrophages showed a greater expression of NLRP3 in response to infection than M2 macrophages, but a lower expression of inflammasome pathway genes AIM2, PANX1, and P2RX7. Genes downregulated in infected M<sup>EP</sup> macrophages compared to M1 macrophages included the inflammasome receptor AIM2, complex component ASC, and CASP5 (caspase-5).

A heatmap of regularized log<sub>2</sub> expression counts for differentially expressed inflammasome genes (Figure 4.13) showed the overall observed trend of expression for uninfected and infected M1, M2, and M<sup>EP</sup> macrophages. Low expression was observed in uninfected M2 macrophages, with moderate expression of many genes on uninfected M1 and infected M2 macrophages. Infected M1 macrophages showed moderate to high expression in these genes, likely contributing to resistance. Both uninfected and infected M<sup>EP</sup> macrophages expressed most inflammasome genes moderately (that is, similarly to uninfected M1 or infected M2 macrophages) aside from a few exceptions such as IL1A and NLRP3. This low number of upregulated inflammasome genes in M<sup>EP</sup> macrophages that are infected with *Salmonella* likely results from the tolerant response to *Salmonella* infection, and may be partly responsible for a loss in *Salmonella* resistance after 4 hours of infection.
**Figure 4.13: Heatmap of inflammasome genes.** Genes shown are those that were differentially expressed between at least two types of differently polarized macrophages (M1, M2, or M\textsuperscript{EP}). The colour indicates the difference in regularized log (rlog) gene expression from the mean. Rlog expression represents the log\(_2\) of counts, adjusted by sequencing depth for each sample and with the values for genes with low counts adjusted towards zero. X-axis labels: “Un” is uninfected, “Inf” is infected, and “ETP” is M\textsuperscript{EP}.

### 4.2.6 Polarization affects expression of genes associated with *Salmonella* infection

*Salmonella* manipulates a variety of genes to suppress host defense or promote SCV formation; this manipulation is important to *Salmonella*’s intracellular survival\(^{369}\). A set of 34 such *Salmonella* target genes was obtained by augmenting a gene list provided by BioCarta\(^{282}\) with additional findings from literature (see Appendix D, Table A.1). Using Roast, this gene set was found to be downregulated in M1, M2, and M\textsuperscript{EP} macrophages in response to infection (Table 4.5), with the greatest downregulation occurring in M1 macrophages (77\% of genes in this set downregulated, p-value <0.001) and the least downregulation in M\textsuperscript{EP} macrophages (38\% downregulated, p-value 0.017). The most downregulated genes in M1 macrophages in response to infection were VPS18 (FC -3.68), PLEKHM1 (FC -2.53), and EEA1 (FC -2.28). VPS18 (a core
HOPS subunit)\textsuperscript{379} and PLEKHM1 both are targeted by \textit{Salmonella} effector SifA in order to recruit microtubules\textsuperscript{11}. EEA1 is an early endosome marker; the retention of EEA1 on the SCV prevents its fusion with the lysosome, thus preventing bacterial killing\textsuperscript{11}. Of these genes, VPS18 was also downregulated in response to infection in M2 macrophages, but to a lesser extent (FC -2.41 rather than -3.68).

Table 4.5: \textbf{Roast gene set enrichment test for set of 31 \textit{Salmonella} targets and SCV-implicated genes}. Columns are as follows. “MDM Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. “% Up-Regulated” and “% Down-Regulated”: percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. “Direction”: whether the gene set was found to be overall up- or down-regulated. “p-value” was calculated by Roast taking into account both percentage of up- and down-regulated genes and magnitude of change. Statistically significant (p<0.05) comparisons are indicated in bold.

<table>
<thead>
<tr>
<th>MDM Types Compared</th>
<th>% Up-regulated</th>
<th>% Down-regulated</th>
<th>Direction</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 vs. M2 uninfected</td>
<td>27</td>
<td>4</td>
<td>Up</td>
<td>0.403</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M1 uninfected</td>
<td>12</td>
<td>23</td>
<td>Down</td>
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<tr>
<td>M\textsuperscript{EP} vs. M2 uninfected</td>
<td>23</td>
<td>27</td>
<td>Down</td>
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<tr>
<td>M1 vs. M2 infected</td>
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<td>31</td>
<td>Down</td>
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<tr>
<td>M\textsuperscript{EP} vs. M1 infected</td>
<td>38</td>
<td>12</td>
<td>Up</td>
<td>0.068</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M2 infected</td>
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<td>35</td>
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<td>0.955</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
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<td>77</td>
<td>Down</td>
<td>&lt;0.001</td>
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<tr>
<td>M2 infected vs. uninfected</td>
<td>4</td>
<td>46</td>
<td>Down</td>
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<tr>
<td>M\textsuperscript{EP} infected vs. uninfected</td>
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<td>38</td>
<td>Down</td>
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<tr>
<td>Infected/uninfected M1 vs. M2</td>
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<td>27</td>
<td>Down</td>
<td>0.058</td>
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<tr>
<td>\textbf{Infected/uninfected M\textsuperscript{EP} vs. M1}</td>
<td>\textbf{31}</td>
<td>\textbf{0}</td>
<td>Up</td>
<td>\textbf{0.036}</td>
</tr>
<tr>
<td>Infected/uninfected M\textsuperscript{EP} vs. M2</td>
<td>12</td>
<td>4</td>
<td>Up</td>
<td>0.743</td>
</tr>
</tbody>
</table>

An upregulation of these genes in M\textsuperscript{EP} compared to M1 macrophages was found when considering the relative polarization effect, indicating that M\textsuperscript{EP} showed higher expression in these \textit{Salmonella} effectors in response to infection than did M1 macrophages. The largest difference was in VPS18 (FC 2.68; padj = 0.0057); this was consistent with the strong downregulation of this gene in M1 macrophages in response to infection.

A modest number of effectors were downregulated when comparing infected M1 and M2 macrophages, both in the simple comparison and the relative polarization effect comparing infection response, but this was not deemed statistically significant by Roast (p=0.058), and despite the identification of this set as a whole as downregulated using the Roast method, none of the genes from this set were individually identified as significantly differentially expressed by the
DESeq2 method. This likely reflects downregulation in these genes that was slightly beneath DESeq2’s cutoff for significance either in terms of magnitude of fold change or adjusted p-value.

The identified Salmonella effector genes related to several pathways (Figure 4.14). TP53 participates in a number of pathways as a part of the stress response, and is activated by Salmonella in epithelial cells\(^{371}\); it is believed that cell cycle arrest caused by TP53 is beneficial to Salmonella\(^{372,373}\). Of the TP53 pathways, “TP53 regulates transcription of cell cycle genes” was upregulated in response to infection in M1 cells, but not other macrophage types. While this is an exception to the general downregulation of Salmonella effectors in M1 macrophages, it is consistent with the observation that IFNα/β induce transcription of TP53 as part of antiviral defenses\(^{374}\). Pathways involving Rab\(^{375}\) deal with the regulation of trafficking, and along with Rho GTPases\(^{376}\) are among those stimulated by Salmonella in the formation of the Salmonella-containing vesicle, which represents the unique intracellular niche for Salmonella growth. Several Rab and Rho pathways were downregulated in M1 macrophages, and to a lesser extent M2 macrophages, in response to infection. The genes in these pathways included TBC1D2, which interacts with the known Salmonella effector Rab7\(^{377}\). This gene was highly downregulated in M1 macrophages in response to infection (FC -16.1) but less so in M2 macrophages (FC -7.89).

Figure 4.14: Enrichment in pathways related specifically to Salmonella infection. The colour of the dots indicate the percentage of the differentially expressed genes found in the pathway which were upregulated: pathways that were largely upregulated are red, while pathways that were largely downregulated are green. The size of the dots indicates the significance level of the pathway enrichment, -log\(_{10}\)(adjusted p-value), such that larger dots have smaller p-values. Abbreviations: “un” for “uninfected”, “inf” for “infected”; “p-adj” refers to the p-value adjusted for multiple testing. Only pathways for which the adjusted p-value was ≤0.05 are shown.
Enrichment was also investigated for a set of 183 genes for which a loss of function results in *Salmonella* resistance\(^{100}\) (Table 4.6). These genes thus appear to result in *Salmonella* susceptibility. The susceptibility genes were not statistically significantly differently regulated in any uninfected macrophage type, and the set was significantly downregulated in all macrophage types in response to infection. This downregulation was largest in M1 macrophages, with 68\% of these genes downregulated in infected M1 macrophages compared to uninfected M1 macrophages. In addition, M1 macrophages downregulate these genes in comparison to infected M2 and M\(^{EP}\) macrophages, with 33\% downregulated compared to M2 macrophages and 51\% downregulated compared to M\(^{EP}\) macrophages (expressed in the table as 51\% upregulated in M\(^{EP}\) macrophages). The relative polarization effect also indicates that the expression of these susceptibility genes in response to infection is lower (further suppressed) in M1 compared to M\(^{EP}\) macrophages.

**Table 4.6: Roast gene set enrichment test for 183 genes for which loss of function results in *Salmonella* resistance\(^{100}\).** Columns are as follows. “MDM Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. “\% Up-Regulated” and “\% Down-Regulated”: percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. “Direction”: whether the gene set was found to be overall up-or down-regulated. “p-value” calculated by Roast taking into account both percentage of up- and down-regulated genes and magnitude of change. Statistically significant (p<0.05) comparisons are indicated in bold.

<table>
<thead>
<tr>
<th>MDM Types Compared</th>
<th>M1 vs. M2 uninfected</th>
<th>M(^{EP}) vs. M1 uninfected</th>
<th>M(^{EP}) vs. M2 uninfected</th>
<th>M1 vs. M2 infected</th>
<th>M(^{EP}) vs. M1 infected</th>
<th>M(^{EP}) vs. M2 infected</th>
<th>M1 infected vs. uninfected</th>
<th>M2 infected vs. uninfected</th>
<th>M(^{EP}) infected vs. uninfected</th>
<th>Infected/uninfected M(^{EP}) vs. M1</th>
<th>Infected/uninfected M(^{EP}) vs. M2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Up-regulated (%)</td>
<td>% Down-regulated (%)</td>
<td>Direction</td>
<td>p-value</td>
<td></td>
<td></td>
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<tr>
<td>M1 vs. M2 uninfected</td>
<td>17</td>
<td>16</td>
<td>Up</td>
<td>0.878</td>
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<td></td>
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<tr>
<td>M(^{EP}) vs. M1 uninfected</td>
<td>31</td>
<td>15</td>
<td>Up</td>
<td>0.311</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>M(^{EP}) vs. M2 uninfected</td>
<td>30</td>
<td>19</td>
<td>Up</td>
<td>0.267</td>
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<td>M1 vs. M2 infected</td>
<td>5.8</td>
<td>33</td>
<td>Down</td>
<td>0.036</td>
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<td>M(^{EP}) vs. M1 infected</td>
<td>51</td>
<td>9.1</td>
<td>Up</td>
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<tr>
<td>M(^{EP}) vs. M2 infected</td>
<td>41</td>
<td>19</td>
<td>Up</td>
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<td>M1 infected vs. uninfected</td>
<td>8.3</td>
<td>68</td>
<td>Down</td>
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<td>M2 infected vs. uninfected</td>
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<td>M(^{EP}) infected vs. uninfected</td>
<td>6.7</td>
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<tr>
<td>Infected/uninfected M(^{EP}) vs. M1</td>
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<td>3.3</td>
<td>Up</td>
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<td>Infected/uninfected M(^{EP}) vs. M2</td>
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<td>Up</td>
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</tbody>
</table>

Genes that have been identified as protective against *Salmonella* infection (see Appendix D, Table A.1) were also investigated (Table 4.7). Uninfected and infected M1 and M\(^{EP}\) macrophages upregulated this gene set when compared to M2 macrophages. Interestingly, when
compared to M2 macrophages, M\textsuperscript{EP} macrophages downregulated a higher number of these resistance genes than did M1 macrophages (15% for uninfected and 23% for infected M\textsuperscript{EP} vs M2, compared with 8% for uninfected and 4% for infected M1 vs M2 macrophages). Infected M\textsuperscript{EP} macrophages also significantly downregulated genes in this set when compared to infected M1 macrophages. Uninfected M\textsuperscript{EP} macrophages did not show a statistically significant difference in expression when compared to uninfected M1 macrophages; since the percentage of genes downregulated in uninfected M\textsuperscript{EP} macrophages was similar to the percentage downregulated in infected M\textsuperscript{EP} macrophages; thus the difference in p-values calculated by Roast must result from a greater magnitude of difference in downregulated genes between M\textsuperscript{EP} and M1 macrophages when infected. Overall, the uninfected M\textsuperscript{EP} macrophages appeared to have M1-like expression of \textit{Salmonella} protective genes, while the infected M\textsuperscript{EP} macrophages had expression levels lying between those of M2 and M1 macrophages.

Table 4.7: Roast gene set enrichment test for set of 33 \textit{Salmonella} resistance genes. Columns are as follows. “MDM Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. “% Up-Regulated” and “% Down-Regulated”: percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. “Direction”: whether the gene set was found to be overall up- or down-regulated. “p-value” was calculated by Roast taking into account both percentage of up- and down-regulated genes and magnitude of change. Statistically significant (p<0.05) comparisons are indicated in bold.

<table>
<thead>
<tr>
<th>MDM Types Compared</th>
<th>% Up-regulated</th>
<th>% Down-regulated</th>
<th>Direction</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
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<td>M1 vs. M2 uninfected</td>
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<td>8</td>
<td>Up</td>
<td>&lt;0.001</td>
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<tr>
<td>M\textsuperscript{EP} vs. M1 uninfected</td>
<td>19</td>
<td>35</td>
<td>Down</td>
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<tr>
<td>M\textsuperscript{EP} vs. M2 uninfected</td>
<td>54</td>
<td>15</td>
<td>Up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M1 vs. M2 infected</td>
<td>58</td>
<td>4</td>
<td>Up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M1 infected</td>
<td>27</td>
<td>38</td>
<td>Down</td>
<td>0.034</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M2 infected</td>
<td>42</td>
<td>23</td>
<td>Up</td>
<td>0.020</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>23</td>
<td>23</td>
<td>Up</td>
<td>0.0501</td>
</tr>
<tr>
<td>M2 infected vs. uninfected</td>
<td>42</td>
<td>12</td>
<td>Up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M\textsuperscript{EP} infected vs. uninfected</td>
<td>19</td>
<td>4</td>
<td>Up</td>
<td>0.324</td>
</tr>
<tr>
<td>Infected/uninfected M1 vs. M2</td>
<td>8</td>
<td>27</td>
<td>Down</td>
<td>0.003</td>
</tr>
<tr>
<td>Infected/uninfected M\textsuperscript{EP} vs. M1</td>
<td>8</td>
<td>19</td>
<td>Down</td>
<td>0.493</td>
</tr>
<tr>
<td>Infected/uninfected M\textsuperscript{EP} vs. M2</td>
<td>4</td>
<td>31</td>
<td>Down</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Reminiscent of primed activation, the M2 macrophages showed a statistically significant upregulation in the \textit{Salmonella} resistance gene set in response to infection, while M1 and M\textsuperscript{EP} macrophages did not. This was also reflected in the relative polarization effect, showing lower
activation in M1 and M\textsuperscript{EP} macrophages in response to infection than in M2 macrophages. As with the other primed activation genes, this would indicate that improved \textit{Salmonella} resistance in M1 macrophages resulted largely from the mobilization of defensive pathways from M1 polarization alone, rather than from polarization producing a different transcriptional response to infection than in M2 macrophages. Meanwhile, the number of downregulated resistance genes in infected M\textsuperscript{EP} macrophages, when compared to M1 macrophages, likely contributed to the loss of \textit{Salmonella} resistance as infection progressed.

Expression of specific differentially expressed \textit{Salmonella} resistance genes was visualized in a heatmap (Figure 4.15). A dramatic increase was observed in the response to infection of genes encoding TNF\(\alpha\) and IFN\(\gamma\). Infected M1 macrophages expressed the highest levels of TNF\(\alpha\) and IFN\(\gamma\). TNF\(\alpha\) is a pro-inflammatory cytokine known to be protective in \textit{Salmonella} infection\textsuperscript{40}. As previously noted, IFN\(\gamma\) is known to be important in defense against intracellular pathogens including \textit{Salmonella}\textsuperscript{378,379} and also promotes M1 activation in what would represent a positive feedback loop\textsuperscript{139}. Infected M1 macrophages also expressed higher (though less dramatic) levels of TLR4, a PRR which detects LPS. Mice deficient in TLR4 are susceptible to \textit{Salmonella} infection\textsuperscript{380}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{heatmap.png}
\caption{Heatmap of expression of \textit{Salmonella} resistance genes. Colour indicates the difference in regularized log (rlog) gene expression from the mean. Rlog expression represents the log\(_2\) of counts, adjusted by sequencing depth for each sample, with values for genes with low counts adjusted towards zero. X-axis labels: “Un” is uninfected, “Inf: is infected, “ETP” is M\textsuperscript{EP}.}
\end{figure}
Salmonella resistance genes also include Guanylate-binding proteins (GBPs), which rupture the SCV\textsuperscript{11,233–236}, exposing *Salmonella* to defense mechanisms. It has been suggested, for example, that SCV rupture exposes *Salmonella* to NADPH oxidase genes, another class of *Salmonella* resistance genes which are involved in oxidative killing of phagocytosed bacteria\textsuperscript{6,28}, though as NADPH oxidase produces reactive oxygen species in intracellular organelles\textsuperscript{381} it is unclear how cytosolic Salmonella would be exposed to this killing mechanism. Infected M1 macrophages upregulated GBP1, 4, and 5, and CYBB, also known as NADPH oxidase 2 (Nox2), and Nox2 complex component NCF2, when compared to both infected M2 and M\textsuperscript{EP} macrophages. Compared to infected M2 macrophages, M1 macrophages also upregulated GBP2-3 and Nox2 complex component NCF4. Uninfected M1 macrophages showed an increase in GBP1-5 and 7 in comparison to M2 macrophages although of these only GBP5 was upregulated in M1 compared to M\textsuperscript{EP} macrophages. Uninfected M1 also showed a slight increase in CYBB/Nox2 expression compared to both M2 and M\textsuperscript{EP} macrophages, though there was only a 1.73-fold change for M\textsuperscript{EP} macrophages, the functional relevance of which is unknown. In response to infection, M1 macrophages, but not M2 or M\textsuperscript{EP} macrophages, upregulated TLR4 and MAP1LC3A (LC3), the latter of which is important in phagocytosis of *Salmonella* in macrophages\textsuperscript{382}, while M2 macrophages upregulated GBPs 2, 4, 5, and 7. Both M1 and M2 macrophages upregulated GBP1.

Both uninfected and infected M\textsuperscript{EP} macrophages expressed high levels of LC3, with FC ranging from 10.2 (when comparing infected M\textsuperscript{EP} and infected M1 macrophages) to 28.4 (comparing uninfected M\textsuperscript{EP} and M2 macrophages). Uninfected M\textsuperscript{EP} macrophages expressed high levels of GBPs and Nox2 complex component NCF1 (FC 7.36) compared to M2 macrophages, but downregulated NCF2 (FC -3.63). Uninfected M\textsuperscript{EP} macrophages also expressed high levels of the notable resistance gene SLC11A1 (NRAMP1) when compared to M2 macrophages (FC 3.38); this divalent-metal efflux pump is important in limiting metals within the macrophage, and its mutation results in *Salmonella* susceptibility in mice\textsuperscript{383}, although mutations in humans do not affect susceptibility to typhoid fever\textsuperscript{384}. Overall, while M\textsuperscript{EP} macrophages upregulated some genes involved in *Salmonella* resistance, they did not do so to the same extent as M1 macrophages.

### 4.2.7 Endotoxin primed cells showed signature genes, and up- and downregulated expression of specific pathways

The endotoxin tolerance signature was identified in our lab\textsuperscript{205} as a gene set that occurred in endotoxin tolerant macrophages (twice treated with LPS, for 24 and 4 hours), but not in
inflammatory (M1) macrophages (once treated with LPS, for 4 hours). It was found to be predictive of human sepsis and multi-organ failure at first clinical presentation in the emergency ward, in both a 500 patient retrospective analysis and a small (72 patient) prospective clinical study. Gene set testing showed that endotoxin primed M\textsuperscript{EP} macrophages upregulated genes from this endotoxin tolerance signature compared to M1 and M2 macrophages, both for infected and uninfected cells (Table 4.8). No other comparison showed an upregulation in these genes.

**Table 4.8: Roast gene set enrichment test for the 103-gene endotoxin tolerance signature.**

Columns are as follows. “MDM Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. “% Up-Regulated” and “% Down-Regulated”: percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. “Direction”: whether the gene set was found to be overall up-or down-regulated. “p-value” was calculated by Roast taking into account both percentage of up- and down-regulated genes and magnitude of change. Statistically significant (p<0.05) comparisons are indicated in bold.

<table>
<thead>
<tr>
<th>MDM Types Compared</th>
<th>% Up-regulated</th>
<th>% Down-regulated</th>
<th>Direction</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 vs. M2 uninfected</td>
<td>31</td>
<td>16</td>
<td>Up</td>
<td>0.158</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M1 uninfected</td>
<td>48</td>
<td>13</td>
<td>Up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M2 uninfected</td>
<td>53</td>
<td>13</td>
<td>Up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M1 vs. M2 infected</td>
<td>21</td>
<td>22</td>
<td>Down</td>
<td>0.830</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M1 infected</td>
<td>49</td>
<td>11</td>
<td>Up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M\textsuperscript{EP} vs. M2 infected</td>
<td>49</td>
<td>16</td>
<td>Up</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>13</td>
<td>22</td>
<td>Down</td>
<td>0.340</td>
</tr>
<tr>
<td>M2 infected vs. uninfected</td>
<td>18</td>
<td>13</td>
<td>Up</td>
<td>0.381</td>
</tr>
<tr>
<td>M\textsuperscript{EP} infected vs. uninfected</td>
<td>11</td>
<td>14</td>
<td>Down</td>
<td>0.644</td>
</tr>
<tr>
<td>Infected/uninfected M1 vs. M2</td>
<td>4</td>
<td>9</td>
<td>Down</td>
<td>0.217</td>
</tr>
<tr>
<td>Infected/uninfected M\textsuperscript{EP} vs. M1</td>
<td>3</td>
<td>3</td>
<td>Up</td>
<td>0.822</td>
</tr>
<tr>
<td>Infected/uninfected M\textsuperscript{EP} vs. M2</td>
<td>5</td>
<td>5</td>
<td>Down</td>
<td>0.316</td>
</tr>
</tbody>
</table>

The genes from this signature that were observed to be upregulated in M\textsuperscript{EP} macrophages were largely similar for both uninfected and infected M\textsuperscript{EP} macrophages. The most upregulated gene was PTGES (prostaglandin E synthase), which produces prostaglandin E2 (PGE2). The fold change for PTGES ranged from 267-fold (when compared to infected M1 macrophages) to 2,590-fold (when compared to infected M2 macrophages).

PGE2 can have a variety of effects depending on factors such as the receptors involved, duration of signaling, and stage of infection. PGE2 can inhibit neutrophil and macrophage activation, inhibit natural killer cell responsiveness to cytokines, attract macrophages, induce M1 polarization markers in macrophages, enhance activation of inflammasomes, inhibit NADPH
oxidase activity and nitric oxides in macrophages, and promote the production of cytokines including IL-10 and IL-17\textsuperscript{385,386}. Immunosuppressive activity is generally associated with the EP2 and EP3 receptors\textsuperscript{386}, while pro-inflammatory effects are reported to result from EP4 activation\textsuperscript{385}. During *Salmonella* infection, the bacterial effector SpiC enhances production of PGE2 in macrophages, leading to immunosuppression and reduced *Salmonella* killing\textsuperscript{386}. Live *Salmonella* promote more PGE2 production than do killed; this could reflect *Salmonella* interference with host defenses such as IL-12 signaling\textsuperscript{387}. Inhibitor studies show that prostaglandin decreases *Salmonella* load in mice during acute infection, but is detrimental during chronic infection\textsuperscript{386}, decreasing long-term survival\textsuperscript{387}. Indeed, PGE2 is suspected to be involved in long-term immunosuppression during sepsis\textsuperscript{388}. Differences in *Salmonella* load in THP-1 cells treated with PGE2 *in vitro* were not observed\textsuperscript{385}, so it is unclear whether PGE2 would have an effect on resistance of M\textsuperscript{EP} MDMs *in vitro*.

Interestingly, while PTGES was upregulated in M\textsuperscript{EP} macrophages, several other genes involved in PGE2 synthesis were downregulated in M\textsuperscript{EP} macrophages (Table 4.9). However, the upregulation in PTGES was of a very large magnitude, with an upregulation from 267-fold up to 2590-fold, while downregulated prostaglandin genes had changes between 2- and 10-fold. This, together with the reported increases of PGE2 in sepsis patients\textsuperscript{389}, make it seem likely that PGE2 production is indeed elevated in M\textsuperscript{EP} macrophages.

**Table 4.9: Fold change in Prostaglandin genes.** Fold change was calculated using DESeq2. Only genes with a p-value < 0.05 and fold change of at least 2 are shown.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Uninfected M\textsuperscript{EP} vs M1</th>
<th>Infected M\textsuperscript{EP} vs M1</th>
<th>Response to Infection M\textsuperscript{EP} vs M2</th>
<th>M\textsuperscript{EP} vs M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTGES</td>
<td>522</td>
<td>581</td>
<td>267</td>
<td>2590</td>
</tr>
<tr>
<td>HPGDS</td>
<td>-4.0</td>
<td>-8.2</td>
<td>-9.6</td>
<td>-9.7</td>
</tr>
<tr>
<td>PTGS1/COX1</td>
<td>-2.7</td>
<td>-8.9</td>
<td>-2.3</td>
<td>-8.2</td>
</tr>
<tr>
<td>PTGS2/COX2</td>
<td>-3.2</td>
<td></td>
<td>238</td>
<td>183</td>
</tr>
<tr>
<td>PTGR2</td>
<td>-2.3</td>
<td></td>
<td>-2.2</td>
<td></td>
</tr>
</tbody>
</table>

Aside from prostaglandins, several lipid pathways were downregulated in M\textsuperscript{EP} macrophages compared to M2 macrophages (Figure 4.16). These notably included cholesterol biosynthesis, “regulation of cholesterol biosynthesis by SREBP (Sterol regulatory element-binding
protein),” and “activation of gene expression by SREBP,” which triggers expression of genes for synthesis of cholesterol and other lipids. Membrane cholesterol promotes the formation of the SCV and decreases autophagy of Salmonella\(^{390}\), so decreased expression of cholesterol synthesis genes in uninfected M\(^{EP}\) compared to M2 macrophages could contribute to their early resistance to Salmonella. Notably, only one of the three cholesterol synthesis pathways was downregulated in infected M\(^{EP}\) when compared to infected M2 macrophages, and it was downregulated to a lesser extent. No lipid pathways were differently regulated in M\(^{EP}\) macrophages in response to infection.

**Figure 4.16: Differential enrichment in lipid metabolism pathways.** The colour of the dots indicate the percentage of the differentially expressed genes found in the pathway which were upregulated: pathways that were largely upregulated are red, while pathways that were largely downregulated are green. The size of the dots indicates the significance level of the pathway enrichment, -log\(_{10}\)(adjusted p-value), such that larger dots have smaller p-values. Abbreviations: “un” is “uninfected”, “inf” is “infected”, and “p-adj” refers to the p-value adjusted for multiple testing. Only pathways for which the adjusted p-value was \(\leq 0.05\) are shown.

Also consistently upregulated from the endotoxin tolerance signature were genes encoding metallothioneins, most prominently MT1M with FCs of 4.88 (infected M1 macrophages) and 8.1 (uninfected M2 macrophages), but also including MT1G, MT1H, MT1X, and MT1F. Metallothioneins have known significance in endotoxin shock and Salmonella infection. They have antioxidant function, and protect macrophages from H\(_2\)O\(_2\) and free oxygen radicals produced in response to LPS\(^{391}\) as well as against inflammatory damage in the lung\(^{392}\). Metallothioneins also chelate metals including zinc. This function inhibits Salmonella growth since in addition to requiring zinc for its own growth, Salmonella can use zinc to inhibit NF-κB signaling and thus reduce production of reactive oxygen species\(^{23}\).
Another significant group of genes in the endotoxin signature are the alarmins S100A8/A9/A12. These alarmins can recruit neutrophils and macrophages, have protective antimicrobial functions, and modulate inflammation, but can also participate in the excessive inflammation that characterizes endotoxin shock. Alarmins S100A8 and S100A9, subunits of calprotectin, sequester zinc; however, in contrast to metallothioneins, calprotectin does not appear to limit *Salmonella* growth, likely due to *Salmonella*’s ability to import sufficient zinc for its needs. Indeed, despite the fact that S100A8 and A9 levels are increased in the plasma of typhoid fever, and that these alarmins can kill *Salmonella in vitro*, mice deficient in S100A9 show no change in their resistance to *Salmonella* or in their inflammatory response. However, introduction of S100A8/A9 does reduce the *Salmonella* load in epithelial cells. Interestingly, S100A8 and S100A9 are also involved in cytoskeletal reorganization through activation of Cdc42 and Rac; there is a possibility that this would interact with the formation of the SCV.

The alarmins S100A8 and S100A12 were upregulated in uninfected M\textsuperscript{EP} macrophages (Table 4.10), and alarmin S100A9 was also upregulated in uninfected M\textsuperscript{EP} compared to M1 macrophages, though only with FC = 2.15, and it was not upregulated compared to M2 macrophages. Although S100A8 and S100A12 were also observed to be upregulated in infected M\textsuperscript{EP} macrophages, the level of upregulation was lower and S100A9 was no longer differentially expressed in infected M\textsuperscript{EP} macrophages when compared to other macrophage types.

**Table 4.10: Fold change in S100A8, S100A9, and S100A12 genes.** Fold change was calculated using DESeq2. Only genes with a p-value < 0.05 and fold change of at least 2 are shown.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Uninfected</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M\textsuperscript{EP} vs M1</td>
<td>M\textsuperscript{EP} vs M2</td>
</tr>
<tr>
<td>S100A8</td>
<td>3.25</td>
<td>15.7</td>
</tr>
<tr>
<td>S100A9</td>
<td>2.15</td>
<td>15.7</td>
</tr>
<tr>
<td>S100A12</td>
<td>5.80</td>
<td>15.7</td>
</tr>
</tbody>
</table>

Pathways differently regulated in M\textsuperscript{EP} macrophages compared to M1 and M2 macrophages were examined thoroughly to identify any pathways particularly associated with endotoxin priming. Looking at the numbers of differently regulated pathways (Table 4.11), uninfected M\textsuperscript{EP} macrophages were more similar to M1 than to M2 macrophages, with a total of 26 pathways up or downregulated compared to M1 macrophages and 55 pathways up or downregulated compared to M2 macrophages. Infected M\textsuperscript{EP} macrophages became more distinct from M1 macrophages, with a total of 44 pathways up or downregulated compared to M1 macrophages, and 49 compared to...
M2 macrophages. Of these pathways, most were downregulated. In particular, while 37 pathways were downregulated in infected M\textsuperscript{EP} macrophages compared to M1 macrophages, only 4 pathways were upregulated, and 4 of these 7 pathways were also upregulated in uninfected M\textsuperscript{EP} macrophages (Figure 4.17).

Table 4.11: Number of pathways down or upregulated in M\textsuperscript{EP} compared to M1 and M2 macrophages. Downregulated pathways are those that are enriched with <50\% of the differentially expressed genes in the pathways being upregulated, while upregulated pathways are those that are enriched with >50\% of the differentially expressed genes being upregulated.

<table>
<thead>
<tr>
<th></th>
<th>Up-regulated in M\textsuperscript{EP} vs. M1</th>
<th>Down-regulated in M\textsuperscript{EP} vs. M1</th>
<th>Up-regulated in M\textsuperscript{EP} vs. M2</th>
<th>Down-regulated in M\textsuperscript{EP} vs. M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>7</td>
<td>37</td>
<td>19</td>
<td>30</td>
</tr>
<tr>
<td>Uninfected</td>
<td>14</td>
<td>12</td>
<td>28</td>
<td>27</td>
</tr>
</tbody>
</table>

Pathways upregulated in M\textsuperscript{EP} when compared to M1 macrophages included “chemokine receptors bind chemokines”, and “Metallothioneins bind metals”, both of which were upregulated in M\textsuperscript{EP} macrophages regardless of infection status (Figure 4.17). The identification of the metallothionein pathway was consistent with the upregulation of multiple metallothionein genes observed when considering the endotoxin tolerance signature. Considering chemokines, as with uninfected M1 macrophages, uninfected M\textsuperscript{EP} macrophages expressed high levels of CXCL11, the receptor for which (CXCR3) is important in \textit{Salmonella} defense\textsuperscript{325,326}. Infection did not increase levels of CXCL11 in M\textsuperscript{EP} macrophages, leading to relatively low expression in infected M\textsuperscript{EP} when compared to that in infected M1 and M2 macrophages. Similarly, uninfected M\textsuperscript{EP} upregulated CCL5 (RANTES), another CXCR3 ligand and inflammatory mediator that attracts monocytes as well as NK cells and activated T cells\textsuperscript{398,399}, but the difference in CCL5 expression disappeared in infected macrophages. Chemokines upregulated in both infected and uninfected M\textsuperscript{EP} included neonatal sepsis biomarker CXCL12\textsuperscript{400}, as well as neutrophil-attracting chemokines CXCL5\textsuperscript{401} and CXCL6 (granulocyte chemotactic protein 2), which also has direct antibacterial killing activity\textsuperscript{402}; and CXCL13, which is downregulated by \textit{Salmonella} in what could represent a strategy for evading host defenses\textsuperscript{403}. 

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Figure 4.17: Pathways upregulated in either uninfected or infected M\textsuperscript{EP} when compared to M1 macrophages. The colour of the dots indicate the percentage of the differentially expressed genes found in the pathway which were upregulated: pathways that were largely upregulated are red, while pathways that were largely downregulated are green. The size of the dots indicates the significance level of the pathway enrichment, -\log_{10}(adjusted p-value), such that larger dots have smaller p-values. “p-adj” refers to the p-value adjusted for multiple testing. Only pathways for which the adjusted p-value was ≤0.05 are shown.

Pathways that were up- or down-regulated in response to infection in M\textsuperscript{EP} macrophages were usually also up- or down-regulated in response to infection in M1 and M2 macrophages (Figure 4.18).
Figure 4.18: The pathways that are differentially expressed in response to infection in M\textsuperscript{FP} were similar to those differentially expressed in M1 and M2 macrophages. The colour of the dots indicate the percentage of the differentially expressed genes found in the pathway which were upregulated: pathways that were largely upregulated are red, while pathways that were largely downregulated are green. The size of the dots indicates the significance level of the pathway enrichment, -log\textsubscript{10}(adjusted p-value), such that larger dots have smaller p-values. Abbreviations: “un” means “uninfected”, “inf” means “infected”, and “p-adj” refers to the p-value adjusted for multiple testing. Only pathways for which the adjusted p-value was ≤0.05 are shown.

4.2.8 Polarization did not affect NF-κB signaling genes

NF-κB is a transcription factor that responds to a variety of endogenous signals and pathogen molecules to induce genes involved in inflammation and both innate and adaptive defense mechanisms, including pro-inflammatory cytokines and genes involved in production of prostaglandins and reactive oxygen species\textsuperscript{404,405}. TLRs, some NLRs, TNF, and IL-1 all signal through NF-κB\textsuperscript{44,110,130}. \textit{Salmonella} infection initially activates NF-κB signaling and the resulting inflammatory response, but upon \textit{Salmonella} entry into the cell, effectors inhibit NF-κB activation\textsuperscript{18–21,313}, dampening host defenses including production of reactive oxygen species\textsuperscript{23}.

NF-κB signaling genes were generally upregulated in all three polarization types during
infection (Table 4.12 and Figure 4.19). Few significant differences in expression of these genes were observed between polarized macrophages whether infected or uninfected (Table 4.12). Minor differences in uninfected cells led to clustering by polarization in the heatmap (Figure 4.19). Uninfected M\(^{EP}\) macrophages upregulated MYD88, NFKB2, and RELA in compared to uninfected M2 macrophages, and uninfected M1 macrophages modestly upregulated ICAM1 compared to uninfected M2 macrophages (FC 2.14, padj = 0.018). In infected cells, the only difference was a 2.09-fold lower expression of NFKB1 in infected M\(^{EP}\) compared to M1 macrophages. The lack of differences in NF-κB signaling genes between the polarized macrophages could suggest that the induction of inflammation resulting from *Salmonella* infection of macrophages is not significantly affected by whether the bacteria is able to replicate within the cell. Further, the expression of NF-κB genes clearly does not contribute to the ability of M1 macrophages to restrict intracellular survival of *Salmonella*, possibly due to *Salmonella*’s ability to reduce activation of NF-κB.

Table 4.12: Change in expression of NF-κB genes in MDM in response to polarization and *Salmonella* infection. Only data for which the Benjamani-Hoffberg adjusted p-value (calculated by DESeq2 using the Wald test) is <0.05 and Fold Change is >2 are shown. *NFKBIA is inhibitory

<table>
<thead>
<tr>
<th>MDM Types Compared</th>
<th>ICAM1</th>
<th>MYD88</th>
<th>NFKB1</th>
<th>NFKB2</th>
<th>NFKBIA*</th>
<th>RELA</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 vs. M2 uninfected</td>
<td>2.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M(^{EP}) vs. M1 uninfected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M(^{EP}) vs. M2 uninfected</td>
<td>2.93</td>
<td>2.20</td>
<td>2.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1 vs. M2 infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M(^{EP}) vs. M1 infected</td>
<td>-2.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M(^{EP}) vs. M2 infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>3.47</td>
<td>7.50</td>
<td>2.89</td>
<td>13.36</td>
<td>3.95</td>
<td></td>
</tr>
<tr>
<td>M2 infected vs. uninfected</td>
<td>6.81</td>
<td>2.58</td>
<td>8.55</td>
<td>3.38</td>
<td>6.32</td>
<td>5.28</td>
</tr>
<tr>
<td>M(^{EP}) infected vs. uninfected</td>
<td>3.47</td>
<td>4.66</td>
<td>2.41</td>
<td>7.88</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.19: Heatmap of expression of NF-κB genes. The colour indicates the difference in regularized log (rlog) gene expression from the mean. Rlog expression represents the log2 of counts, adjusted by sequencing depth for each sample, with the values for genes with low counts adjusted towards zero. X-axis labels: “Un” is uninfected, “Inf” is infected, and “ETP” is MEP.

4.3 Discussion

4.3.1 The relationships between samples and changes in expression of polarization signatures indicate correct polarization in M1/M2, and tolerance in MEP macrophages

The separation of samples on the PCA plot (Figure 4.2) according to infection in the first component, and polarization in the second component, reflected the fact that both of these situations should have a strong transcriptional effect on cells. The stronger effect of infection was likely a result of the larger number of signals received as a result of Salmonella exposure compared to polarization. Furthermore, a macrophage detecting Salmonella would need to induce extensive gene expression changes in order to defend against the pathogen. The location on the PCA plot of M0 between M1 and M2 macrophages is consistent with a lack of activation in either direction. The intermixing of M1 and M2 macrophages on the heatmap (Figure 4.3), but not on the PCA plot (Figure 4.2), likely reflects the fact that the heatmap represents the relationship between each pair of samples with a single number representing the distance between those samples (indicated by their colour), while the PCA plot renders the relationship between samples in two components (indicated as the samples’ relative positions on the X and Y axes). In this case, the second component discriminated between M1 and M2 macrophages. The observed clustering, as well as the upregulation of the M1(IFNγ+LPS/TNFα) gene signature261 in M1 macrophages (Table 4.1) and the M2(IL4/IL-13) gene signature261 in M2 macrophages (Table 4.2), indicate a correct polarization of these cells.
The location, on the PCA plot (Figure 4.2), of M\textsuperscript{EP} macrophages closer to M1 than to M2 macrophages is consistent with an initial M1-like activation of these endotoxin-primed M\textsuperscript{EP} macrophages. This is consistent with the observation that M\textsuperscript{EP} macrophages showed statistically similar enrichment of M1 and M2 signature gene sets (i.e., no statistically significant differences in enrichment) when compared to M1 macrophages (Table 4.1 and Table 4.2), although there were obvious differences to M1 macrophages (Table 4.1). On the other hand, the clustering of uninfected and infected M\textsuperscript{EP} macrophages into their own separate groups on the heatmap, while M1 and M2 macrophages were more intermixed (Figure 4.3), indicated that M\textsuperscript{EP} macrophages had a distinct transcriptional profile. The statistically significant upregulation of the endotoxin tolerance signature in both infected and uninfected M\textsuperscript{EP}, but not in M1 or M2 macrophages (Table 4.8), was in keeping with the identification of M\textsuperscript{EP} macrophages as a distinct phenotype.

Furthermore, the partial upregulation of this signature (~50% of genes upregulated) in uninfected M\textsuperscript{EP} macrophages indicated that the 24-hour endotoxin priming favoured the induction of an endotoxin tolerance-like phenotype or reprogramming in the M\textsuperscript{EP} macrophages, even prior to a second LPS stimulation. The introduction of Salmonella considerably reduced the number of M1 signature genes that were upregulated in M\textsuperscript{EP} macrophages compared to M2 macrophages by nearly half, while leading to downregulation of 12% of M1 signature genes (Table 4.1). Infection also increased the number of upregulated M2 signature genes in M\textsuperscript{EP} macrophages when compared to M1 and M2 macrophages (Table 4.2), with M\textsuperscript{EP} macrophages overall becoming somewhat more M2-like. However, infection did not substantially alter the expression of the endotoxin tolerance signature, either in enrichment of the gene set or the identity of the most highly expressed signature genes.

These shifts in the M1 and M2 signatures could indicate that the change in Salmonella resistance in M\textsuperscript{EP} macrophages between 2 and 4 hours of infection resulted from the endotoxin primed cells becoming relatively more M2-like in response to Salmonella infection, while M1 and M2 macrophages both become more M1-like. In keeping with this concept, infection of M\textsuperscript{EP} macrophages did not cause the same statistically significant enrichment of upregulated M1 signature genes as did infection of both M1 and M2 macrophages (Table 4.1). This is consistent with an altered, tolerant response to Salmonella infection in M\textsuperscript{EP} macrophages. The fact that the transcriptomes of uninfected and infected M\textsuperscript{EP} macrophages appeared to be related by hierarchical clustering of the heatmap results (Figure 4.3) was also consistent with the suggestion that there
were fewer differences resulting from infection of $M^{EP}$ macrophages than for the other two polarization states.

Overall, the conclusions from these observations are that these three types of macrophages are correctly polarized, and that the change in resistance for $M^{EP}$ macrophages between 2 hours and 24 hours after infection is not due to the induction of tolerance signature genes during *Salmonella* infection but rather due to a failure to activate certain M1 processes as a result of cellular reprogramming. To explore this proposition, it is necessary to take a closer look at changes in other genes and pathways involved in infection.

### 4.3.2 Primed activation pathways and genes

The main differences observed in this study after pathway enrichment generally reflected a pattern that I term “primed activation”, which reflects, for some genes, a smaller difference in the expression of immune genes and pathways between infected M1, M2, and $M^{EP}$ macrophages than between uninfected M1, M2, and $M^{EP}$ macrophages (represented in Figure 4.5 and Figure 4.6a). The primed upregulation in uninfected M1 macrophages of genes in immune pathways affecting interferon, C-type lectin receptors (CLRs), and JAK-STAT signaling (Figure 4.6b, Figure 4.8, Figure 4.9b, and Figure 4.20) suggests that M1 macrophages were pre-primed for an anti-infective response, even in absence of *Salmonella*. In contrast, M2 macrophages only activated these pathways when *Salmonella* was introduced. Despite in many cases reaching similar pathway expression levels after infection when compared to M1 macrophages, M2 macrophages were still not able to resist *Salmonella* infection. This suggests that these pathways might contribute to *Salmonella* resistance in M1 macrophages (e.g. during the initial stages of *Salmonella* uptake and growth) as a result of priming of host defence mechanisms prior to contact with the pathogen, rather than as a result of differences in activation of these pathways by infection.

Primed activation was also observed in tryptophan catabolism and nicotinamide salvaging pathways (Figure 4.6b), suggesting that, prior to contact with *Salmonella*, M1 macrophages may also have stockpiled NAD$^+$ to fuel autophagy$^{331}$, and restricted the supply of nutrients important to *Salmonella* by altering the levels of the molecules tryptophan and nicotinamide mononucleotide from which *Salmonella* synthesizes its own NAD$^+$. While the expression of the tryptophan catabolism gene IDO has previously been associated with IFN$\gamma$ stimulation$^{170}$, to my knowledge the association of nicotinamide salvaging with M1 activation is a novel finding of this study.
Figure 4.20: Summary of changes in primed activation pathways resulting from polarization in infected and uninfected MDM. The colour of the dots indicate the percentage of the differentially expressed genes found in the pathway which were upregulated: pathways that were largely upregulated are red, while pathways that were largely downregulated are green. The size of the dots indicates the significance level of the pathway enrichment, $-\log_{10}(\text{adjusted p-value})$, such that larger dots have smaller p-values. “p-adj” refers to the p-value adjusted for multiple testing. Only pathways with an adjusted p-value <0.05 are shown.

While the JAK-STAT signalling gene set showed a primed activation pattern overall (Table 4.3), the specific JAK-STAT genes that were upregulated in uninfected M1 macrophages rather than in infected M1 macrophages are particularly interesting. JAK-STAT signaling participants were primarily upregulated in uninfected M1 macrophages (Figure 4.9b), while JAK-STAT effectors were more upregulated in infected M1 macrophages (Figure 4.9c). This indicated that JAK-STAT signaling might be a central component of the primed activation response, with priming of the overall pathway leading to an elevated upregulation of specific effector genes in M1 macrophages upon encountering Salmonella.

Notably, primed activation pathways were also often primed in $M^{EP}$ as well as M1 macrophages (Figure 4.6b and Figure 4.9b), consistent with the fact that $M^{EP}$ were primed by LPS treatment which also generates M1 macrophages. While this RNA-Seq experiment did not assess
a time course, it seems possible that the activation of anti-infective pathways in absence of *Salmonella* reflects activation of these pathways before the introduction of *Salmonella*, i.e. by the end of the 24-hour pre-polarization period. In this case, this primed activation is likely to have been responsible for the early resistance of M$^{\text{EP}}$ macrophages to *Salmonella* infection, as reflected with M1-like killing at 2 hours after invasion. Since there is little difference in expression of these pathways between infected M1 and M$^{\text{EP}}$ macrophages, the loss of resistance in M$^{\text{EP}}$ after 4 hours likely results from differences in other pathways, such as inflammasomes or *Salmonella* resistance genes.

### 4.3.3 Interleukin and inflammasome pathways

Two types of pathways with significance in innate immunity were noticeably absent from the primed activation pathways: inflammasomes, and most interleukin pathways. Interleukins are key regulators of the immune response, with activities that include activating various innate immune cells including macrophages and pro- and anti-inflammatory effects (Table 1.2). The specific interleukins IL-12, IL-15, and IL-23 have identified roles in defense against *Salmonella*. Inflammasomes are multi-protein complexes that include one of several pathogen recognition receptors, and which cause the processing and release of IL-1β and IL-18, pyroptosis, and exposure of intracellular *Salmonella* to killing by neutrophils.

In contrast to the primed activation pathways, interleukin and inflammasome pathways generally showed no statistically significant difference between uninfected M1 and uninfected M2 macrophages; they were primarily enriched when comparing infected to uninfected cells (Figure 4.11). Thus, while these pathways were of mechanistic interest, any effect they would have on differential resistance of polarized macrophages did not derive from activation prior to infection.

However, compared to infected M2 macrophages, infected M1 macrophages upregulated a small set of 10 genes that included key genes at multiple levels of the inflammasome process (Figure 4.12 and Figure 4.13): initiating receptors AIM2 and NLRP3, facilitator PELI2, central pyroptosis coordinator CASP1 (caspase-1), pyroptosis mediator Gasdermin-D (GSDMD) and effector cytokines IL-1A and IL-18. Infected M1 macrophages also downregulated CARD9, which inhibits the NLRP3 inflammasome during *Salmonella* infection. In response to infection, as represented by the relative polarization effect, M1 macrophages showed a greater gene expression of two downstream effectors connected to inflammasome signaling, IL-1α and IL-1β, than did M2 macrophages.
Infected MEP macrophages showed moderate expression of inflammasome genes (Figure 4.12 and Figure 4.13), with more downregulated genes compared to M1 and M2 macrophages than are seen in uninfected MEP macrophages (Table 4.4). They expressed greater levels of inflammasome receptor NLRP3 than did M2 cells, but lower levels of AIM2, and did not show a relative difference in IL-1A and IL-1B expression in response to infection. Compared to infected M1 macrophages, MEP macrophages downregulated 8 genes including receptor AIM2, complex component ASC, and CASP5. Furthermore, since MEP macrophages downregulated receptor IL-1R1 and accessory IL-1RAP when compared to M2 macrophages, they may be poorly responsive to IL-1 produced by inflammasome activation; this would result in a decreased efficacy of inflammasome activation in defense for a population of MEP macrophages.

Overall, these data suggest that when exposed to Salmonella, M1 macrophages increase their expression of genes involved in inflammasome formation and pyroptotic signaling, which would promote resistance to infection. Lower expression of these genes could contribute to the lack of such resistance in M2 macrophages, and in MEP macrophages at time points later than 4 hours. It is of particular interest that the NLRP3 inflammasome was upregulated in M1 macrophages. Since Salmonella evades detection by NLRC4 as a result of suppressing flagellin, NLRP3 would provide an alternative route for inflammasome activation in response to this pathogen. This study therefore provides novel evidence in favor of the significance of NLRP3 in the current debate regarding the role of this inflammasome in Salmonella infection.

### 4.3.4 Differential modulation of Salmonella targets and resistance genes

*Salmonella* effectors are known to influence infection by variously manipulating actin, promoting microtubule association with the SCV, and preventing SCV fusion with pathogen-digesting lysosomes. The list of *Salmonella* targets consists of a variety of macrophage genes involved in this process. All macrophage types downregulated this gene set in response to infection, which would have the potential to interfere with the ability of *Salmonella* to reorganize the cytoskeleton to its benefit.

Based on Roast analysis, M1 macrophages most significantly downregulated this gene set in response to infection, with 77% of these genes downregulated compared to 46% in M2 macrophages (Table 4.5). DESeq2 also identified downregulation of many of these genes in response to infection in M1 macrophages, with VPS18 showing the greatest fold change. This downregulation was also seen at the pathway level with the Rho GTPase and Rab pathways.
which are targeted by *Salmonella* in the formation of the SCV and which were more
downregulated in M1 macrophages in response to infection than M2 macrophages, with lower
adjusted p-values and a greater percentage of pathway genes downregulated (Figure 4.14). M^{EP}
macrophages showed little downregulation of these *Salmonella* target pathways. This greater
downregulation in *Salmonella* effectors in M1 macrophages suggests that M1 macrophages may
restrict the ability of *Salmonella* to target the cytoskeleton, while M2 and M^{EP} are relatively
vulnerable to *Salmonella*’s cytoskeletal manipulations. Downregulation in a wider set of 183
*Salmonella* susceptibility genes identified through genetic screening also indicated a greater
suppression of these genes in response to infection in M1 macrophages than in M2 or M^{EP}
macrophages, based on smaller p-values and greater percentages of downregulated genes (Table
4.6). Notably, Yeung *et al*’s screen was performed at a time point of 30 minutes after
intracellular infection; further testing would be required to determine the relevance of these
genes to resistance at the 4-hour time point at which these changes in expression were observed.

Mirroring the decreased expression of *Salmonella* targets, a set of genes involved in
*Salmonella* resistance was statistically significantly upregulated in M1 when compared to M2
macrophages both in uninfected and infected cells (Table 4.7). Some of these upregulated
*Salmonella* resistance genes (Figure 4.15) encoded general immune system effectors such as
TNFα, TLR4, and IFNγ, as well as phagocyte NADPH oxidase complex components, which are
involved in the killing of pathogens, including *Salmonella*. Other such genes were more
specifically *Salmonella*-associated. For example, both uninfected and infected M1 macrophages
upregulated an assortment of Guanylate Binding Proteins (GBPs), which rupture the SCV and
participate in inflammasome activation. This upregulation is known to be dependent on JAK-
STAT signaling in response to IFNγ. While uninfected M^{EP} macrophages express M1-like
levels of *Salmonella* resistance genes, the expression of these genes in infected M^{EP} macrophages
was intermediate, being higher than M2 but lower than M1 macrophages. This might have
contributed to the lower resistance of M^{EP} than M1 macrophages after 4 hours of infection.

### 4.3.5 Endotoxin genes

The most differentially expressed endotoxin signature gene observed in both uninfected
and infected M^{EP} macrophages was PTGES (prostaglandin E synthase). Prostaglandin E (PGE2)
has a variety of immune effects; overall it is protective during acute *Salmonella* infection but
detrimental during chronic infection, and is believed to be involved in immunosuppression
associated with sepsis. The 4-hour period of Salmonella in this experiment is likely too short to represent a transition from acute to chronic infection, and thus it seems unlikely that PTGES activity is responsible for the transition from resistance to susceptibility in M\textsuperscript{EP} macrophages; however its presence is consistent with gene expression in M\textsuperscript{EP} macrophages being relevant to sepsis.

Notably, infected M\textsuperscript{EP} macrophages exhibited decreased expression of two genes involved in the NADPH oxidase complex, CYBB/Nox2 and NCF2, compared to infected M1 macrophages (see section 4.2.6 and Figure 4.11). This could result from decreased NF-κB activation resulting from endotoxin tolerance, as has previously been reported\textsuperscript{406}, independent of Salmonella infection.

At the pathway level, overall the pathways that were up- or downregulated in M\textsuperscript{EP} macrophages in response to infection were similarly regulated in M1 and M2 macrophages (Figure 4.18). This suggests that there does not exist a particular “M\textsuperscript{EP} response to infection” that would result in their loss of resistance to Salmonella after 4 hours of infection. For example, no key immune pathways were uniquely downregulated in M\textsuperscript{EP} macrophages in response to infection. Rather, the loss of resistance likely resulted from a failure to up- or down-regulate some combination of genes related to inflammasomes, Salmonella resistance, and/or Salmonella manipulation of the cytoskeleton, as previously described.

However, some interesting differences in pathway expression were observed when comparing M\textsuperscript{EP} macrophages with M1 and M2 macrophages. First, uninfected M\textsuperscript{EP} macrophages downregulated several pathways involved in cholesterol synthesis compared to uninfected M2 macrophages; this downregulation was largely eliminated in infected M\textsuperscript{EP} macrophages (Figure 4.16). Since cholesterol promotes SCV formation and decreases Salmonella autophagy\textsuperscript{390}, the downregulation of these genes in uninfected M\textsuperscript{EP} macrophages could contribute to early Salmonella resistance. The loss of Salmonella resistance after 4 hours of infection, however,
indicates that metallothioneins are insufficient to control *Salmonella*.

M<sup>EP</sup> macrophages also upregulated the “chemokine receptors bind chemokines” pathway both in uninfected and infected cells (Figure 4.17). The significance of this pathway is particularly interesting with respect to *Salmonella* resistance. *Salmonella* effectors SipA and SopABDE2 induce the expression of chemokines including CXCL6, which could represent an attempt to recruit additional host cells, or to trigger gut inflammation, which provides a competitive advantage to *Salmonella* relative to the host microbiota. The expression of chemokines such as CXCL6 by M<sup>EP</sup> could thus be a result of *Salmonella* manipulating the host immune system.

However, strong evidence exists supporting a protective role for these chemokines. Chemokine CCL2(MCP-1) and chemokine receptor CXCR3 are both known to be important components in host defense against *Salmonella*. Upregulation of CXCR3 ligands CXCL11 and CCL5 in uninfected M<sup>EP</sup> compared to M1 and M2 macrophages could thus be protective; relatively low expression of these chemokines in infected M<sup>EP</sup> could contribute to loss of resistance observed after 4 hours of infection. In addition, *Salmonella* suppresses neutrophil chemotaxis, possibly as a defense against intestinal neutrophils which prevent systemic *Salmonella* infection by efficiently killing bacteria and limiting the spread of *Salmonella* from the gut. Indeed, the *in vivo* resistance of endotoxin tolerant mice to *Salmonella* infection is attributed in part to early accumulation of leukocytes and to neutrophil recruitment. A strong upregulation of chemokines, particularly neutrophil targeting chemokines CXCL5 and CXCL6, in both uninfected and infected M<sup>EP</sup> macrophages, could improve recruitment of key anti-pathogenic neutrophils. This could drive *Salmonella* resistance at the level of the endotoxin tolerant host animal even if individual M<sup>EP</sup> macrophages are permissive to intracellular *Salmonella* replication.

### 4.3.6 Summary of analysis

Overall, the transcriptional data suggest that M1 macrophage resistance to *Salmonella* infection results from a combination of four elements. First, primed activation of key immune pathways and genes, including JAK-STAT signaling, likely reflected an improved ability to mount a rapid immune response to *Salmonella*. Second, upregulation of inflammasomes and particularly the NLRP3 inflammasome in response to infection might contribute to pyroptosis. Third, downregulation of *Salmonella* target genes could interfere with *Salmonella*’s ability to manipulate the cytoskeleton to promote SCV survival. Fourth, upregulation of resistance genes, including GBPs and NADPH oxidase, would promote the killing of *Salmonella*. Observation of primed
activation in tryptophan and nicotinate pathways may further indicate a significant role for NAD$^+$ in *Salmonella* infection, as suggested previously$^{329-331}$, and a novel importance for this pathway in M1 macrophages. It is worth mentioning however that this systems biology examination of *Salmonella* infection has demonstrated numerous pathways/mechanisms with known supportive and inhibitory functions on such infections, and thus it seems likely that it is the overall integration of these pathways that critically determines the fate of *Salmonella*.

For M$^{\text{EP}}$ macrophages, the difference in bacterial killing at the early 2-hour and later 24-hour time points likely reflect the differences between M1 and M$^{\text{EP}}$ macrophage activation. M$^{\text{EP}}$ macrophages activated certain primed pathways in a manner similar to M1 macrophages. Given that these pathways were induced in the absence of *Salmonella*, they may have been active at earlier phases of infection and contributed to early killing. In keeping with this idea that genes upregulated in uninfected MDM might contribute to early resistance, uninfected M$^{\text{EP}}$ macrophages showed a similar expression of *Salmonella* resistance genes when compared to uninfected M1 macrophages. However, greater differences were observed in activated genes and pathways when comparing infected M$^{\text{EP}}$ with infected M1 macrophages. In particular, infected M$^{\text{EP}}$ macrophages showed lower expression of inflammasome and *Salmonella* resistance genes than did infected M1 macrophages, and M$^{\text{EP}}$ macrophages did not downregulate *Salmonella* target genes as extensively as did M1 macrophages. In combination, these transcriptional effects could have been responsible for the change in permissiveness for intracellular *Salmonella* persistence of M$^{\text{EP}}$ macrophages as the infection progressed.

### 4.3.7 Limitations and future directions

Though gene expression in uninfected macrophages is used to infer which genes and pathways are active in absence of *Salmonella* infection, and thus which are likely to be active at early stages of infection, the experiments performed do not represent a time course. In order to obtain a more detailed picture of changes in gene expression in the course of infection, a future experiment might include RNA samples from the end of the 24-hour polarization phase (immediately prior to infection), 2 hours (while M$^{\text{EP}}$ are resistant to *Salmonella*), 4 hours, and 24 hours. In addition, gene expression does not always correspond with the activity of a pathway. Additional experiments are necessary to verify differences in protein expression. In some cases localization (e.g., nuclear localization of NF-κB) or metabolite quantification (e.g. tryptophan and nicotinamide mononucleotide) would be necessary to confirm pathway activity.
The bioinformatics methods used to analyze the data have their own limitations. I minimized the effects of these limitations by examining the data at multiple levels, examining pathways, gene sets, and individual genes. For example, Sigora identifies pathway enrichment using gene-pair signatures, which indicate a particular pathway when they are co-expressed. This enables targeted identification of biologically relevant pathways with fewer overlapping pathways than alternative pathway analysis methods\textsuperscript{288}. However, because the input is simply the list of differentially expressed genes, it is sensitive to the utilized cut-offs for fold change and p-value of the differentially expressed genes and does not differentiate between the magnitude of the fold change above the cut-off value. Meanwhile, Roast gene set enrichment analysis takes into account the magnitude of fold changes of genes, such that sets with genes that were highly differentially expressed were more likely to be detected as enriched. However, Roast is intended for testing small numbers of gene sets that are known to be of prior interest\textsuperscript{280} (such as, shown here, polarization signatures or known \textit{Salmonella} susceptibility genes), and thus cannot be used in the same way as Sigora to identify pathways with the greatest biological significance selected from a comprehensive database.

Additionally, the Sigora method for assessing pathway enrichment does not take into account whether a particular gene promotes or suppresses the function of a particular pathway. Because of this, it is possible to, for example, identify an immune pathway as upregulated even if the upregulated genes within that pathway primarily serve to suppress the activity of the pathway. It is not practical to identify every differentially expressed suppressor gene within every pathway in an analysis. However, the risk of drawing inappropriate conclusions from pathway enrichment is minimized by identifying the most highly differentially expressed genes for pathways of particular interest (e.g., IDO1 for Tryptophan pathways, or NLRP3 and CASP1 for inflammasome pathways) and ensuring these were consistent with activation of the pathway. Ultimately, this Chapter represents a hypothesis-generating investigation, and has enabled the generation of several hypotheses relating to the differences in resistance between M1, M2, and M\textsuperscript{EP} macrophages. Based on the strengths of the data and opportunity to build on existing literature, I could, in particular, list five substantive hypotheses as candidates for future work.

First, I propose that primed activation of JAK-STAT signalling genes results in M1 resistance, and early M\textsuperscript{EP} resistance, to \textit{Salmonella} infection and thus tested this hypothesis in Chapter 6 using an inhibitor of JAK2, Ruxolitinib. As a comparison, I also observed the effect of
a different inhibitor, Parthenolide. Unlike Ruxolitinib, Parthenolide would be expected to reduce Salmonella resistance in all three polarization types due to its inhibitory effect on NF-κB. NF-κB genes were similarly expressed and upregulated in response to infection in all three macrophages types (Table 4.12 and Figure 4.19), so observing a decrease in resistance in all macrophage types, or no change in resistance, as a result of Parthenolide infection, would indicate that any M1-specific decrease in resistance from Ruxolitinib treatment resulted from the specific effect of JAK-STAT priming.

As a second hypothesized mechanism, I propose that NAD+ production resulting from primed activation of tryptophan and nicotinate pathways results in M1, and early MEP, resistance to Salmonella. This hypothesis could be tested by depleting NAD+ in M1 and MEP macrophages, prior to infection, or introducing inhibitory RNAs for the NAD+ producing genes IDO1 and NAMPT into M2 macrophages, and measuring whether this could reduce the difference in resistance between polarization types.

As a third hypothesis, I propose that the activation of the NLRP3 inflammasome contributes to Salmonella resistance in M1 macrophages. This could be tested by determining whether the difference in resistance between polarization types is reduced in NLRP3 knockout macrophages. A fourth hypothesis concerning mechanism would be that downregulation of Salmonella target genes contributes to resistance in M1 macrophages. This could be tested using a knockout of the most significantly downregulated gene, VPS18, given that a conditional knockout of this gene is available in mice414.

A fifth hypothesis, I propose that the upregulation of Salmonella resistance genes in M1 macrophages contributes to resistance. With respect to this hypothesis, it would be of great interest to measure whether the induction of these resistance genes in MEP or M2 macrophages after infection would influence Salmonella killing.

Given the likely importance of integration of multiple pathways for resistance, the effect of combinations of these pathways could also be tested; for example, it is possible that a knockout of VPS18 in combination with induction of GBPs would confer a synergistic effect on destabilization of SCV and impact on Salmonella killing.

Notably, the investigation of many of these hypotheses would be aided by the identification of a rich, renewable source of knockout human macrophages. Such a source was investigated in Chapter 5.
Chapter 5: Transcriptomic comparisons of induced pluripotent stem-cell derived macrophages and monocyte-derived macrophages in response to polarization and *Salmonella* infection

5.1 Introduction and rationale

Human induced pluripotent stem-cells (iPS) represent a source of derived macrophages (iPSDM) with lower variability and greater potential for genetic manipulation than monocyte-derived macrophages (MDM)\(^{240,243}\). Furthermore, these could potentially be transfused therapeutically in order improve immunity or healing\(^{138,244}\). In order to realize this potential, it is necessary to have a detailed understanding of the similarities and any differences between iPSDM and MDM.

Previous studies have established that iPSDM express similar surface markers and cytokines to MDM in response to polarization\(^{255,257}\), and show similar expression of a subset of 32 genes that are affected by polarization\(^{240}\). iPSDM can be infected by *Salmonella*\(^{255}\), and murine embryonic stem-cell derived macrophages (ESDM) and bone-marrow derived macrophages (BMDM) have a similar transcriptional response to *Salmonella* infection\(^{254}\). A strong overall similarity has also been reported between iPSDM and MDM gene expression in response to LPS\(^{258}\) and to *Chlamydia*\(^{248}\) infection, with some notable differences in pathway enrichment in chemokine expression and antigen presentation. However, the effect of polarization on the complete iPSDM transcriptome had not been assessed, nor had the transcriptional response of human iPSDM to *Salmonella* been determined. The current analysis presented in this Chapter provides insights into the suitability of iPSDM as a model to study the effect of macrophage M1/M2 polarization on resistance to *Salmonella* infection.

The RNA-Seq dataset discussed in Chapter 4 was compared with an iPSDM RNA-Seq dataset that was collected in collaboration with Dr. Chris Hale, Wellcome Trust Sanger Institute, using similar protocols, and measured at the same 4-hour time point. Correlations between gene expression and the overlap in differentially expressed genes, especially functional genes such as JAK-STAT, indicated a broad similarity between iPSDM and MDM in their transcriptional responses to polarization and infection. iPSDM also showed similar enrichment in pathways involved in the response to infection, including a similar “primed activation” pattern to MDM. However differences were observed in some functions such as metabolic pathways that were not
suspected to be involved in infection. In addition, iPSDM displayed greater transcriptional differences between uninfected M1 and M2 macrophages than did MDM. Overall, this analysis reinforces the importance of the primed activation pathways. It also demonstrates that while iPSDM and MDM are broadly similar, and have similar immunological responses to infection, caution should be exercised in extending definitive conclusions about other transcriptional responses such as metabolism from iPSDM studies to MDM studies.

5.2 Results

5.2.1 Overview of iPSDM data

Differentially expressed genes for these two macrophage types were computed using the general scheme described in Chapter 4 and illustrated in Figure 4.1 and Figure 4.4. In brief, DESeq2 estimates Log2 fold change in gene expression between two conditions, adjusted such that genes with lower counts, or greater variability, are reported as having a lower estimated fold change. A p-value is derived using the Wald test, and corrected for multiple testing. Genes are considered significantly differentially expressed if both the magnitude of the fold change is greater than 2 (Log2 fold change > 1), and the Benjamani-Hoffberg-adjusted p-value is lower than 0.05. Comparisons were made between uninfected M1 and M2 macrophages (providing a baseline), infected M1 and M2 macrophages (difference between infected macrophages of each type), and between uninfected and infected macrophages of both polarization types (response to infection). A relative polarization effect (statistical “interaction term”) was also calculated, reflecting the difference in response to infection between M1 and M2-polarized macrophages.

Using DESeq2, a total of 1870 genes were identified as differentially expressed by unpolarized (M0) iPSDM macrophages in response to Salmonella infection (Table 5.1); 2,124 genes were identified in M1 macrophages, and 2,006 in M2 macrophages. M2 macrophages upregulated the greatest number of genes in response to infection, with 1,274 upregulated genes and 732 downregulated genes. Of these differentially expressed genes, 998 represented a common response to infection between all three types of macrophage (Figure 5.1a). A large overlap of 524 differentially expressed genes was observed between M0 and M2 macrophages, while 837 genes were uniquely differentially expressed in M1 macrophages.

1,942 genes were differentially expressed between uninfected M1 and M2 macrophages (Table 5.1). Only 1,464 differentially expressed genes were identified when comparing infected M1 and M2 macrophages. In both cases, more genes were upregulated in M1 macrophages than
downregulated. Of these genes, 860 were differentially expressed in both infected and uninfected macrophages (Figure 5.1b). This indicates that while infected M1 and M2 macrophages had somewhat fewer differences than uninfected M1 and M2 macrophages, there were still many differences that persisted during infection of polarized cells.

Table 5.1: Number of genes differentially expressed in iPSDM. Differentially expressed genes were determined using DESeq2, cutoffs corrected p-value < 0.05 and fold change > 2. Abbreviations: “un” = “uninfected,” “inf” = “infected.”

<table>
<thead>
<tr>
<th>Direction of regulation</th>
<th>M0 inf vs. un</th>
<th>M1 inf vs. un</th>
<th>M2 inf vs. un</th>
<th>M1 vs. M2. Un</th>
<th>M1 vs. M2 inf</th>
<th>M1 vs. M2 in/un effect</th>
</tr>
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<tr>
<td>Total</td>
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<td>2124</td>
<td>2006</td>
<td>1942</td>
<td>1464</td>
<td>364</td>
</tr>
<tr>
<td>Up-regulated</td>
<td>1166</td>
<td>1247</td>
<td>1274</td>
<td>1089</td>
<td>870</td>
<td>114</td>
</tr>
<tr>
<td>Downregulated</td>
<td>704</td>
<td>877</td>
<td>732</td>
<td>853</td>
<td>594</td>
<td>250</td>
</tr>
</tbody>
</table>

![Figure 5.1: Venn diagram of differentially expressed genes in iPSDM. Genes were determined to be differentially expressed by DESeq2, cutoffs corrected p-value < 0.05 and fold change > 2. [a] Genes differentially expressed in response to infection (infected vs uninfected) for M0, M1, and M2 macrophages. [b] Genes differentially expressed in M1 vs M2 macrophages, for uninfected and infected macrophages.](image)

Pathway enrichment analysis was performed as in Chapter 4. M1 macrophages showed the most enriched pathways in response to infection (Table 5.2), with 28 of these 61 pathways being unique to the M1 response to Salmonella (Figure 5.2a). A further 24 pathways were part of the shared response to infection between M0, M1, and M2 macrophages, and 13 were shared between M0 and M2 macrophages. 56 pathways were enriched in uninfected M1 vs. M2 macrophages, with only 35 identified comparing infected M1 and M2 macrophages (Table 5.2); of these, 24 were shared (Figure 5.2b). As with the DE genes, this indicates that there are more differences between
uninfected polarized cells, but still some distinct differences between M1 and M2 macrophages even during infection.

Table 5.2: Number of pathways differentially enriched in iPSDM. Pathways were identified using the Reactome database with Sigora, or with InnateDB for the relative polarization effect, with corrected p-value < 0.05. Abbreviations: “un” = “uninfected,” “inf” = “infected.”

<table>
<thead>
<tr>
<th>Pathways</th>
<th>M0 inf vs. un</th>
<th>M1 inf vs. un</th>
<th>M2 inf vs. un</th>
<th>M1 vs. M2 un</th>
<th>M1 vs. M2 inf</th>
<th>M1 vs. M2 in/un effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathways</td>
<td>43</td>
<td>61</td>
<td>53</td>
<td>56</td>
<td>35</td>
<td>113</td>
</tr>
</tbody>
</table>

Figure 5.2: Venn diagram of pathways differentially enriched in iPSDM. Enriched pathways were determined using Sigora, corrected p-value < 0.05. [a] Pathways enriched in response to infection (infected vs uninfected) for M0, M1, and M2 macrophages. [b] Pathways enriched in M1 vs M2 macrophages, for uninfected and infected macrophages.

5.2.2 Overall patterns of gene expression in iPSDM are similar to those of MDM

As the first step in comparing iPSDM with MDM, the broad patterns of gene expression were analyzed, to confirm that the clustering of the iPSDM data based on polarization and infection conditions aligned with expectations based on MDM data. As in Chapter 4, Principal Component Analysis and a sample-distance heatmap were used to represent the high-dimensional variability of the gene expression data in two-dimensional space.

Using Principal Component Analysis (Figure 5.3a), the first component (representing 66% of the sample variation) separated the polarized iPSDM primarily by infection status, though uninfected M1 iPSDM macrophages also showed a shift in this direction. The second component (representing 17% of the variation) separated M1 from M0 and M2 iPSDM. These patterns were similar to those observed in MDM (Figure 5.3b). This established general similarity between the
two datasets with respect to macrophage responses to polarization and *Salmonella* infection.

![PCA plot comparing clustering of normalized gene expression in iPSDM with that of MDM.](image)

**Figure 5.3**: PCA plot comparing clustering of normalized gene expression in iPSDM with that of MDM. The first two principal components, plotted on x and y axes, summarize the greatest sources of variation between samples. [a] iPSDM [b] MDM, with principal components computed without M\(^{\text{EP}}\) samples.

As for the PCA plot, the heatmap separated samples by infection status and by M1 vs. M0 and M2 macrophages (Figure 5.4). These analyses provided a preliminary indication that iPSDM responded similarly to these stimuli when compared to MDM. When compared to MDM, iPSDM showed an overall greater differentiation of M1 from M2 and M0 macrophages, and greater overlap between the M2 and M0 macrophages. This is not entirely unexpected, as these M0 macrophages were differentiated using M-CSF and such macrophages have been found to be transcriptionally M2-like\(^{167}\). Indeed the differentiation protocol for iPSDM used a higher concentration of M-CSF in the final stages of maturation when compared to the MDM protocol (100 ng/mL cf. 50 ng/mL).
Figure 5.4: Sample distance heatmap showing clustering of gene expression for differently activated iPSDM. The colour of the heatmap indicates the distance between samples.

GeneOverlap was used to test the similarity in lists of differentially expressed genes identified in iPSDM with those identified in MDM. This method asks the question: do we observe that a larger number of genes are present on both lists than would be expected by chance? GeneOverlap uses Fisher’s exact test to calculate a p-value for the overlap between the two gene lists, an odds ratio describing the strength of association, with higher odds ratios (greater than 1) indicating stronger association, and a Jaccard index, where values closer to 1 indicate better similarity. Statistically significant overlap was observed in the lists of differentially expressed genes when comparing uninfected and infected M0, M1, or M2 macrophages, and when comparing M1 and M2 macrophages both before and after infection, as well as in the relative polarization effect (Table 5.3). Filtering the iPSDM gene counts to only include genes that were present in the MDM datasets produced only a modest improvement in the p-values for the overlap in differentially expressed gene lists. This indicates that genes that were expressed only in iPSDM did not have a large distorting effect on the overall analysis, despite their somewhat different origins.
Table 5.3: Overlap in lists of differentially expressed genes in MDM and iPSDM, assessed by GeneOverlap\(^{415}\). For columns labelled “All”, overlap was tested comparing an iPSDM differentially expressed (DE) gene set, while for columns labelled “MDM”, overlap was calculated using all iPSDM genes and a DE gene set calculated using only iPSDM genes that were also observed in MDM studies.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>P-value</th>
<th>Odds Ratio</th>
<th>Jaccard Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>MDM</td>
<td>All</td>
</tr>
<tr>
<td>M0 infected vs. uninfected</td>
<td>2.75x10^{-264}</td>
<td>8.79x10^{-291}</td>
<td>6.8</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>1.70x10^{-222}</td>
<td>5.38x10^{-259}</td>
<td>5.3</td>
</tr>
<tr>
<td>M2 infected vs. uninfected</td>
<td>1.91x10^{-299}</td>
<td>&lt;5x10^{-259}</td>
<td>7.7</td>
</tr>
<tr>
<td>M1 vs. M2 (uninfected)</td>
<td>1.00x10^{-169}</td>
<td>3.76x10^{-198}</td>
<td>9.4</td>
</tr>
<tr>
<td>M1 vs. M2 (infected)</td>
<td>2.62x10^{-86}</td>
<td>1.39x10^{-102}</td>
<td>7.7</td>
</tr>
<tr>
<td>M1 vs. M2 (infected/uninfected)</td>
<td>1.78x10^{-37}</td>
<td>1.21x10^{-36}</td>
<td>73.9</td>
</tr>
</tbody>
</table>

In addition, the percentage of genes differentially expressed in both MDM and iPSDM that were regulated in the same direction (both up-regulated or both down-regulated) was at least 96.7% (Table 5.4). This is similar to the >95% similarity that was reported when comparing the response of iPSDM and MDM to *Chlamydia* infection\(^{248}\). However, the numbers of genes identified as differentially expressed in both iPSDM and MDM was lower. Yeung *et al* identified 2,029 differentially expressed genes for unpolarized macrophages in response to *Chlamydia*, while this study identified 865 for the M0 macrophages in response to *Salmonella*. One reason for this is that Yeung’s study used a cutoff for differential gene expression of a fold change ≥1.5, while this study used a more stringent cutoff of a fold change >2, which results in fewer genes being recorded as differentially expressed. Another reason for the difference might be that the RNA-Seq datasets for this study were generated in two different laboratories, leading to potential batch effects, modest differences in *Salmonella* growth state, and RNA-Seq parameters such as depths of sequencing or reference genome, which would introduce sources of variation between the datasets.

Table 5.4: Numbers of genes modulated in the same or different direction between iPSDM and MDM. Genes shown are those that were determined to be differentially expressed in both iPSDM and MDM using DESeq2, cutoffs corrected p-value < 0.05 and fold change > 2. Abbreviations: “un” = “uninfected,” “inf” = “infected.”

<table>
<thead>
<tr>
<th>Direction of regulation</th>
<th>M0 inf vs. un</th>
<th>M1 inf vs. un</th>
<th>M2 inf vs. un</th>
<th>M1 vs. M2. un</th>
<th>M1 vs. M2 inf</th>
<th>M1 vs. M2 in/un effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulated Similarly</td>
<td>865</td>
<td>912</td>
<td>862</td>
<td>390</td>
<td>205</td>
<td>30</td>
</tr>
<tr>
<td>Both Up</td>
<td>244</td>
<td>550</td>
<td>631</td>
<td>309</td>
<td>154</td>
<td>4</td>
</tr>
<tr>
<td>Both Down</td>
<td>621</td>
<td>362</td>
<td>231</td>
<td>81</td>
<td>51</td>
<td>26</td>
</tr>
<tr>
<td>Different</td>
<td>11</td>
<td>26</td>
<td>6</td>
<td>12</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>% Same</td>
<td>98.7</td>
<td>97.2</td>
<td>99.3</td>
<td>97.0</td>
<td>96.7</td>
<td>100.0</td>
</tr>
</tbody>
</table>
Similarity in gene expression was then assessed at the level of log fold change in expression for these comparisons. Linear regression was used to calculate the correlation between log fold change of gene expression observed in iPSDM and the change of expression in the same gene observed in MDM. This produces both a p-value for the correlation, and a measure of the strength of correlation in the form of an $R^2$ value.

For all gene expression correlations, the correlation between log fold change in MDM and iPSDM was highly statistically significant ($p < 0.001$) (Figure 5.5 and Table 5.5). These correlations were similar whether or not the iPSDM gene expression list was filtered to include only genes that were observed to be expressed in MDM, as seen for uninfected vs. infected M0 macrophages in Figure 5.5. For all comparisons, the $R^2$ values with or without filtering based on the MDM gene universe were within 0.006 of each other (Table 5.5). As for the p-values in overlap between differentially expressed gene lists, the similarity in $R^2$ values for log fold change correlation indicates that genes that were expressed only in iPSDM did not distort the overall analysis.

Figure 5.5: Correlation between the log$_2$ fold change in gene expression during infection in M0 MDM and change of expression during infection in M0 iPSDM. Dots indicate genes, with colour indicating whether the gene was upregulated in both iPSDM and MDM, downregulated in both, or regulated in different directions. Lines indicate a linear regression, with the adjusted $R^2$ value and p-value for the correlation indicated above the plot. iPSDM differential expression was calculated [a] using all genes expressed in iPSDM; or [b] by filtering out genes not observed to be expressed in MDM, leaving only the MDM gene universe.
Table 5.5: Correlation between log: fold change in gene expression for iPSDM and MDM as assessed using linear regression (R^2 values) for all comparisons between polarization and infection states. Data labelled (All) indicate the calculation included all genes expressed in iPSDM, while data labelled (MDM) included only the genes also differentially expressed in MDM. Column labelled “correlation for” indicates whether the lists of genes for which the log fold change correlation were calculated using changes of all genes, only genes differentially expressed in iPSDM whether or not they were differentially expressed in MDM, or genes differentially expressed in both iPSDM and MDM. All correlations were statistically significant (p<0.001) but of different magnitudes as represented by the given R^2 values.

<table>
<thead>
<tr>
<th>Correlation for:</th>
<th>Comparison</th>
<th>R^2 (All)</th>
<th>R^2 (MDM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All genes</td>
<td>M0 infected vs. uninfected</td>
<td>0.403</td>
<td>0.402</td>
</tr>
<tr>
<td></td>
<td>M1 infected vs. uninfected</td>
<td>0.388</td>
<td>0.388</td>
</tr>
<tr>
<td></td>
<td>M2 infected vs. uninfected</td>
<td>0.460</td>
<td>0.459</td>
</tr>
<tr>
<td></td>
<td>M1 vs. M2 uninfected</td>
<td>0.318</td>
<td>0.318</td>
</tr>
<tr>
<td></td>
<td>M1 vs. M2 infected</td>
<td>0.186</td>
<td>0.186</td>
</tr>
<tr>
<td></td>
<td>M1 vs. M2 infected/uninfected</td>
<td>0.081</td>
<td>0.081</td>
</tr>
<tr>
<td>Genes differentially expressed in iPSDM</td>
<td>M0 infected vs. uninfected</td>
<td>0.544</td>
<td>0.544</td>
</tr>
<tr>
<td></td>
<td>M1 infected vs. uninfected</td>
<td>0.558</td>
<td>0.553</td>
</tr>
<tr>
<td></td>
<td>M2 infected vs. uninfected</td>
<td>0.603</td>
<td>0.601</td>
</tr>
<tr>
<td></td>
<td>M1 vs. M2 uninfected</td>
<td>0.503</td>
<td>0.509</td>
</tr>
<tr>
<td></td>
<td>M1 vs. M2 infected</td>
<td>0.414</td>
<td>0.415</td>
</tr>
<tr>
<td></td>
<td>M1 vs. M2 infected/uninfected</td>
<td>0.368</td>
<td>0.378</td>
</tr>
<tr>
<td>Genes differentially expressed in both MDM and iPSDM</td>
<td>M0 infected vs. uninfected</td>
<td>0.658</td>
<td>0.658</td>
</tr>
<tr>
<td></td>
<td>M1 infected vs. uninfected</td>
<td>0.720</td>
<td>0.717</td>
</tr>
<tr>
<td></td>
<td>M2 infected vs. uninfected</td>
<td>0.704</td>
<td>0.702</td>
</tr>
<tr>
<td></td>
<td>M1 vs. M2 uninfected</td>
<td>0.681</td>
<td>0.685</td>
</tr>
<tr>
<td></td>
<td>M1 vs. M2 infected</td>
<td>0.721</td>
<td>0.721</td>
</tr>
<tr>
<td></td>
<td>M1 vs. M2 infected/uninfected</td>
<td>0.709</td>
<td>0.709</td>
</tr>
</tbody>
</table>

The overlap in the lists of differentially expressed genes and the correlation in log fold change of gene expression provided further evidence as to the relevance of iPSDM as a model for MDM in experiments involving polarization and *Salmonella* infection. The strength of the observed correlations depended on the comparison (polarization or infection status) and on whether all genes were considered or only a subset of genes (Table 5.5 and Figure 5.6). The weakest correlation was for the list of all genes. Progressively stronger correlation was observed when considering only genes that were differentially expressed in iPSDM, and differentially expressed in both iPSDM and MDM. Generally, the change in gene expression in response to infection showed a better correlation than differences between polarization types, particularly comparing infected M1 and M2. This effect disappeared when only genes differentially expressed in both MDM and iPSDM were considered. Overall these data suggest that iPSDM are an excellent
model for *Salmonella* infection of differentially polarized macrophages.

**Figure 5.6:** Strength of correlation between the fold changes for genes in MDM and iPSDM, based on type of comparison between polarized and infected cells. R² values were calculated by linear regression for the log₂ fold changes in MDM vs fold changes in iPSDM. The lists of genes for which the log fold change correlation was calculated included, for the three facets of the graph: “All Genes”, changes of all genes, “DE in iPSDM”, genes differentially expressed in iPSDM whether or not they are differentially expressed in MDM, and “DE in Both”, genes differentially expressed in both iPSDM and MDM. Abbreviations used: “un” = “uninfected”, “inf” = “infected”, and “inf/un” = relative polarization effect (“interaction term”).

The fact that the correlation in log fold change between the two macrophage cell types was stronger for genes that were differentially expressed (Figure 5.6), than for the total set of genes, indicates that genes identified as meaningful in a differential expression analysis were likely to demonstrate similar behavior in the two cell types.

In particular, for various comparisons between cells of different polarization and infection states, a good correlation (linear regression R² values of 0.37 - 0.60; mean of 0.50; p<0.001) was
observed between the log fold change of genes that were differentially expressed in iPBSDM, and the log fold changes observed in MDM (regardless of whether those genes were differentially expressed or not in iPBSDM). This indicates that iPBSDM are likely able to adequately model *Salmonella* infections and macrophage phenotypes, even in the absence of MDMs for comparison.

While significant, this correlation in log fold changes as measured by $R^2$ values was somewhat weaker than has been previously reported when comparing the response of iPBSDM and MDM to LPS\textsuperscript{258}. Alasoo *et al* found that for genes differentially expressed in either iPBSDM or MDM, the correlation of log fold change of genes in response to LPS had an $R^2$ value of 0.82, compared to 0.54 that I observed here for the correlation between log fold change of genes in M0 macrophages in response to *Salmonella*. As with the number of differentially expressed genes identified, this difference might result from the generation of RNA-Seq data by two different laboratories, introducing additional sources of variation such as batch effects, modest differences in *Salmonella* growth state, and depths of sequencing. It also seems likely that the response to a single molecule LPS might be more consistent between MDM and iPBSDM when compared to the transcriptional response to *Salmonella* or to polarization. Nevertheless, the substantial overlaps observed here and reported for the two cell types indicate that overall these data reflect biological changes rather than the source of macrophages.

5.2.3 Genes differentially expressed in iPBSDM had functional significance and related to those differentially expressed in MDM.

To better assess the functional similarity between iPBSDM and MDM, I investigated differential expression in iPBSDM of specific genes (assessed using DESeq2) and gene sets of interest (assessed using Roast, with p-values calculated by simulation). Both uninfected and infected M1 iPBSDM upregulated the NFkB-associated genes, RELA, UBD, and STAT5A, Interferon regulatory factor transcriptional regulatory genes, IRF1 and IRF8, the gene for pro-inflammatory cytokine TNF, and the antigen presentation genes B2M and TAP1-2. Uninfected M1 iPBSDM upregulated the genes for TNF Superfamily member TNFSF10 (32.3 FC), antiviral protein RSAD2 (25.5 FC), the peptidoglycan recognition receptor NOD2 (10.3 FC), and apolipoproteins APOL3 and APOL6 (15.8 and 9.1 FC) which were all previously associated with M1 activation\textsuperscript{167}. The most highly upregulated genes in infected M1 macrophages included the genes encoding Type 1 interferons IFNL1 and IFNB1 (17.5 and 11.2 fold change) consistent with the known role of Type 1 interferon signaling in intracellular infection\textsuperscript{356}. 

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Roast was used to assess the expression of specific gene sets, starting with the M1 (IFNγ+LPS/TNF) and M2 (IL-4/IL-13) signatures derived by Becker et al using integrative transcriptomics\(^{261}\) and discussed in Chapter 4 (see Appendix D, Table A.1 for lists of genes). In line with expectations and the MDM data, genes from the M1 signature were upregulated in M1 compared to M2 macrophages in uninfected and infected iPSDM (Table 5.6), and genes from the M2 signature were upregulated in M2 iPSDM (Table 5.7).

**Table 5.6: Enrichment in the 100-gene M1 signature\(^{261}\) in iPSDM compared with enrichment in MDM.** Enrichment was calculated from normalized gene counts using Roast. MDM data are copied from Table 4.1. Columns are as follows. “Macrophage Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. iPSDM or MDM “% Up-Regulated” and “% Down-Regulated”: the percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. iPSDM or MDM “p-value” was calculated by Roast using simulations taking into account both percentage of up- and down-regulated genes and direction of change. Statistically significant comparisons (p<0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Macrophage Types Compared</th>
<th>iPSDM</th>
<th>MDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Up-regulated</td>
<td>% Down-regulated</td>
</tr>
<tr>
<td>M1 vs. M2 uninfected</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>44</td>
<td>21</td>
</tr>
<tr>
<td>M2 infected vs. uninfected</td>
<td>84</td>
<td>8</td>
</tr>
<tr>
<td>Infected/uninfected M1 vs. M2</td>
<td>2</td>
<td>82</td>
</tr>
<tr>
<td>M1 vs. M2 infected</td>
<td>68</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 5.7: Enrichment in the 58-gene M2 signature\(^{261}\) in iPSDM compared with enrichment in MDM.** Enrichment was calculated from normalized gene counts using Roast. MDM data are copied from Table 4.2. Columns are as follows. “Macrophage Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. iPSDM or MDM “% Up-Regulated” and “% Down-Regulated”: the percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. iPSDM or MDM “p-value” was calculated by Roast using simulations taking into account both percentage of up- and down-regulated genes and direction of change. Statistically significant comparisons (p<0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Macrophage Types Compared</th>
<th>iPSDM</th>
<th>MDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Up-regulated</td>
<td>% Down-regulated</td>
</tr>
<tr>
<td>M1 vs. M2 uninfected</td>
<td>2</td>
<td>79</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>12</td>
<td>48</td>
</tr>
<tr>
<td>M2 infected vs. uninfected</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>Infected/uninfected M1 vs. M2</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>M1 vs. M2 infected</td>
<td>6</td>
<td>75</td>
</tr>
</tbody>
</table>
M1 markers upregulated in M1 iPSDM included IL-12A, IL-12B (infected only), CXCL9, SOCS1, and SOCS3. M2 markers upregulated in M2 iPSDM included CD209, CD200R1, CCL26, CCL13, CCL18, CCL22, and ALOX15. The observation that M1 iPSDM upregulate the 100-gene M1 signature and that M2 iPSDM upregulate the 58-gene M2 signature builds on previous observations that iPSDM express polarization-associated cytokines and surface markers such as the M1 markers HLA-DR, IL-6, and TNFα, and M2 marker CD206255,416 and a panel of 32 gene markers such as CXCL-10 and CXCL-11 (M1) and CCL-17 and CCL-22 (M2)240.

As observed for MDM, in response to infection, M2 iPSDM upregulated genes from the M1 signature (Table 5.6). M2 and M1 macrophages of both origins downregulated genes from the M2 signature in response to infection (Table 5.7). The single difference between the cell types with respect to these signatures was that in MDM, the upregulation of the M1 signature in M1 macrophages in response to infection reached statistical significance (p-value 0.002) but did not in iPSDM (p-value 0.07) (Table 5.6). This appeared to be due to an increase in downregulated genes in iPSDM; however, the percentage of upregulated genes was almost the same in the two cell types (44% in iPSDM and 45% in MDM), and the p-value for iPSDM was close to the cutoff. In both types the relative polarization effect showed a downregulation (or reduced upregulation in response to infection) in M1 signature genes in M1 compared to M2 macrophages. These similarities in the effect of Salmonella infection on the expression of M1 and M2 signatures suggested that iPSDM are a good model for MDM in the context of polarization and infection.

Like MDM, iPSDM also showed a primed activation pattern in JAK-STAT pathway genes (determined using the KEGG283 pathway scheme; see Appendix D, Table A.1). The primed activation pattern is represented conceptually in Figure 4.5. Primed activation features observed in the JAK-STAT genes were: uninfected M1 macrophages upregulating the genes/pathway compared to uninfected M2 macrophages, M2 macrophages upregulating the genes/pathway in response to infection, and no statistically significant upregulation in M1 in response to infection, or difference between infected M1 and M2 macrophages (Table 5.8). There was also statistically significant downregulation in the relative polarization effect for response to infection in M1 vs. M2 macrophages. As JAK-STAT signaling pathways are important in translating cytokine signaling is translated into immune responses121,341, including macrophage activation196, a similar pattern in the expression of these genes has significant functional significance.
Table 5.8: Primed enrichment in KEGG\textsuperscript{283} JAK-STAT genes in iPSDM and MDM. Set contains 155 genes. Enrichment was calculated from normalized gene counts using Roast. MDM data are copied from Table 4.3. Columns are as follows. “Macrophage Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. iPSDM or MDM “% Up-Regulated” and “% Down-Regulated”: the percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. iPSDM or MDM “p-value” was calculated by Roast using simulations taking into account both percentage of up- and down-regulated genes and direction of change. Statistically significant comparisons (p<0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Macrophage Types Compared</th>
<th>% Up-regulated</th>
<th>% Down-regulated</th>
<th>p-value</th>
<th>% Up-regulated</th>
<th>% Down-regulated</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1 vs. M2 uninfected</td>
<td>32</td>
<td>25</td>
<td>&lt;0.001</td>
<td>31</td>
<td>13</td>
<td>0.061</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>34</td>
<td>38</td>
<td>0.61</td>
<td>33</td>
<td>45</td>
<td>0.996</td>
</tr>
<tr>
<td>M2 infected vs. uninfected</td>
<td>45</td>
<td>30</td>
<td>&lt;0.001</td>
<td>36</td>
<td>30</td>
<td>0.010</td>
</tr>
<tr>
<td>Infected/uninfected M1 vs. M2</td>
<td>15</td>
<td>26</td>
<td>0.012</td>
<td>11</td>
<td>21</td>
<td>0.11</td>
</tr>
<tr>
<td>M1 vs. M2 infected</td>
<td>32</td>
<td>26</td>
<td>0.085</td>
<td>16</td>
<td>14</td>
<td>0.878</td>
</tr>
</tbody>
</table>

iPSDM displayed a more significant relative upregulation of JAK-STAT genes in uninfected M1 macrophages, with a p-value < 0.001 compared to 0.061 in MDM (Table 5.8). Recall that the Roast method calculates enrichment in a pathway based both on the number of differentially expressed genes in the pathway, and the magnitude of change of those genes. Given the similar percentages of upregulated JAK-STAT genes in uninfected M1 macrophages (32\% in iPSDM and 31\% in MDM), this difference in significance must be due to a greater magnitude of change in these upregulated genes in iPSDM. iPSDM also showed a stronger difference between the response of M1 and M2 macrophages to infection, as the relative polarization effect showed a statistically significantly lower upregulation in M1 compared to M2 macrophages.

Similarly to MDM, the genes upregulated in M1 iPSDM before infection included key signal transduction genes such as JAK2, STAT1/2, and SOCS1. These genes are important in interferon signaling, which as noted previously is important in defense against intracellular pathogens\textsuperscript{356}. IFN\(\gamma\) signals through JAK2 and STAT1 (as well as JAK1), and type I interferons additionally signal through STAT2\textsuperscript{343,346,348}. STAT2 also mediates expression of cytokines in response to TLR agonists including LPS and flagellin\textsuperscript{417}. STAT1 deficient mice are defective in immunity to intracellular bacteria\textsuperscript{350}. SOCS1 is a feedback inhibitor of STAT1\textsuperscript{173}; its upregulation in uninfected M1 macrophages could result in decreased upregulation of JAK-STAT genes in infected M1 relative to M2 macrophages. As seen in MDM, those genes that were upregulated in
infected M1 macrophages primarily represented cytokines, such as the interferons IFNL1 (IFNλ) and IFNB1 (IFNβ), and IL-12 and IL-23. Similarly to the interferons, IL-12 and IL-23 are both required for Salmonella defense²,³⁹.

Overall, the observation that JAK and STAT genes are upregulated in uninfected M1 macrophages suggests that they are primed to respond to further activation with interferons or LR agonists. This priming may lead to a greater upregulation of JAK-STAT responsive cytokines in infected M1 macrophages compared to M2 macrophages, even as the levels of the JAK and STAT genes themselves become similar in the two macrophage types. This reflects an additional functional similarity between iPSDM and MDM.

The change in iPSDM expression of a list of 34 Salmonella target genes (see Appendix D, Table A.1) was also assessed. These genes are involved in remodelling actin and endocytic compartments, and are known to be manipulated by Salmonella in the process of establishing the Salmonella-containing vacuole (SCV) and preventing SCV fusion with lysosomes¹¹,²⁸². Similarly to MDM, both M1 and M2 iPSDM downregulated the set of Salmonella target genes in response to infection (Table 5.9). For both iPSDM and MDM, M1 macrophages also showed a statistically significantly greater downregulation in these genes in response to infection than M2 macrophages, as reported by the relative polarization effect. In iPSDM, this resulted in lower expression of these genes in infected M1 than infected M2 macrophages. In MDM, this difference in expression of Salmonella effector genes between infected M1 and M2 macrophages was not statistically significant (p-value 0.064).

The genes from this set that were most downregulated in M1 iPSDM in response in infection were SCV-stabilizing genes¹¹,³⁷⁰,⁴¹⁸ VPS18, VPS33A, and MTMR4. VPS18 and VPS33A are core components of the HOPS complex which is targeted by Salmonella effector SifA¹¹,³⁷⁰. The downregulation of these genes would be expected to reduce stability of the SCV, resulting in exposure of Salmonella to pathogen-killing mechanisms; indeed, depletion of MTR4 reduces survival of intracellular Salmonella⁴¹⁸. Thus it seems likely that reduced expression of these SCV-stabilizing Salmonella target genes in M1 macrophages upon infection would contribute to their non-permissiveness to intracellular Salmonella survival.
Table 5.9: Enrichment in down-regulated genes in the 31-gene *Salmonella* target set in iPSDM compared with MDM. Enrichment was calculated from normalized gene counts using Roast. MDM data are copied from Table 4.5. “Macrophage Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. iPSDM or MDM “% Up-Regulated” and “% Down-Regulated”: the percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. iPSDM or MDM “p-value” was calculated by Roast using simulations taking into account both percentage of up- and down-regulated genes and direction of change. Statistically significant comparisons (p<0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Macrophage Types Compared</th>
<th>iPSDM</th>
<th>MDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Up-regulated</td>
<td>% Down-regulated</td>
</tr>
<tr>
<td>M1 vs. M2 uninfected</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>0</td>
<td>85</td>
</tr>
<tr>
<td>M2 infected vs. uninfected</td>
<td>11</td>
<td>56</td>
</tr>
<tr>
<td>Infected/uninfected M1 vs. M2</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>M1 vs. M2 infected</td>
<td>4</td>
<td>56</td>
</tr>
</tbody>
</table>

Next, the set of 183 susceptibility genes, for which loss of function results in *Salmonella* resistance\(^{100}\), was analyzed. In iPSDM, this gene set was downregulated in uninfected as well as infected M1, compared to M2 macrophages (Table 5.10). Otherwise, the differences in gene expression were similar to those that were observed in MDM, with both types of macrophages downregulating this gene set in response to infection, and a downregulation in the set in infected M1 compared to infected M2 macrophages.

Table 5.10: Enrichment in down-regulated genes in the 183-gene *Salmonella* susceptibility set\(^{100}\) in iPSDM compared with MDM. Enrichment was calculated from normalized gene counts using Roast. MDM data are copied from Table 4.6. “Macrophage Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. iPSDM or MDM “% Up-Regulated” and “% Down-Regulated”: the percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. iPSDM or MDM “p-value” was calculated by Roast using simulations taking into account both percentage of up- and down-regulated genes and direction of change. Statistically significant comparisons (p<0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Macrophage Types Compared</th>
<th>iPSDM</th>
<th>MDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Up-regulated</td>
<td>% Down-regulated</td>
</tr>
<tr>
<td>M1 vs. M2 uninfected</td>
<td>15</td>
<td>51</td>
</tr>
<tr>
<td>M1 infected vs. uninfected</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td>M2 infected vs. uninfected</td>
<td>11</td>
<td>52</td>
</tr>
<tr>
<td>Infected/uninfected M1 vs. M2</td>
<td>13</td>
<td>18</td>
</tr>
<tr>
<td>M1 vs. M2 infected</td>
<td>18</td>
<td>49</td>
</tr>
</tbody>
</table>
The final gene set investigated was the set of 35 genes identified as important in *Salmonella* resistance (see Appendix D, Table A.1 for a list). This set includes guanylate-binding proteins (GBPs), which rupture the SCV and can participate in inflammasome activation\(^{11,233-236}\), as well as NADPH oxidase genes such as Nox1, CYBB/Nox2, and NCF2; and additional innate immune response genes specifically identified as important in *Salmonella* defense, such as TLR4\(^{419}\) and IL12\(^{39}\).

For these *Salmonella* resistance genes iPSDM again showed a similar pattern to MDM (Table 5.11). Both uninfected and infected M1 macrophages upregulated these genes when compared to M2 macrophages. M2 macrophages also upregulated *Salmonella* resistance genes in response to infection, and a lower response was noted in M1 macrophages (represented by downregulation of the relative polarization effect = infected/uninfected). In MDM, M1 macrophages appeared to upregulate this gene set somewhat in response to infection, though this was not quite statistically significant (p-value 0.0501). However, in iPSDM the upregulation was clearly not significant with a p-value of 0.49 and more down-regulated than up-regulated genes.

**Table 5.11: Primed enrichment in set of 33 Salmonella resistance genes in iPSDM and MDM.** Enrichment was calculated from normalized gene counts using the roast method. MDM data are copied from Table 4.7. Columns are as follows. “Macrophage Types Compared”: cell types for which gene expression counts were compared to determine enrichment. “Infected/uninfected”: the relative polarization effect. iPSDM or MDM “% Up-Regulated” and “% Down-Regulated”: the percentage of the genes in the gene set that were found to be up- or down-regulated for the comparison in question by Roast. iPSDM or MDM “p-value” was calculated by Roast using simulations taking into account both percentage of up- and down-regulated genes and direction of change. Statistically significant comparisons (p<0.05) are indicated in bold.

<table>
<thead>
<tr>
<th>Macrophage Types Compared</th>
<th>M1 vs. M2 uninfected</th>
<th>M1 infected vs. uninfected</th>
<th>M2 infected vs. uninfected</th>
<th>Infected/uninfected M1 vs. M2</th>
<th>M1 vs. M2 infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Up-regulated</td>
<td>% Down-regulated</td>
<td>p-value</td>
<td>% Up-regulated</td>
<td>% Down-regulated</td>
</tr>
<tr>
<td>iPSDM</td>
<td>59</td>
<td>19</td>
<td>&lt;0.001</td>
<td>54</td>
<td>8</td>
</tr>
<tr>
<td>MDM</td>
<td>30</td>
<td>41</td>
<td>0.49</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>FDR %</td>
<td>8</td>
<td>27</td>
<td>0.003</td>
<td>4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

In iPSDM, as in MDM, despite the fact that M1 macrophages did not upregulate the *Salmonella* resistance set in response to infection, the resistance gene set was still upregulated in infected M1 macrophages when compared to infected M2 macrophages. Thus the M2
Macrophages were unable to “close the gap” in gene expression that was created by primed activation of these genes in uninfected M1 macrophages. Indeed, uninfected M1 macrophages upregulated 59 genes, while M2 macrophages upregulated only 48 genes in response to infection. The fact that the primed activation of *Salmonella* resistance genes has a greater magnitude than the response of either M1 or M2 macrophages to infection indicates that primed activation of these genes in M1 macrophages is likely to have a strong impact on host defense.

Ultimately, the conditions under which I observed statistically significant changes in *Salmonella* resistance gene expression were identical for iPSDM and MDM, increasing confidence that iPSDM are a good model for *Salmonella* infection and the effect of polarization on *Salmonella* resistance.

Seven of the ten genes that were upregulated in M1 iPSDM compared to M2 iPSDM were GBPs 1-7; NADPH oxidase genes are notably absent. This predominance of GBPs over NADPH oxidase genes in the iPSDM differentially expressed *Salmonella* resistance genes has particularly interesting implications for the mechanism of resistance of M1 macrophages to *Salmonella* infection. It would be reasonable to expect that differences in *Salmonella* resistance between M1 and M2 macrophages might result from differences in oxidative killing. Indeed, differential phagosome activity has been observed in M1 and M2 macrophages, at least partly attributed to post-transcriptional regulation of CYBB/Nox2. However, it has also been observed that *Salmonella* has the ability to prevent fusion of the SCV with the NADPH oxidase complex. Thus, it seems likely that any upregulation in NADPH oxidase genes would be futile without the capacity to disrupt the SCV.

The increased expression of SCV-rupturing GBP genes in M1 macrophages suggested a greater capacity to expose *Salmonella* to the cytosol, and to defensive mechanisms including autophagy and pyroptosis. Recall as well that M1 macrophages were found to downregulate SCV-stabilizing *Salmonella* target genes (Table 5.9). These SCV-disrupting mechanisms would thus lead to increased bacterial clearance even in the absence of transcriptional upregulation of NADPH oxidase genes in M1 iPSDM. Indeed, it is possible that the disruption of the SCV is the “rate determining step,” or a bottleneck limiting bacterial killing, though this cannot be confirmed without further mechanistic studies that take post-transcriptional regulation of NADPH oxidase into account.

Overall, investigation of expression of specific gene sets indicated a strong functional
similarity between iPSDM and MDM with respect to polarization and Salmonella infection. This increases our confidence that iPSDM are a good model for MDM.

5.2.4 Enrichment of many immune pathways, but not all pathways, was similar in iPSDM and MDM.

Analysis of change in expression of functional genes in iPSDM was continued using pathway enrichment analysis with Sigora\textsuperscript{288} (Table 5.12). Sigora determines whether the list of differentially expressed genes includes gene-pair signatures from known pathways at a greater rate than would be expected by chance, after multiple test correction.

Table 5.12: Number of overlapping significantly enriched pathways between MDM and iPSDM. Shown are the numbers of pathways that were enriched in both cell types (“Shared”), observed only in MDM, and observed only in iPSDM. Both the number of pathways and the percentage of pathways for each comparison, e.g. between uninfected M1 and uninfected M2 macrophages, are shown.

<table>
<thead>
<tr>
<th># Pathways</th>
<th>M1 vs. M2 Uninfected</th>
<th>M1 vs. M2 Infected</th>
<th>M1 Uninfected vs. Infected</th>
<th>M2 Uninfected vs. Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shared</td>
<td>17 (27%)</td>
<td>10 (23%)</td>
<td>32 (28%)</td>
<td>32 (34%)</td>
</tr>
<tr>
<td>MDM-Only</td>
<td>7 (11%)</td>
<td>9 (20%)</td>
<td>55 (47%)</td>
<td>42 (44%)</td>
</tr>
<tr>
<td>iPSDM-Only</td>
<td>39 (62%)</td>
<td>25 (57%)</td>
<td>29 (25%)</td>
<td>21 (22%)</td>
</tr>
</tbody>
</table>

Similarity in pathway expression was first assessed by determining, for each comparison between infection and polarization conditions, how many of the pathways observed in total for iPSDM and MDM were enriched in both cell types. This percentage ranged from 23% (when comparing infected M1 vs. infected M2 macrophages) to 34% (comparing uninfected M2 vs. infected M2 macrophages) of total pathways (Table 5.12). To better assess the relevance of these numbers, it was useful to investigate pathways of particular interest. Similarly to MDM, primed activation (Figure 5.7a) of several immune pathways was observed in iPSDM (Figure 5.7b).

Recall that the characteristics of the primed activation pattern are as follows. First, compared to uninfected M2 iPSDM macrophages, uninfected M1 macrophages showed upregulation in genes involved in these primed activation pathways. Second, these pathways were generally more upregulated in M2 than M1 macrophages in response to infection. Third, as a result, for infected cells, the differences in expression of these pathways became less evident, although M1 macrophages still showed increased expression of some pathways such as the IFN\(\gamma\) and \(\alpha/\beta\) signaling pathways and “chemokine receptors bind chemokines” when compared to M2 macrophages. Finally, the infection-associated enrichment of genes in the Interferon and IFN\(\gamma\)
signaling pathways was more significant for M2 than M1 macrophages, as assessed by the Benjamani-Hoffberg-adjusted p-value.

![Diagram](image)

**Figure 5.7: Visualization of pathways showing primed activation in iPSDM.** The colour of the dots indicate the percentage of the differentially expressed genes found in the pathway which were upregulated: pathways that were largely upregulated are red, while pathways that were largely downregulated are green. The size of the dots indicates the significance level of the pathway enrichment, -log10(adjusted p-value), such that larger dots have smaller p-values. [a] Diagram representing the primed activation pattern as it appears on a pathway dot plot: (1) upregulation in M1 vs M2 uninfected macrophages (2) little or no upregulation in M1 infected vs uninfected macrophages (3) upregulation in M2 infected vs uninfected macrophages (4) little or no upregulation in M1 vs M2 infected macrophages (5) possibly a downregulation in the pathway in the relative polarization effect, reflecting lower response to infection in M1 macrophages. [b] Pathways that showed this pattern in iPSDM data. Abbreviations: “un” means “uninfected”, “inf” means “infected”, and “inter” or “Interaction” refers to the relative polarization effect (the difference between the response to infection – infected/uninfected – in different polarization types). “p-adj” refers to the p-value adjusted for multiple testing. Only pathways with an adjusted p-value <0.05 are shown.

These primed activation pathways include Interferon signaling, IL-15 signaling, “Chemokine receptors bind chemokines”, and pathogen recognition receptor pathways “negative regulators of RIG-I/MDA5 signaling”, “NLR signaling pathways” and “C-lectin type receptors”. The primed activation of these immune pathways was consistent with the idea that M1 macrophages are largely...
prepared to respond to *Salmonella* infection even before encountering the pathogen, while M2 macrophages did not upregulate these genes until exposed to *Salmonella*.

As shown in Figure 5.8, these pathways were mostly enriched under similar conditions in iPSDM and MDM, with 27 instances of a pathway enriched in both iPSDM and MDM, 7 instances of enrichment in iPSDM only, and 4 in MDM only. As for MDM (Figure 4.11), iPSDM (Figure 5.9b) showed upregulation in Inflammasome pathways and IL-1 signaling, in infected macrophages when compared to uninfected macrophages. However, unlike MDM, iPSDM showed an upregulation in the “NLRP3 inflammasome” and “Inflammasomes” pathways in M1 compared to M2 macrophages for both infected and uninfected cells; this is reminiscent of primed activation (Figure 5.9a).

![Figure 5.8: Co-occurrence of enrichment in primed activation pathways in the same polarization/infection comparisons for iPSDM and MDM.](image)

The shown pathways contain 593 unique genes.
Figure 5.9: Visualization of inflammasome and IL-1 pathways in iPSDM. The colour of the dots indicate the percentage of the differentially expressed genes in the pathway which were upregulated: those largely upregulated are red, while those largely downregulated are green. The size of the dots indicates the significance level of the pathway enrichment, $-\log_{10}(\text{adjusted } p\text{-value})$. [a] Diagram showing example of the primed activation pattern as it appears in a pathway dot plot: (1) upregulation in M1 vs M2 uninfected macrophages (2) little or no upregulation in M1 infected vs uninfected macrophages (3) upregulation in M2 infected vs uninfected macrophages (4) little or no upregulation in M1 vs M2 infected macrophages. [b] Pathway enrichment in iPSDM. For x-axis abbreviations, “un” means “uninfected” and “inf” means “infected.” “p-adj” refers to the p-value adjusted for multiple testing. Only pathways with an adjusted p-value <0.05 are shown.

Protein-protein interaction networks (Figure 5.10) were used to visualize the connections between differentially expressed inflammasome genes. Minimum networks were used in order to visualize connections between differentially expressed genes that were linked by a single non-differentially expressed gene. These networks showed that M1 iPSDM upregulated important inflammasome genes such as IL-1A and IL-1B (in uninfected M1), NLRP3 (in infected M1), and CASP1 (in both uninfected and infected M1). In iPSDM a greater number of these genes were upregulated in uninfected cells (18 uninfected vs 10 infected), in contrast to MDM (Figure 4.12) where more of these genes were upregulated in infected M1 macrophages (9 infected vs 10 infected). This suggests that iPSDM may have a stronger primed activation of defensive pathways than MDM.
Figure 5.10: Protein-protein interaction networks of inflammasome genes in uninfected and infected iPSPDM. Gene expression data were rendered as a protein-protein interaction minimum network using NetworkAnalyst and visualized using Cytoscape. Grey circles indicate genes which were not significantly differentially expressed, but which connect two differentially expressed genes in the network. Networks include genes differentially expressed for [a] uninfected M1 iPSPDM in red vs. uninfected M2 iPSPDM in green. [b] infected M1 iPSPDM in red vs. infected M2 iPSPDM in green.

Interleukin signaling pathways, which are involved in regulating inflammation and the
immune response, showed a substantial degree of agreement between the two cell types, with 15 instances of pathway enrichment in both iPSDM and MDM, 9 in iPSDM only, and 6 in MDM only (Figure 5.11). In addition to the previously discussed IL-1 signaling pathways, both cell types similarly regulated IL-15 pathways, including the IL-15 receptor and IL-15 itself; this cytokine activates natural killer cells, and has been implicated in *Salmonella* killing and resistance to *Salmonella* colonization in the gut\(^{36,424}\). They also similarly regulated IL-10, IL-4, and IL-13 pathways, which could reflect a negative feedback mechanism limiting inflammation.

![Figure 5.11: Co-occurrence of enrichment in interleukin pathways in the same polarization/infection comparisons for iPSDM and MDM.](image)

Enriched in: ○ BOTH ☢ iPSDM blue MDM purple

Figure 5.11: Co-occurrence of enrichment in interleukin pathways in the same polarization/infection comparisons for iPSDM and MDM. Dots represent pathways that were enriched in both iPSDM and MDM (orange), iPSDM only, or MDM only, for each indicated comparison (in columns) between cell types. A large portion of orange dots in a column indicates good agreement between iPSDM and MDM regarding the effect of polarization or infection described by the comparison. For x-axis abbreviations, “un” means “uninfected”, “inf” means “infected”, and “inter” or “Interaction” refers to the relative polarization effect (the difference between the response to infection – infected/uninfected – in different polarization types). The pathways shown contained 438 unique genes.

The iPSDM data revealed two additional pathways differently modulated between uninfected M1 and M2 macrophages, IL-2 family signaling and IL-12 signaling. MDM
demonstrated four more pathways enriched in M1 and/or M2 macrophages in response to infection: IL-17 signaling, IL-12 signaling and IL-12 family signaling, and gene protein expression by JAK-STAT signaling after IL-12 stimulation (Figure 5.11). IL-2, IL-12, and IL-17 are all involved in Salmonella resistance. IL-12 activates natural killer cells and a Th1 response\(^{30}\), and its deficiency results in susceptibility to salmonellosis\(^{2}\). IL-17 is involved in preventing Salmonella dissemination from the gut\(^{125}\), and IL-2 production is protective in Salmonella infection\(^{426}\). As observed in MDM, the enrichment in these pathways represents upregulation, both in M1 iPSDM and in response to infection (Figure 5.12b). Thus, while iPSDM and MDM had some differences in the specific pathways that were identified, both upregulated Salmonella-protective interleukins in M1 macrophages.

**Figure 5.12: Visualization of interleukin pathways in iPSDM.** The colour of the dots indicate the percentage of the differentially expressed genes in the pathway which were upregulated: those largely upregulated are red, while those largely downregulated are green. The size of the dots indicates the significance level of the pathway enrichment, -log\(_{10}\)(adjusted p-value). [a] Diagram showing example of the primed activation pattern as it appears in a pathway dot plot: (1) upregulation in M1 vs M2 uninfected macrophages (2) little or no upregulation in M1 infected vs uninfected macrophages (3) upregulation in M2 infected vs uninfected macrophages (4) little or no upregulation in M1 vs M2 infected macrophages. [b] Pathway enrichment in iPSDM. For x-axis abbreviations, “un” means “uninfected” and “inf” means “infected.” “p-adj” refers to the p-value adjusted for multiple testing. Only pathways with an adjusted p-value <0.05 are shown.
As with the inflammasome pathways, the fact that more of these pathways were activated in response to M1 polarization in uninfected iPSDM, while more were activated in response to infection in MDM, might indicate a stronger primed activation of defensive pathways in M1 iPSDM than in MDM. However, the primed activation pattern (Figure 5.12a) was not consistent in interleukins for iPSDM; for example, it was not observed in IL-6 or IL-7. The identification of interleukin and inflammasome pathways as upregulated in both M1 and infected iPSDM, relative to M2 and uninfected iPSDM, indicates that the iPSDM are still a reasonable model for MDM.

To complete an analysis of the effects of polarization and infection on innate immune responses in iPSDM compared to MDM, I considered the differentially enriched innate immune pathways that did not fall into the categories of primed activation pathways, inflammasomes, or interleukins. These pathways primarily include a variety of pathogen recognition receptors such as TLR4, TLR9, NOD1/2, NLRs, Dectin-1 and Dectin-2, and the complement cascade.

In these innate immune pathways, iPSDM and MDM showed some similar pathway enrichment (Figure 5.13). There were 7 instances of pathways being enriched in both iPSDM and MDM, 11 in iPSDM only, and 5 in MDM only. The Dectin-2 family was notably enriched in both M1 iPSDM and MDM. Dectin-2 is a CLR that can detect Salmonella O-antigen\textsuperscript{322} and thus likely has a role in Salmonella defense. The pathway “cytosolic sensors of pathogen-associated DNA” was also similarly regulated; again, this pathway would be relevant to defense against intracellular pathogens such as Salmonella\textsuperscript{82,427}. These observations suggest that, looking at changes of gene expression due to polarization and Salmonella infection, there is better agreement between iPSDM and MDM in pathways likely to be involved in Salmonella infection in particular rather than necessarily strong agreement in all immune pathways.

The agreement between iPSDM and MDM was strongest when looking at genes changing in M2 macrophages in response to infection, with 3 of 7 pathways enriched in both iPSDM and MDM. In contrast, 5 of the 6 pathways differently modulated between uninfected M1 and M2 macrophages were enriched in only the iPSDM dataset, and 3 of the 6 pathways that changed expression in M1 macrophages in response to infection were enriched in only MDM. As seen with the inflammasome pathways, these other innate immune pathways indicate that iPSDM appeared to show stronger primed activation than MDM – having a greater portion of defensive pathways activated in M1 macrophages in response to polarization, rather than in response to infection.
Several metabolic pathways were observed to be enriched in the iPSDM data. These showing limited agreement with those observed in MDM (Figure 5.14), but the pathways that were similarly enriched in both iPSDM and MDM were relevant to host defense.

Both cell types showed an enrichment in genes involved in Tryptophan catabolism in uninfected M1 macrophages. For both iPSDM and MDM, IDO1 and IDO2 were upregulated in M1 macrophages while MDM also upregulated TDO2 and iPSDM also upregulated KYNU (kynureninase). This is consistent with previous observations that upregulation of IDO has previously been associated with IFNγ stimulation\(^{170}\). Further, a study by Blohmke \textit{et al} found that IDO1 and KYNU expression increases in IFNγ-primed (M1) iPSDM, but not in response to \textit{Salmonella} infection\(^ {337}\). Blohmke \textit{et al} did not consider M2 iPSDM\(^ {337}\). I found that M2 MDM, but
not M2 iPSDM, had this pathway enriched in response to infection; as a result, only infected M1 iPSDM, and not infected M1 MDM, demonstrated an enrichment of this pathway compared to infected M2 cells. As discussed in Chapter 4, tryptophan catabolism and IDO enhance *Salmonella* killing\(^{337}\) through some combination of nutrient restriction, and immunomodulatory properties of Tryptophan catabolites\(^{330,337}\), and tryptophan catabolites kynurenine and quinolate\(^{330}\) are increased in patients with typhoid fever. The activation of the tryptophan catabolism pathway in uninfected M1 macrophages in both iPSDM and MDM is consistent with the proposition\(^{339}\) that M1 macrophages represent a metabolically non-permissive environment for *Salmonella* replication.

**Figure 5.14:** Comparison of enrichment in metabolic pathways in iPSDM and MDM. Dots represent pathways that were enriched in both iPSDM and MDM (orange), iPSDM only, or MDM only, for each indicated comparison (in columns) between cell types. For x-axis abbreviations, “un” means “uninfected”, “inf” means “infected”, and “inter” or “Interaction” refers to the relative polarization effect (the difference between the response to infection – infected/uninfected – in different polarization types). The shown pathways contain 612 unique genes.

KYNU, kynurenine, and quinolate are part of the pathway by which tryptophan is converted to NAD\(^+\)\(^{329,330}\), linking tryptophan catabolism to two other enriched pathways that are involved in NAD\(^+\) production\(^{428}\): Nicotinate metabolism, and Nicotinate salvaging. iPSDM and
MDM showed differential expression of genes in Nicotinamide salvaging such as NAMPT in uninfected M1 macrophages, and in M2 cells in response to infection; MDM also showed differential expression in genes in Nicotinate metabolism in uninfected M1 and in M2 in response to infection (a primed activation pattern) and in Nicotinamide salvaging in M1 macrophages in response to infection. These observations are consistent with a role for NAD\(^+\) in *Salmonella* infection. As noted in Chapter 4, NAD\(^+\) promotes monocyte and neutrophil mobilization, is necessary for autophagy, and protects macrophages from damage due to oxidative killing of bacteria\(^{330,331}\). It is also involved in the regulation of inflammation\(^{333,334}\), and since *Salmonella* imports nicotinamide salvaging intermediates to produce NAD\(^{335,336}\), the pathway could also affect *Salmonella*’s access to this important nutrient.

The final metabolic pathways with relevance to *Salmonella* infection involve glycosaminoglycans (GAGs): a group of complex linear polysaccharides including heparin sulfate (HS), dermatan sulfates, and hyaluronan. iPSDM and MDM both had these pathways enriched, but in different ways. Dermatin sulfate biosynthesis was enriched in uninfected M1 iPSDM, but not uninfected M1 MDM. Conversely, in response to infection, MDM, but not iPSDM, showed enrichment in HS-GAG biosynthesis and glycosaminoglycan metabolism. Altered expression of GAGs such as heparin sulfate/HS and dermatan sulfates at the cell membrane has been associated with macrophage polarization\(^{429}\), and *Salmonella* binds to GAGs during the process of attachment and invasion\(^{430}\). M2 MDM were observed to have a higher rate of *Salmonella* association at the 0-hour time point (Figure 3.5b), in addition to lower bacterial killing (Figure 3.5c); differences in GAGs could contribute to this higher rate of association.

Hyaluronan is a particularly interesting GAG where host defense is concerned. *Salmonella* can bind to hyaluronan, possibly contributing to invasion\(^{431}\). Further, high molecular weight hyaluronan has been associated with the suppression of inflammatory responses\(^{432,433}\) and of M1 polarization\(^{434}\). While reports of low molecular weight hyaluronan inducing inflammation are likely due to contamination with endotoxin\(^{432}\), low molecular weight hyaluronan does induce expression of host defense peptide β-defensin and protect epithelial cells from *Salmonella* infection\(^{435}\). Hyaluronan metabolism was enriched in response to infection in M2 macrophages for both cell types. iPSDM, but not MDM, additionally showed enrichment in hyaluronan metabolism in M1 compared to M2 macrophages, and in M1 macrophages in response to infection; upregulated genes include HAS1, HAS2, and HAS3.
These results indicated that while metabolic responses overall may differ, pathways with immunologic relevance are similarly regulated between MDM and iPSDM. The observed differences in metabolic pathways may result from the fact that iPSDM are still capable of expanding (personal communication, R.E.W. Hancock) while MDM are not, which would be expected to result in metabolic differences.

Similarly to previous studies in *Chlamydia*\(^{248}\), several extracellular matrix organization pathways were found to be differentially expressed in iPSDM (Figure 5.15), but only integrin cell surface interactions were enriched in uninfected and infected M1 macrophages in both cell types.

**Figure 5.15: Comparison of enrichment in extracellular matrix organization pathways in iPSDM and MDM.** Dots represent pathways that were enriched in both iPSDM and MDM (orange), iPSDM only, or MDM only, for each indicated comparison (in columns) between cell types. For x-axis abbreviations, “un” means “uninfected”, “inf” means “infected”, and “inter” or “Interaction” refers to the relative polarization effect (the difference between the response to infection – infected/uninfected – in different polarization types). The shown pathways contain 301 unique genes.
5.3 Discussion

Overall, gene expression in iPSDM was similarly affected by polarization and infection to MDM, though iPSDM appeared to show greater differences between M1 and M2 macrophages than did MDM, possibly due in part to the higher levels of M-CSF used in maturation. The lists of differentially expressed genes showed a statistically significant overlap between iPSDM and MDM for all comparisons between different infection or polarization conditions (Table 5.3). Further, for all comparisons the log fold changes in expression of the set of all genes differentially expressed by iPSDM showed good correlation with the log fold changes for those genes in MDM, as assessed by linear regression, with a mean $R^2$ value of 0.50 (Table 5.5 and Figure 5.6). The analysis of overlap in differentially expressed gene lists and correlation of log fold changes in gene expression between iPSDM and MDM was not greatly affected by the inclusion of genes that were expressed only in iPSDM, but not in MDM. This indicates that iPSDM-only genes do not distort the overall analysis of differentially expressed genes. Thus a study using iPSDM, which would provide a bountiful and continuous source of genetically similar human macrophages, would likely be able to adequately model Salmonella infections and macrophage phenotype even in the absence of MDMs for comparison. An additional advantage of such cells is that iPSDM are genetically malleable enabling knockout and knock-in studies\textsuperscript{248,436}.

Roast enrichment analysis showed similar changes in functionally interesting sets of genes between M1 and M2 macrophages. Genes from the M1 and M2 signatures were upregulated in M1 and M2 macrophages, respectively (Table 5.6 and Table 5.7). In response to infection, M2 cells upregulated the M1 signature (Table 5.6), and both infected M1 and M2 macrophages downregulated the M2 signature (Table 5.7). JAK-STAT pathways showed a primed activation pattern, with M1 macrophages upregulating more genes than were downregulated and M2 macrophages upregulating these genes in response to infection (Table 5.8). Both M1 and M2 macrophages downregulated Salmonella targets in response to infection, but M1 macrophages did so to a greater extent as described by the relative polarization effect (Table 5.9). Similarly, both M1 and M2 macrophages downregulate the set of 183 Salmonella susceptibility genes in response to infection, but infected M1 macrophages show lower expression in this gene set than infected M2 macrophages (Table 5.10). Finally, Salmonella resistance genes showed a primed activation pattern in iPSDM as well as MDM (Table 5.11).

Roast enrichment did not identify any gene sets that were enriched in opposite directions.
between iPSDM and MDM. However, for five tests of a gene set for a given comparison, the gene set was enriched in only one cell type. For four out of five of these cases, this resulted in gene set enrichment trending similarly in both types of macrophages but reaching statistically significant enrichment in only one cell type. For example, in iPSDM, the upregulation of M1 signature genes in M1 macrophages in response to infection did not reach significance (p=0.07), while it did for MDM (p=0.02). Conversely, upregulation of JAK-STAT genes in uninfected M1 iPSDM was significant (p<0.001), while it did not meet the threshold for MDM (p=0.06), the relative polarization effect for JAK-STAT genes was significant in iPSDM (p=0.012) but not in MDM (p=0.11), and a statistically significant downregulation of Salmonella targets was observed for infected M1 iPSDM compared to infected M2 iPSDM (p <0.001), whereas this fell short of the significance cutoff for MDM (p=0.064). In all of these cases, the p-values for the comparison that did not meet significance was very close to the cutoff, with the largest being p=0.11. Thus, these discrepancies more likely reflect the limitations inherent in setting specific cutoffs for p-values, rather than assessing functional differences between iPSDM and MDM in these pathways.

Pathway analysis revealed a similar patterns in enrichment of primed activation pathways in iPSDM and MDM, including interferon pathways, CLR, IL-15, and nicotinamide signaling (Figure 5.7 Figure 5.8, and Figure 5.16), which would, as discussed in Chapter 4, result in M1 macrophages being prepared to mount a defensive response to Salmonella even in absence of contact with the pathogen. iPSDM also showed generally similar enrichment to MDM in inflammasome (Figure 5.9 and Figure 5.10), interleukin (Figure 5.11 and Figure 5.12), and some other innate immune pathways (Figure 5.13) including the Dectin-2 family; Dectin-2 recognizes Salmonella O-antigen. Inflammasome genes upregulated in iPSDM included IL-1A, IL-1B, NLRP3, and CASP1 (caspase-1) (Figure 5.10). These genes are involved in raising an inflammatory response to Salmonella in the cytosol, and deficiencies in caspase-1, and IL-1β result in susceptibility to Salmonella in mice. Interleukins upregulated in iPSDM (Figure 5.12) include IL-15 and IL-12, both of which activate natural killer cells and are known to be significant in defenses against Salmonella, as well as IL-10, IL-4, and IL-13, which also promote M2 macrophage polarization and could be involved in a switch to M2 activation during resolution of inflammation. Similar enriched expression was also observed in tryptophan metabolism, nicotinamide salvaging, and hyaluronan metabolism (Figure 5.14), metabolic pathways with...
These observations increase confidence that iPSDM are a good model for MDM in terms of polarization and *Salmonella* infection.

**Figure 5.16: Summary of changes in primed activation pathways resulting from polarization in infected and uninfected iPSDM.** The colour of the dots indicate the percentage of the differentially expressed genes found in the pathway which were upregulated: pathways that were largely upregulated are red, while pathways that were largely downregulated are green. The size of the dots indicates the significance level of the pathway enrichment, -log_{10}(adjusted p-value), such that larger dots have smaller p-values. “p-adj” refers to the p-value adjusted for multiple testing. Only pathways with an adjusted p-value <0.05 are shown.

However, there was a general trend for iPSDM to enable identification of more of these pathways as enriched in uninfected M1 than in M2 macrophages. For example, uninfected M1 iPSDM upregulated anti-*Salmonella* cytokine IL-12, while in MDM this pathway was upregulated in macrophages in response to infection (Figure 5.11). This type of discrepancy was also observed in the only Roast gene set enrichment test for which a similar trend was not observed in both cell types: a suppression of the 183 susceptibility genes was observed in uninfected M1 iPSDM, but not uninfected M1 MDM (Table 5.10). These observations may reflect the greater separation that was observed between M1 and M2 iPSDM macrophages in the PCA plots (Figure 5.3) and particularly the heatmap (Figure 5.4), suggesting a greater distinction between polarized M1 and M2 macrophages for iPSDM than for MDM. This, in turn, likely results from certain differences that could exist between the way that these macrophages of two different origins respond to...
infection. In particular, iPSDM are known to developmentally resemble, more closely, tissue macrophages. Since tissue macrophages tend to be M2-like in absence of infection, stimulation with IFNγ could result in a higher number of differences in polarized iPSDM than in MDM.

Some differences in pathway expression were also observed. This is consistent with previous comparisons of the response to iPSDM and MDM to LPS and to Chlamydia, which found different expression in iPSDM and MDM in some immunologically relevant pathways including chemokine expression and antigen presentation.

Overall, a greater similarity was observed in immune system pathways than in other types of pathways such as metabolism, and a very strong similarity was observed in the pattern of enrichment for specific gene sets known to be involved in polarization or Salmonella infection. In particular, iPSDM and MDM both showed similar primed expression of a subset of immune pathways in response to infection (Figure 5.7 and Figure 5.8), including upregulation of JAK2, STAT1, and STAT2. In considering these similarities of expression, it is notable that like M1 MDM, M1 iPSDM are resistant to intracellular Salmonella proliferation. While it cannot be ruled out that M1 macrophages from both origins are resistant to Salmonella due to different mechanisms, it is more likely that the shared resistance of M1 macrophages to Salmonella results from elements of the response to polarization and infection that are shared between iPSDM and MDM. Thus, this analysis demonstrates that iPSDM are a good model for MDM, but also provides some additional confidence that the gene expression patterns that were conserved in iPSDM and MDM, including upregulation of inflammasome genes, downregulation of Salmonella targets, and primed activation of JAK-STAT genes and Salmonella resistance genes, are likely to be generally significant in the resistance of M1 macrophages to Salmonella infection.

5.3.1 Limitations and future directions

As many of the bioinformatics methods used are similar to those used in Chapter 4, the limitations and strategies to mitigate those limitations also apply to the work done in this chapter. For this chapter, the use of data obtained in collaboration with another laboratory in comparison with data generated in our lab, could introduce a potential batch effect as well as reflecting modest differences in Salmonella growth state, and depths of sequencing. Together, this variation prevents a direct calculation of changes in gene expression between the iPSDM and MDM data (e.g. in uninfected M1 iPSDM compared to uninfected M1 MDM). In lieu of direct comparisons, I relied
on a thorough comparative analysis of the outputs of these two studies based on changes in expression of specific sets of genes in iPSDM and MDM to enable a characterization of the extent of similarity between these cell types.

The iPSDM dataset did not include M\textsuperscript{EP} macrophages, and it would be of interest to obtain gene expression data from M\textsuperscript{EP} iPSDM in order to establish iPSDM as a useful model for studying sepsis. As a result of my conclusion that iPSDM are a good comparator for M1 and M2 polarization, and data from other lab members (B. Baquir and R.E.W. Hancock, personal communication) indicating that iPSDM can be reprogrammed to give similar endotoxin tolerant cytokine responses, it would be of great interest to use knockout or knock-in iPSDM to test the mechanisms underlying resistance of M1 macrophages that were identified by this study. Thus although beyond the scope to the current study, the genetic malleability of stem cells could be exploited to test whether upregulation of inflammasome genes (particularly NLRP3), downregulation of \textit{Salmonella} targets (e.g. VPS18), and primed activation of JAK-STAT genes, tryptophan and nicotinamide genes (e.g. IDO1 and NAMPT) and \textit{Salmonella} resistance genes (particularly GBPs) are indeed important in \textit{Salmonella} resistance. Based on the thesis work described here, I would expect that the knockout of NLRP3, STAT1/2, JAK2, IDO1, or NAMPT would increase susceptibility of M1 macrophages to \textit{Salmonella}, while a knock-out of VPS18 or a knock-in of GBPs might increase \textit{Salmonella} resistance in M2 macrophages. Based on the lack of NADPH oxidase gene upregulation observed in M1 iPSDM, I would also expect that a knock-in of NADPH oxidase gene CYBB would not increase \textit{Salmonella} resistance in M2 macrophages.
Chapter 6: Investigation of mechanisms for resistance

6.1 Introduction and rationale

In chapters 4 and 5, it was observed that in both monocyte-derived macrophages (MDM) and induced pluripotent stem cell-derived macrophages (iPSDM), M1 macrophages displayed increased expression of several immune genes and pathways in absence of Salmonella. Notably, these genes included key components of the JAK-STAT signaling pathway such as JAK2, STAT1, and STAT2. In the case of MDM, these three genes were only seen to be highly expressed in the Salmonella-resistant M1 macrophages, and not M2 macrophages or M^{EP} macrophages at the susceptible 4-hour time point. M^{EP} macrophages otherwise displayed primed activation of many pathways, including the JAK-STAT gene set as a whole. This upregulation of JAK-STAT signaling genes in uninfected M1 macrophages was paired with an upregulation of JAK-STAT effectors in infected M1 macrophages. This leads me to hypothesize that priming of JAK-STAT signaling pathways as a result of polarization led to more efficient expression of their effectors upon introduction of Salmonella, resulting in Salmonella resistance in M1, but not in M2 or in M^{EP} macrophages.

To test this hypothesis, JAK1/2 inhibitor Ruxolitinib was applied to MDM during the polarization phase of the experiment, suppressing JAK-STAT signalling before but not during infection). Ruxolitinib is used to treat myelofibrosis and may have applications in treating psoriasis and rheumatoid arthritis. While JAK1 and 2 are important participants in IFN\(\gamma\) signaling, inhibition of these genes by Ruxolitinib is not expected to reverse all effects of M1 polarization. There is strong and growing evidence that IFN\(\gamma\) participates in signaling pathways beyond JAK-STAT signaling. In particular, IFN\(\gamma\) can demonstrably act as a signal in a JAK-independent fashion, resulting in PI3K and ERK activation in embryonic fibroblasts. PI3K signalling is involved in regulating macrophage polarization, and can induce an M1 phenotype under conditions such as viral infection.

In addition to JAK-STAT inhibition by Ruxolitinib, inhibition with Parthenolide was also tested. Parthenolide inhibits both NF-\(\kappa\)B signaling and the inflammasome. NF-\(\kappa\)B is a key mediator of the response to TLR ligands such as LPS, and is also involved in transmitting the response to TNF\(\alpha\) and IL-1\(\beta\) and mediating the effect of alarmins. Inhibition of NF-\(\kappa\)B favours M2 polarization. In contrast to JAK-STAT signaling, NF-\(\kappa\)B genes are upregulated primarily in response to Salmonella (Table 4.12 and Figure 4.19), and in all three polarization
types. Thus, inhibition of NF-κB during infection might be expected to reduce *Salmonella* resistance in all three cell types. It is possible that Parthenolide would have a stronger effect on M1 and M\textsuperscript{EP} macrophages due to its inhibitory effect on inflammasomes (which were upregulated in M1 macrophages) and the role of NFκB in responding to alarmins (which were upregulated in M\textsuperscript{EP} macrophages).

Given that the rate of infection of macrophages observed in Chapter 3 was modest relative to other *Salmonella* replication studies\textsuperscript{4}, it was considered possible that I might see an increase in *Salmonella* susceptibility in M2 or M\textsuperscript{EP} macrophages as a result of Ruxolitinib JAK1/2 inhibition or Parthenolide NF-κB inhibition in the event that these pathways were important to infection of these macrophages.

JAK1 and JAK2 inhibition resulted in an increase in bacterial load in M1 macrophages, but no change in bacterial load in M2 or M\textsuperscript{EP} macrophages. The effect of this inhibition on cytokine expression in response to infection indicated that JAK1/2 inhibition does not appear to simply nullify polarization of M1 macrophages. Parthenolide treatment during infection resulted in a decreased bacterial load in M1 macrophages, suggesting a complex role for NF-κB in *Salmonella* infection. Overall these inhibition experiments supported the important role of primed activation of JAK-STAT pathways on resistance of macrophages to Ruxolitinib, and provided interesting information about the role of NF-κB in *Salmonella* infection.

6.2 Results

6.2.1 M1, but not M2 and M\textsuperscript{EP} macrophages, became more *Salmonella* permissive after JAK/STAT inhibition during polarization

When JAK1 and JAK2 were inhibited using Ruxolitinib during the polarization phase, the *Salmonella* load in M1 macrophages increased significantly when compared to the DMSO control (Figure 6.1). An increase in the load in M2 macrophages was observed as a result of the DMSO treatment, but no further increase in load resulted from JAK inhibition, and no effect was observed in M\textsuperscript{EP} macrophages. As a result of this increase in bacterial load in M1 macrophages, the statistically significant difference between M1 and M2 macrophages for DMSO-treated cells (p=0.009) was no longer significant when comparing M1 and M2 macrophages treated with Ruxolitinib (p=0.180). As in Chapter 3, presented data are indexed in order to minimize the effect of donor variation. Raw data are presented in Appendix B (Figures A.6-A.10).
Figure 6.1: Effect of JAK inhibition during polarization on *Salmonella* resistance. Colour and shape reflect polarization states as labelled and are used for emphasis. For each donor, data were expressed relative to CFU in untreated M2 macrophages from the same donor; lines represent the means of 6 biological replicates (BR). 100% reflects a median 3.4x10^7 CFU. Significance was determined relative to the DMSO control for each polarization state. Statistics were calculated by Wilcoxon test, with p-values indicated as * (< 0.05).

JAK inhibition resulted in a decrease in MCP-1 and IL-6 production during the polarization phase in all three macrophage types (Figure 6.2). JAK inhibition also decreased expression of IL-10 production in M2 and M^{EP} macrophages whereas M1 macrophages did not produce significant quantities of this cytokine in response to polarization alone, even in absence of inhibition. No change in TNFα production was observed. IL-1β was not expressed in absence of *Salmonella*; indeed, levels of TNFα, IL-10, and IL-6 were all very low prior to infection (median expression of 462pg/mL or less).
Figure 6.2: Effect of JAK inhibition on cytokine and chemokine expression measured after polarization.  [a] TNFα, [b] IL-6, [c] IL-10, and [d] MCP-1. Colour and shape reflect polarization states as labelled and are used for emphasis. For each donor, data were expressed relative to the expression level in the M1 DMSO control from the same donor. 100% represents a median 220pg/mL TNFα, 462pg/mL IL-6, 68pg/mL IL-10, and 58,900pg/mL MCP-1. Lines represent the median of 6 BR. Statistical significance was determined relative to the DMSO control for each polarization state. Statistics were calculated by Wilcoxon test, with p-values indicated as * (<0.05), **(<0.01).

In M<sup>EP</sup> macrophages, the inhibitor reduced MCP-1 expression in response to *Salmonella* infection (Figure 6.3a), and there was also a trend towards lower production of IL-1β, though this was not significant (Figure 6.3c). In all three cell types the inhibitor reduced TNFα and IL-6 expression due to *Salmonella* (Figure 6.3b and d). There was an increase in the median production of IL-10, particularly for M1 macrophages, but there was substantial donor variability and the difference was not statistically significant (Figure 6.3e). Interestingly, while Ruxolitinib treatment resulted in a decrease in MCP-1 production in infected M2 macrophages, there was a modest increase in MCP-1 production in infected M1 macrophages. This led to a significant difference in MCP-1 production between Ruxolitinib-treated M1 and M2 macrophages (p=0.015).
Figure 6.3: Effect of JAK inhibition on cytokine and chemokine expression measured after infection. [a] MCP-1 [b] IL-1β [c] TNFα [d] IL-6 [e] IL-10. Colour and shape reflect polarization states as labelled and are used for emphasis. For each donor, data were expressed relative to the expression level in the DMSO-treated M1 macrophages from the same donor. 100% represents a median 9,610pg/mL MCP-1, 123pg/mL IL-1β, 42,100pg/mL TNFα, 11,300pg/mL IL-6, 1,400pg/mL IL-10. Lines represent the median of 6 BR, except for the untreated IL-6 and IL-10 samples, which contain 3 BR. Statistics were calculated by Wilcoxon test, with p-values indicated as * (< 0.05), **(<0.01).
6.2.2 M1, but not M2 and M\textsuperscript{EP} macrophages, became less *Salmonella* permissive when NF-κB and caspase-1 were inhibited during infection.

Parthenolide binds and inhibits the IKK complex member IKKβ. Since the IKK complex is responsible for allowing NF-κB to enter the nucleus, Parthenolide has been used in several studies as an NF-κB inhibitor. Parthenolide was added 1 hour in advance of infection, and maintained during *Salmonella* infection. The inhibition of NF-κB using Parthenolide during infection led to a reduced bacterial load in M1 macrophages (Figure 6.4). Due to a small number of replicates, the Wilcox test could not determine statistical significance for this experiment, but the trend was clear, with a median bacterial load in the Parthenolide pretreated M1 macrophages that was 22.4% the level of the DMSO control, and the highest bacterial load for the Parthenolide pretreated sample was still less than the lowest load for the DMSO control. In contrast, no change was observed in M\textsuperscript{EP} macrophages and if anything a slight increase was observed in load for M2 macrophages.

![Figure 6.4: Effect of NF-κB and caspase-1 inhibition during infection on *Salmonella* resistance](image)

Colour and shape reflect polarization states as labelled and are used for emphasis. For each donor, data were expressed relative to CFU in untreated M2 macrophages from the same donor. 100% reflects approximately 7.0x10\textsuperscript{6} CFU. Lines represent the medians of 3 BR.

This reduced bacterial load was observed despite the successful inhibition of cytokine production in M1 macrophages as a result of the Parthenolide pretreatment (Figure 6.5).
Figure 6.5: Effect of NF-κB and caspase-1 inhibition during infection on cytokine and chemokine expression. [a] MCP-1 [b] TNFα [c] IL-1β [d] IL-6 [e] IL-10. Colour and shape reflect polarization states as labelled and are used for emphasis. For each donor, data were expressed relative to the expression level in untreated M1 macrophages from the same donor. 100% represents a median 9,230pg/mL MCP-1, 100,000pg/mL TNFα, 103pg/mL IL-1β, 4490pg/mL IL-6, 361pg/mL IL-10. Lines represent the medians of 3 BR.

MCP-1 production in Parthenolide-treated M1 macrophages was reduced to 52.7% that of the DMSO control (Figure 6.5a), TNFα was reduced to 25.1% (Figure 6.5b), and IL-1β, IL-6, and IL-10 production were almost eliminated (reduced to 7.0%, 5.6%, and 6.2% respectively) (Figure
6.5c-e). M2 macrophages also showed strong inhibition of IL-1\(\beta\) (14.6% of the DMSO control for M2 macrophages) and IL-10 (6.3%), and a moderate inhibition of IL-6 (30.3%). Only a minor effect was observed in M2 macrophages on MCP-1 production (91.1%) and TNF\(\alpha\) production (70.2%). The effect of Parthenolide on M\(^{\text{EP}}\) macrophages was even more modest, with only partial inhibition of IL-1\(\beta\) (27.6%), IL-6 (25.1%), and IL-10 (34.0%), and little inhibition of MCP-1 (66.9%), although the TNF\(\alpha\) production, already reduced by endotoxin priming, was further suppressed by Parthenolide (to 49.8% of the level of DMSO-treated M\(^{\text{EP}}\) macrophages).

The reduced bacterial load in M1 macrophages was accompanied by a moderate increase in cytotoxicity for M1 macrophages treated with Parthenolide, from 8.7% for M1 macrophages that were treated with DMSO, to 20.1% for M1 macrophages treated with Parthenolide (Figure 6.6). This represented a reduction from 91% survival to 80% survival of M1 macrophages. M2 and M\(^{\text{EP}}\) macrophages did not show a similar increase in cytotoxicity upon Parthenolide treatment.

Figure 6.6: Effect of NF-\(\kappa\)B and caspase-1 inhibition during infection on cytotoxicity. Colour and shape reflect polarization states as labelled and are used for emphasis. Lines represent the medians of 3 BR.

6.3 Discussion

As a result of the transcriptional analysis in Chapters 4 and 5, I hypothesized that primed activation of JAK-STAT, i.e. increased expression and signaling occurring specifically before the encounter with \textit{Salmonella}, would result in a more efficient response to infection and thus \textit{Salmonella} resistance. To test this hypothesis, JAK inhibitor Ruxolitinib was applied to macrophages during the polarization phase but not during the infection phase, to suppress any effects of increased JAK-STAT expression occurring before infection but not effects of upregulation of these genes in response to infection. This inhibition produced a statistically
significant increase in the bacterial load in M1, and not in M2 or M\textsuperscript{EP} macrophages (Figure 6.1). This is consistent with the Salmonella resistance observed in M1 macrophages, relative to M2 and M\textsuperscript{EP} macrophages at this time point, resulting at least in part from primed activation of JAK-STAT signalling, prior to Salmonella exposure.

The effect of JAK-STAT inhibition by Ruxolitinib on cytokine expression during the polarization phase resulted in a decrease in MCP-1 and IL-6 expression in all three macrophage types, an increase in TNF\textalpha expression in M2 macrophages, and a decrease in IL-10 production in M2 and M\textsuperscript{EP} macrophages (Figure 6.2). This suppression was partial in M\textsuperscript{EP} macrophages, consistent with the fact that LPS signals partially through JAK2 through production of IFN-\textbeta. Similarly, suppression of cytokine signaling in M2 macrophages likely results from the fact that IL-4 signals partly through JAK1. Lack of a reduction in TNF\textalpha, and of IL-10 expression in M1 macrophages at this stage likely reflects the low baseline levels of expression of these cytokines in absence of Salmonella infection. In response to infection, suppression of Salmonella-induced TNF\textalpha and IL-6 was observed for all three polarization types (Figure 6.3). This was accompanied in some donors with an increase in IL-10 production, which can inhibit TNF\textalpha production, but variability was high.

Interestingly, Ruxolitinib treatment had a divergent effect on MCP-1 production as a result of infection. Co-stimulation with IL-4 is known to reduce LPS-induced MCP-1 production in monoblasts, and in M2 macrophages the application of Ruxolitinib also led to a statistically significant decrease in MCP-1 production (Figure 6.3a). In contrast, IFN\textgamma leads to a modest induction of MCP-1, and despite JAK inhibition during polarization, an increased MCP-1 response was observed in M1 macrophages (polarized with IFN\textgamma) as a result of Ruxolitinib treatment (Figure 6.3a). This led to a significant difference in MCP-1 production between the Ruxolitinib-treated M1 and M2 macrophages. The increased expression of MCP-1 in Ruxolitinib-treated M1 (IFN\textgamma-polarized) macrophages indicates an effect of IFN\textgamma stimulation on polarization that is not reversed by JAK inhibition; the M1 cells are not becoming in all respects more M2-like. This apparently JAK-independent effect on MCP-1 could involve a JAK-independent effect of PI3K signaling, or pathways such as MyD88/NF-\textkappaB, since MCP-1 can be induced through multiple pathways, including MyD88/NF-\textkappaB, during infection with Listeria. MCP-1 is important in recruiting macrophages to tissues during Salmonella infection, and its deficiency in mice results in susceptibility to salmonellosis. Osteoblasts secrete MCP-1 in response to
Salmonella infection\textsuperscript{452}, and Salmonella-infected macrophages produce exosomes which induce secretion of cytokines including MCP-1 in naive macrophages\textsuperscript{453}.

Ruxolitinib treatment prior to infection is known to exacerbate sepsis\textsuperscript{454,455}. However, in this experiment treatment of M\textsuperscript{EP} macrophages with Ruxolitinib prior to infection did not result in further sensitivity to Salmonella replication (Figure 6.1). This may suggest that the effect of Ruxolitinib on sepsis risk is not mediated directly by macrophages. However, it is also possible that the effect is not captured at the time point measured. Given that M\textsuperscript{EP} macrophages are resistant to Salmonella at the 2 hour time point, it would be interesting to see if Ruxolitinib reduced this resistance. This would be consistent with the primed activation of JAK-STAT genes in uninfected M\textsuperscript{EP} macrophages, although as noted JAK2 itself was only upregulated in M1 macrophages.

Interestingly, the DMSO vehicle treatment resulted in an increase in bacterial load in M2 macrophages, but did not produce a statistically significant effect in either M1 or M\textsuperscript{EP} macrophages. While DMSO is known to repress pro-inflammatory cytokine production in macrophages\textsuperscript{456}, no statistically significant effect was observed on cytokine production as a result of DMSO at the concentration used (Figure 6.2 and Figure 6.3).

NF-κB genes, unlike JAK-STAT, were largely upregulated in response to infection in all three types of macrophages (Table 4.12 and Figure 4.19). Thus, to test the expectation that NF-κB inhibition would similarly affect Salmonella resistance in all three types of macrophages, I applied the inhibitor Parthenolide during the infection. Only a partial effect of inhibition was observed when the inhibitor was added simultaneously with infection (Appendix E, Figure A.13), so in accordance with previous protocols using Parthenolide to inhibit inflammatory responses to infection\textsuperscript{444,445,447}, the inhibitor was added 1 hour prior to infection.

I did not anticipate that Parthenolide would sharply decrease the bacterial load in M1, but not in M\textsuperscript{EP} and M2 macrophages, when pre-administered 1 hour before infection, and maintained through the infection (Figure 6.4). Given the broad effects of Parthenolide on immune signaling, through both NF-κB inhibition and inhibition of the inflammasome, as well as the observed reductions in production of inflammatory cytokines (Figure 6.5) it was anticipated that Parthenolide would increase bacterial load in all three types of macrophages. There are several explanations for this observation. First, the increased cytotoxicity in M1 macrophages (Figure 6.6) might have impacted on the reduced bacterial load in these macrophages. NF-κB promotes survival in the presence of otherwise pro-apoptotic stimuli\textsuperscript{457}. It is possible that without this effect, M1

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macrophages would be particularly susceptible to death in response to *Salmonella* invasion.

The measured increase in cytotoxicity from ~10% to 20%, or reduction in survival from 90% to 80%, was not in itself enough to account for the roughly 3-fold reduction in bacterial load. However, the experimental method consisted of a 30-minute initial exposure to *Salmonella* followed by a replacement of the medium with media containing gentamicin to remove and kill extracellular bacteria. If the invasion of *Salmonella* resulted in increased M1 macrophage death in that period, then a large number of *Salmonella* may have become unable to initiate intracellular infection and would not persist to be measured in the bacterial load counts. Further, any LDH released by M1 macrophages prior to the media replacement would not have been taken into account in the cytotoxicity assay, indicating it may be an underestimate of actual cytotoxicity.

Other alternatives exist. Since NFκB has pleiotropic impacts on the function of cells, it is possible that it has some effect that is deleterious to *Salmonella* resistance in the context of M1 macrophages, with the result that inhibition would be protective in these macrophages. Intriguingly, *Salmonella* itself inhibits NFκB responses through Type III secretion effectors, and some of these effectors target specific arms of the NFκB response, such as TNFα signaling. This would tend to limit the effects on host defense of NFκB inhibition by Parthenolide. If not all effects of NFκB are equally inhibited by *Salmonella*, this could result in a predominately harmful NFκB response in M1 macrophages.

Conversely, Parthenolide has complex effects, including inhibition of the inflammasome and stabilization of microtubules. These complex effects might have led to reduced *Salmonella* invasion, reducing the bacterial load even if NFκB inhibition would otherwise decrease *Salmonella* killing. Indeed, *Salmonella* promotes its own uptake by inducing membrane ruffling and micropinocytosis, and the stabilization of microtubules would reduce bacterial invasion through this method. With respect to this mechanism, differences in the effects of Parthenolide between M1, M2, and M<sup>EP</sup> macrophages could result from differences in expression of genes involved in cytoskeletal manipulation as previously observed in the case of genes involved in formation of the *Salmonella*-containing vesicle (Table 4.5). Overall, the M1-specific reduction in bacterial load upon Parthenolide treatment presents intriguing information regarding the mechanisms of *Salmonella* infection, but this requires further investigation to confirm which of these processes are involved.
6.3.1 Limitations and future directions

The statistical power in these experiments was modest to low as a result of the small number of biological replicates (six for the Ruxolitinib experiment, and three for Parthenolide). Indeed, the Parthenolide experiments lacked the power to determine any statistical significance below the p=0.05 significance threshold, despite a very clear trend. Additional replicates would improve the reliability of the results. In addition, the effect of Ruxolitinib was measured solely by assessing cytokine expression. In order to more directly confirm JAK1/2 inhibition, it would be necessary to measure STAT phosphorylation. Further, use of additional JAK inhibitors would rule out possible off-target effects of Ruxolitinib and strengthen the evidence indicating the importance of primed activation of the JAK-STAT pathway.

To more thoroughly explore the effect of Ruxolitinib on infection, additional experiments could be performed. In particular, primed activation of JAK-STAT was also observed in M Ep macrophages (Figure 4.8 and Table 4.3). Thus, it would be valuable to measure bacterial load after 2 hours of infection, when M Ep were resistant to Salmonella, in order to determine if Ruxolitinib reduced this resistance similarly to its effects on M1 macrophages. In addition, it has not been determined which of the effectors downstream of JAK were responsible for producing resistance in M1 macrophages. Measuring the effect of JAK-STAT inhibition on transcription in M1 macrophages would provide additional information regarding the role of this pathway in Salmonella resistance.

Further experiments could also be performed in order to assess the mechanism by which Parthenolide increases Salmonella resistance. The first step would be to examine the dynamics of Salmonella infection under Parthenolide inhibition at earlier time points, preferably using live cell microscopy to visualize the fate of individual macrophages upon association with GFP-Salmonella. Measurement of cell death and Salmonella load beginning immediately after the 30-minute “infection phase” would distinguish between rapid death of infected Parthenolide-treated cells, inhibition of Salmonella entry, or enhanced killing. One possible mechanism is through Salmonella targeting of the NF-κB response via Type III secretion effectors resulting in a predominantly harmful effect of NF-κB signalling within M1 macrophages. This scenario could be tested by determining whether Parthenolide inhibition had a different effect on resistance of polarized macrophages to Salmonella that lack these NF-κB-targeting effectors.
Chapter 7: Conclusion

7.1 Main findings

To date, studies on the effect of endotoxin tolerance on clearance of various pathogens have produced contradictory results\(^{207,211,214,226}\). While polarization is known to have an effect on the resistance of macrophage to *Salmonella* infection\(^{3,4}\), the effect of endotoxin priming on the resistance of monocyte-derived macrophages (MDM) was undetermined, and a whole-transcriptome comparison of the response of differently polarized MDM to infection had not been performed. This research provided the first determination of *Salmonella* resistance and susceptibility in endotoxin primed (M\(^E\)P) MDM. It also provided a comprehensive picture of differing transcriptional responses in M\(^E\)P, M1 and M2 macrophages to *Salmonella* infection, identifying likely mechanisms for differing resistance to the pathogen. Similar responses were also observed in induced pluripotent stem-cell derived macrophages (iPSDM). This transcriptomic comparison provided evidence that iPSDM are good models of MDM for polarization and *Salmonella* infections, supporting the use of iPSDM as a rich source of genetically similar macrophages that can be manipulated to create knock-out and knock-in macrophages\(^{248,436}\). Finally, based on a hypothesis generated as a result of transcriptional analysis, this thesis provided evidence that JAK-STAT signaling is mechanistically important for the response of M1 macrophages to *Salmonella* infection, but not for M2 or for M\(^E\)P macrophages at the 4-hour time point. An interesting M1-specific effect of Parthenolide was also observed, but further research is necessary to confirm the mechanism of this effect.

7.1.1 M\(^E\)P macrophages initially controlled *Salmonella* similarly to M1 macrophages, but in the long term were susceptible like M2 macrophages.

Expected polarization and infection was confirmed using cytokine measurements and microscopy. M\(^E\)P macrophages showed a similar level of bacterial internalization and phagocytosis after a 30 minute “infection phase” in which the macrophages were exposed to extracellular *Salmonella*. Since extracellular *Salmonella* was eliminated through washing and gentamicin treatment at the end of this phase, it is referred to as the 0 hour time point for intracellular *Salmonella* infection. M\(^E\)P also showed a similar load to M1 macrophages at the 2 hour time point, which reflects greater bacterial killing in these first two hours than observed with M2 macrophages. However, at the 4 hour and 24 hour time points, M\(^E\)P macrophages had a similar bacterial load to M2 macrophages reflecting a long-term failure to kill intracellular *Salmonella*. 
This finding has interesting implications when considering that, while the risk of mortality in early stages of sepsis derives from high inflammation and organ failure, mortality in later stages results from immune suppression\textsuperscript{201}. Specifically, the lack of inflammatory responses by M\textsuperscript{EP} macrophages would be protective in early sepsis, and the M1-like early killing reflects the possibility that some antimicrobial defenses might have been preserved. However, the inability to control replication of \textit{Salmonella} beyond 4 hours of exposure was consistent with the profound and deadly immunosuppression of late-stage sepsis.

7.1.2 \textbf{Transcriptional analysis reveals genes likely involved in M1 macrophage \textit{Salmonella} resistance, and the transition in M\textsuperscript{EP} macrophages from resistance to susceptibility.}

Gene set analysis using Roast confirmed the expression of M1, M2, and endotoxin tolerance signatures in these macrophages. DESeq2 and Sigora/InnateDB were used to identify differentially expressed genes, and enriched pathways containing high numbers of differentially expressed genes. Uninfected M1 and M\textsuperscript{EP} macrophages had a high baseline expression of key immune genes and pathways, referred to as “primed activation.” These primed genes include JAK2, STAT1, and STAT2 in M1 macrophages, and genes involved in NAD\textsuperscript{+} production. While nicotinamide metabolism is upregulated in response to short-term IFN\textalpha treatment\textsuperscript{462}, NAD\textsuperscript{+} has not yet been associated with M1 macrophage polarization, making this a novel finding. Since primed genes represent immune defenses present in absence of \textit{Salmonella}, they may enable a more rapid response to infection upon encountering the pathogen, and thereby contribute to resistance. In contrast, M2 macrophages only upregulated these genes in response to infection, which may represent a slower immune response to invading \textit{Salmonella}, resulting in susceptibility to infection. This would then permit \textit{Salmonella} to grow intracellularly to a point where it could potentially overwhelm immune defences.

Additional pathways relating to \textit{Salmonella} resistance were observed to differ between M1 and M\textsuperscript{EP} macrophages. These differences may explain why M1 macrophages showed sustained resistance to \textit{Salmonella} while M\textsuperscript{EP} macrophages eventually failed to control \textit{Salmonella}. These pathways included: upregulation of inflammasome pathways, downregulation of genes involved in stabilization of the \textit{Salmonella}-containing vacuole, and upregulation of \textit{Salmonella} resistance genes such as guanylate binding proteins and NADPH oxidase. While uninfected M\textsuperscript{EP} macrophages showed M1-like expression of these resistance genes, expression was reduced in infected macrophages; this is consistent with early \textit{Salmonella} resistance followed by a reduction
in antimicrobial activity resulting from a tolerant response to infection. This transcriptional analysis of M\textsuperscript{EP} thus provides novel mechanistic insights in addition to the finding that these cells transition from Salmonella resistance to susceptibility.

Interestingly, while gene expression was generally down-regulated in infected M\textsuperscript{EP} macrophages compared to M1 macrophages, the pathway “chemokine receptors binding chemokines” was observed to be upregulated. This finding may explain why this study determined that M\textsuperscript{EP} macrophages are ultimately susceptible to Salmonella, while a protective effect was previously observed as a result of endotoxin priming in mice \textit{in vivo}\textsuperscript{207}. The previous study attributed Salmonella protection to improved leukocyte and neutrophil recruitment\textsuperscript{207}, which is consistent with increased chemokine production by M\textsuperscript{EP} macrophages. Thus, M\textsuperscript{EP} macrophages could contribute in some way to defense against Salmonella even if they are permissive to Salmonella replication.

7.1.3 iPSDM gene expression was largely similar to MDM in response to polarization and Salmonella infection, and included key immune genes and pathways.

iPSDM gene expression correlated well with MDM gene expression by several measures. A statistically significant overlap was detected in the lists of differentially expressed genes for iPSDM and MDM for all comparisons between infection and polarization types, and a good correlation, based on R\textsuperscript{2} values, between log fold change of expression was also observed when performing linear regression on all genes that were differentially expressed in iPSDM and MDM. Enrichment in gene sets and pathways involved in infection was also similar, including those defining differences in expression of M1/M2 signatures, Salmonella targets, and Salmonella resistance genes, as well as priming of JAK/STAT genes and NAD\textsuperscript{+} production genes. This comparison between iPSDM and MDM provides validation of the use of iPSDM both as a model for macrophage polarization, and a model for Salmonella infection.

The differences that were identified in gene and pathway expression were comparable with those reported in previous studies\textsuperscript{248,258}. The observed differences in metabolism, and the identification of stronger differences between uninfected M1 and M2 iPSDM than M1 and M2 MDM, may reflect findings that iPSDM ontologically and functionally resemble tissue macrophages\textsuperscript{247,249}, which have distinct transcriptomes\textsuperscript{138,149}.

7.1.4 Inhibition of JAK1/2 confirms significance of JAK-STAT signaling in resistance of
M1 to *Salmonella*, while NF-κB inhibition with Parthenolide suggests a complex response.

M1, but not M2 and $M^{EP}$ macrophages, became more *Salmonella* permissive after JAK-STAT inhibition during polarization. This supported the hypothesis, developed from transcriptomic analysis, that a primed activation of JAK-STAT was important for the resistance of M1 macrophages to *Salmonella*, but did not have a significant effect on defenses for M2 or $M^{EP}$ macrophages. An upregulation of MCP-1 in JAK-inhibited M1 macrophages, paired with a downregulation of that chemokine in JAK-inhibited M2 macrophages, indicated that the effect of JAK inhibition was not simply to make the M1 macrophages more M2-like, and provided novel evidence of JAK-independent signaling in response to IFNγ.

In contrast, M1, but not M2 and $M^{EP}$ macrophages, became increasingly resistant when NF-κB was inhibited using Parthenolide 1 hour prior to, and during infection, as measured by decreased recovery of live *Salmonella* from lysed macrophages. This novel finding of an M1-specific protective effect of Parthenolide is interesting given the significant role of NF-κB in mobilizing immune defenses. Possible reasons for this effect included: (1) reduced invasion resulting from microtubule stabilization and/or some other effect of NF-κB on bacterial uptake\textsuperscript{332,461,463}, (2) a putative harmful effect of NF-κB signaling that becomes specifically relevant during M1 polarization\textsuperscript{464,465}, (3) inhibition of inflammasomes\textsuperscript{442,466}, (4) targeting of NF-κB by *Salmonella* mitigates any harmful effects of its inhibition\textsuperscript{18–22}, and/or (5) rapid host cell death during the 30 minute “infection phase” of the experiment resulting in a failure of *Salmonella* to colonize M1 macrophages\textsuperscript{467}. Further research differentiating between these possibilities would provide further insights into the dynamics of *Salmonella* infection in M1 macrophages, and possibly indicate a role for Parthenolide, or a similarly acting agent, as a treatment for *Salmonella* infection.

### 7.2 Strengths and limitations

The most significant limitation of my experiments is that they reflect a simplified *in vitro* model, involving a single cell type and polarization with individual molecules IFNγ, IL-4, or LPS. *In vivo*, macrophages would be exposed to multiple cytokines and bacterial molecules with differing or competing effects on activation resulting in non-binary activation states. For example, IFNγ and IL-4 each partially repress the transcriptional programs of the other cytokine, resulting in intermediate states\textsuperscript{468}, stimulation with cytokines such as IFNγ+TNFα or IL-10 can produce variations on the M1 or M2 transcriptional programs, stimulation with IFNγ and LPS together can
create a more distinctly M1 state of polarization than IFNγ alone\textsuperscript{178}, and stimulation with substances such as free fatty acids can lead to activation states outside the M1/M2 spectrum\textsuperscript{178}. During 	extit{Salmonella} infection \textit{in vivo}, macrophages might be exposed to a combination of IFNγ (produced by neutrophils, NK cells, NKT cells, or ILC3s)\textsuperscript{30–35}, TNFα (produced by neutrophils as well as other activated macrophages)\textsuperscript{33}, and various alarmins and danger signals released through necroptotic or pyroptotic cell death\textsuperscript{54}. Nutrient levels \textit{in vivo} might also affect polarization compared to growth media, as glucose levels are known to have an effect on inflammatory cytokine production\textsuperscript{469}. Thus, while the macrophages seen \textit{in vivo} are expected to be M1- or M2-like\textsuperscript{173}, their responses will not be identical to those of macrophages stimulated with only a single cytokine. Nevertheless the types of macrophage models utilized here are common surrogates for \textit{in vivo} behaviour of these cells types.

A second limitation is that macrophage resistance and transcriptional activation was assessed at the population level. Each sample consisted of a large number of cells, of which some may be effectively killing intracellular 	extit{Salmonella}, while others allow 	extit{Salmonella} to proliferate, and others have not become invaded by 	extit{Salmonella} at all. Polarization with IFNγ, IL4, and LPS shifts the distribution of the population’s resistance and gene expression patterns, but the methods used are unable to detect heterogeneity within the population. It was not possible, for example, to detect any differences in gene expression between IFNγ-stimulated M1 macrophages which resist and do not resist 	extit{Salmonella} infection.

Interestingly, single-cell RNA-Seq in murine bone marrow derived macrophages (BMDM) shows that macrophages containing proliferating 	extit{Salmonella} have a more M2-like profile than those restricting 	extit{Salmonella}, or those that have not been invaded by 	extit{Salmonella}. The researchers attribute this to successful macrophage reprogramming by the proliferating 	extit{Salmonella}\textsuperscript{3}. However, this reprogramming occurred in macrophages that had not been activated by any cytokine stimulation prior to infection. A single-cell RNA-Seq analysis of polarized macrophages would indicate the extent to which 	extit{Salmonella} is able to reprogram cells that have already been polarized to an M1 state, as opposed to preventing M1 activation in unpolarised macrophages. Such an analysis would also allow comparison of macrophages within each polarization state that are more or less effective in restricting 	extit{Salmonella}, potentially revealing additional relevant pathways that may not be detected at the level of the entire M1 vs M2 vs M\textsuperscript{EP} macrophage populations.

The bioinformatics methods used in the RNA-Seq analysis have their own strengths and
limitations, and this experiment used several methods in order to obtain a well-rounded analysis, as described in detail in Chapter 4. The observations of patterns such as primed activation at the levels of pathway enrichment, gene set enrichment, and differential expression of specific genes of interest within these pathways and sets increases overall confidence that these transcriptional changes are robust to the methodology used.

Overall, the greatest strength of this work is its representation of a complete transcriptomic picture of gene expression in M1, M2, and M^{EP} macrophages, both MDM and iPSDM, that are both infected with *Salmonella* and uninfected. This allows for the detection of patterns involving multidimensional comparisons between infected and uninfected cells of different polarizations. For example, the primed activation pattern in M1 and M^{EP} macrophages was characterized not only by the activation of pathways in uninfected cells, but also by the activation of these pathways in response to infection, and reduced differences between polarized cells when infected. Similarly, the upregulation of the chemokine pathway in M^{EP} macrophages was notable largely because it was observed in infected as well as uninfected M^{EP}, and the expression in *Salmonella* resistance genes in uninfected, but not infected M^{EP} is consistent with the existence of a mechanism promoting the loss of *Salmonella* resistance. Thus, the picture produced by this research provides a greater degree of context than a study examining gene expression only in response to polarization, or only in response to infection.

### 7.3 Applications and future directions

By improving our understanding of the mechanisms of differing *Salmonella* resistance in polarized and endotoxin primed macrophages, this work contributes to the development of macrophage-targeting immunomodulatory treatments for *Salmonella* infection and sepsis. When considering the fact that tissue resident macrophages are M2-like\(^{145}\), the first macrophages that encounter *Salmonella* are likely susceptible to infection. M1 activation depends on the production of IFNγ, which in primary *Salmonella* infection is derived from neutrophils\(^{32}\), natural killer cells\(^{30,31}\), or ILC3s\(^{34}\). *Salmonella*’s tendency in systemic infection to spread to areas that have not yet activated an inflammatory response\(^2\), as well as its ability to suppress antimicrobial activation\(^3,18–21,23\), would limit the chances that this pathogen would encounter *Salmonella*-resistant M1 macrophages. A better understanding of the mechanisms involved in M1 resistance to *Salmonella* infection could lead to immunomodulatory treatments capable of activating these functions.
Immunomodulatory treatments are most likely to be relevant in a timeframe of several days to a week into an infection, at which point *Salmonella* have transitioned to intracellular infection\(^2,29\) and persister cells complicate antibiotic treatment\(^{470}\). Moreover, endotoxin tolerance can persist for weeks after the initial exposure to LPS\(^{206,208}\) and the majority of sepsis patients currently die in the immunosuppressive phase, 3 days to several weeks after onset of sepsis\(^2^{201}\). Treatments targeting mechanisms that are not inherently inflammatory, for example changes in gene expression resulting in destabilization of the *Salmonella*-containing vesicle, would be particularly beneficial as chronic inflammation resulting from *Salmonella* infection is associated with carcinogenesis\(^471\). There also exists a potential to extend these findings to other pathogens capable of surviving intracellularly, such as *Neisseria meningitides*; formation of a “*Neisseria*-containing vacuole” is linked with Lamp-1\(^{472}\), which is recruited to the SCV by *Salmonella*\(^473\). Further work is required to realize therapeutic benefits.

The most immediate extension of this research would be to more fully characterize the effects of JAK-STAT and NF-κB inhibition, as described in 6.3.1. While the results presented here confirm that signalling through JAK1/2 prior to *Salmonella* infection is important in enabling resistance of M1 macrophages to *Salmonella*, measuring bacterial load after 2 hours and observing the effect of JAK-STAT inhibition on transcription in M1 macrophages would provide additional information regarding the role of this pathway in *Salmonella* resistance. It is also of interest to confirm and investigate the potential mechanisms by which NF-κB inhibition with Parthenolide leads to *Salmonella* resistance in M1 macrophages. Depending on the results, *in vivo* investigation of the effects of Partheolide on *Salmonella* infection in mice may be warranted. Notably, given the importance of macrophages in spreading systemic *Salmonella* infection\(^2\), even without a beneficial effect on macrophage killing, restricting *Salmonella* entry into macrophages might be protective *in vivo* in the event that neutrophils or antibiotics are able to clear the extracellular *Salmonella*.

Secondly, the analysis identified additional possible mechanisms involved in resistance to *Salmonella* that have yet to be investigated. Of these, I believe that the downregulation of *Salmonella* effectors is of most interest for further confirmation. The expression of SCV-disrupting Guanylate-binding proteins has been previously linked to IFNγ stimulation of macrophages\(^{11,233}\). However, there has also been, to my knowledge, no study reporting differential expression of SCV-promoting *Salmonella* effectors in the macrophage response to polarization or infection. Given the importance of SCV formation to the process of *Salmonella* persistence in macrophages, it would
be of interest to see if actin manipulation by *Salmonella* is impaired in M1 macrophages, and whether this impairment can be induced in differently polarized macrophages.

Finally, experiments more closely modelling the conditions in the gut could be performed. One such experiment could involve investigating macrophage activation by commensal bacteria or probiotics. Commensal bacteria represent a common stimulus found in the intestine, and are known to modulate immune responses and to have a role in controlling *Salmonella* infection. It would be of interest to observe the effects of a 24-hour prestimulation with a probiotic, with or without additional IFNγ or IL-4, in order to determine how this impacts *Salmonella* resistance and expression of significant genes compared to simple polarization and endotoxin tolerance.

A second experiment that would more closely model in vivo conditions would be the use of intestinal tissue macrophages, or of induced pluripotent stem cells (iPSC) that have been induced to more closely resemble intestinal tissue macrophages. Intestinal macrophages are known to have a microbicidal but non-inflammatory activation, specialized for keeping commensal bacteria from escaping the gut. Since this represents a hybrid M1/M2 activation, it would be of interest to examine *Salmonella* replication in such tissue macrophages. Given the successful differentiation of human stem cells into microglia in organ-like culture, it is likely that stem cell derived macrophages resembling intestinal tissue macrophages could be produced. Moreover, stem-cell derived intestinal organoids have been produced, and co-cultures of intestinal epithelial organoid monolayers with MDM have been used to study host-pathogen interactions in the gut. Together with my findings that iPSDM are a good model of polarization and *Salmonella* infection, it is likely that a co-culture of stem-cell derived intestinal-like macrophages with intestinal organoids would provide an excellent model of *Salmonella* infection in the gut.

In addition to their research applications, iPSDM are of therapeutic interest, since stem cell derived macrophages have been shown to integrate into liver, brain, and lung tissue and relieve disease symptoms. Transfusion of activated *Salmonella*-resistant stem cell-derived macrophages could be used to control infection under extreme situations such as chronic or relapsing infection.

The research presented in this thesis provides novel insights into the transcriptional changes occurring in differently polarized macrophages, including endotoxin primed macrophages, in response to *Salmonella* infection. Extending this analysis could lead to improved treatments for *Salmonella* infection and sepsis, either through immunomodulatory treatments to
improve *Salmonella* resistance or by transfusion of stem-cell derived macrophages with a better ability to resist infection. Such treatments would be particularly important in cases of antibiotic resistance.
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Appendices

Appendix A  Confirmation of macrophage markers by flow cytometry

Figure A.1 Verification of macrophage surface markers CD14, CD16, and CD64 using flow cytometry.
Appendix B  Raw data for ELISA and CFU measurements

Figure A.2: Production of cytokines in polarized MDM uninfected or infected with S. Typhimurium for 4 hours. Colour indicates polarization as labelled and is used for emphasis. Shown are 4 biological replicates for uninfected cells, and for infected cells 16 biological repeats (BR) for TNFα, 13 BR IL-6 and IL-10, 9 BR IL-1β, 6 BR MCP-1. Statistics were calculated by paired Wilcoxon test, with p-values indicated as * (< 0.05), ** (<0.01), *** (<0.001).

Figure A.3: Production of cytokines in polarized MDM infected with S. Typhimurium at MOI 20 for 24 hours. Data are from 8 biological repeats and expressed in pg/mL. Statistics were calculated by paired Wilcoxon test, with p-values indicated as * (< 0.05), ** (<0.01), *** (<0.001).
Figure A.4: Intracellular bacteria load in polarized cells after 4 hours intracellular infection. Lines represent the means of 16 BR. Statistics were calculated by paired Wilcoxon test. P-values indicated as * (<0.05), **(<0.01), ***(<0.001).

Figure A.5: Intracellular bacterial load in polarized cells at 0, 2, and 24 hours after infection. Data represent 8 biological repeats. Statistics were calculated by paired Wilcoxon test, with p-values indicated as * (<0.05), **(<0.01), ***(<0.001). [a] CFU at all three time points; lines represent the mean. [b] Bacterial load at 2 hours after infection, expressed as % bacterial survival. [c] Bacterial load at 24 hours after infection, expressed as % bacterial survival.
Figure A.6: Effect of JAK inhibition during polarization on *Salmonella* resistance. Lines represent the means of 6 biological replicates (BR). Statistics were calculated by paired Wilcoxon test, with p-values indicated as * (< 0.05).

Figure A.7: Effect of JAK inhibition on cytokine and chemokine expression measured after polarization. [a] TNFα, [b] MCP-1, [c] IL-6, and [d] IL-10. Colour represents polarization as labelled and is used for emphasis. Lines represent the mean of 6 BR. Statistics were calculated by paired Wilcoxon test, with p-values indicated as * (< 0.05).
Figure A.8: Effect of JAK inhibition on cytokine and chemokine expression measured after infection. [a] MCP-1 [b] IL-1β [c] TNFα [d] IL-6 [e] IL-10. Colour reflects polarization as labelled and is used for emphasis. Lines represent the mean of 6 BR. Statistics were calculated by paired Wilcoxon test, with p-values indicated as * (< 0.05).
Figure A.9: Effect of NF-κB and caspase-1 inhibition during infection on *Salmonella* resistance. Colour reflects polarization as labelled and is used for emphasis. Lines represent the means of 3 BR.
Figure A.10: Effect of NF-κB and caspase-1 inhibition during infection on cytokine and chemokine expression. [a] MCP-1 [b] TNFα [c] IL-1β [d] IL-6 [e] IL-10. Colour reflects polarization as labelled and is used for emphasis.
Figure A.11: Quantification of *Salmonella* load within MDM as visualized by microscopy. For each donor, data are expressed relative to the value for M2 macrophages for the same donor. Lines represent the mean of 6 biological replicates.
Appendix D  Gene signatures and sets used for enrichment analysis

Table A.1: Genes for the M1, M2, and Endotoxin signatures, the JAK-STAT pathway, and the sets of Salmonella protective genes and effectors used for roast analysis.

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<th>Salmonella Effectors</th>
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* gene has multiple haplotypes present in the signature
Figure A.13: Effect of timing of Parthenolide on inhibition of cytokine expression. [a] MCP-1, [b] TNFa, [c] IL-1b [d] IL-6, [e] IL-10. Colour reflects polarization and is used for emphasis. For each donor, data are expressed relative to the value for untreated M1 macrophages for that donor. X-axis abbreviations used: “Par. Infect” indicates Parthenolide added at the time of infection, while “Par. Adv” indicates Parthenolide added 1 hour in advance of infection and maintained throughout infection.