

Placental infection by *Salmonella* Typhimurium in a murine
model: the role of innate immune mediators in cell death at the fetal-
maternal interface

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Abstract

Maternal tolerance during pregnancy increases the risk of infection with certain intracellular pathogens such as *Salmonella enterica* serovar Typhimurium (S.Tm). Systemic S.Tm infection during pregnancy in normally resistant 129X1/SvJ mice, with a functional natural resistance-associated macrophage protein-1 (*Nramp1*), leads to severe placental infection followed by fetal and maternal death. We hypothesized infection-induced inflammatory trophoblast cell death contributes to adverse pregnancy outcomes. We therefore investigated the kinetics of systemic and oral S.Tm infection in wild-type and gene deficient mice with defects in specific inflammatory pathways. Systemic infection with S.Tm resulted in preferential placental replication compared to other tissues in *Nramp1*^{+/+} mice. At 24 hours, <25% of individual placentas per mouse were infected, progressively increasing to >75% by 72 hours which correlated with a steady increase in resorption rates. Moreover, placental infection was associated with increased neutrophils, macrophages and natural killer cells whereas neutrophil numbers in the spleen remained unchanged, suggesting dichotomous modulation of inflammation in the systemic compartment compared to the feto-maternal interface. Oral infection resulted in systemic dissemination of the bacteria, substantial placental colonization and fetal loss five days post-infection in C57BL/6J mice. Systemic infection in pregnant cell death deficient *Rip3*^{-/-}*Nramp1*^{+/+} mice (with defective necroptosis) resulted in decreased fetal demise relative to *Nramp1*^{+/+} and *Caspase-1,11*^{-/-}*Nramp1*^{+/+} mice (with defective pyroptosis) suggesting a role for necroptotic inflammation. This study provides insight into the kinetics and mechanism of inflammation and cell death during placental S.Tm infection. Such studies may assist in the rational management of foodborne pathogens contracted during pregnancy.

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List of Abbreviations

ANOVA	Analysis of Variance
APAF-1	Apoptotic Protease-Activating Factor One
APC	Allophycocyanin
APCs	Antigen Presenting Cells
APC-Cy7	Allophycocyanin with cyanine dye Cy7 (Tandem fluorochrome)
ASC	Apoptosis-Associated Speck Protein Containing a CARD
AV	Anchoring Villi
BAK	BCL-2 Antagonist/Killer
BAX	BCL-2-Associated X protein
BCL-2	B Cell Lymphoma Two
BHI	Brain Heart Infusion
BID	BH-3 Interacting Domain Death Agonist
BM	Basement Membrane
BSA	Bovine Serum Albumin
BUV395	Brilliant Ultra Violet fluorochrome with emission maximum at 395 nm
BV421	Brilliant Violet fluorochrome with emission maximum at 421 nm
BV605	Brilliant Violet fluorochrome with emission maximum at 605
B6	C57BL/6J inbred mouse strain
CARD	Caspase Activation and Recruitment Domain
CD	Cluster of Differentiation
CFUs	Colony Forming Units
CMV	Cytomegalovirus
CTB	Cytotrophoblast
DAMPs	Danger Associated Molecular Patterns
DC	Dendritic Cell
DISC	Death Inducing Signalling Complex
DNA	Deoxyribonucleic Acid
EVT	Extravillous Trophoblast
FADD	Fas-Associated Death Domain
FASL	Tumour Necrosis Factor Ligand Superfamily member 6
FASR	Tumour Necrosis Factor Receptor Superfamily Member 6
FB	Fetal Blood
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
Foxp3	Forkhead Box P3
FV	Floating Villi
G-CSF	Granulocyte-Colony Stimulating Factor
gd	Gestational Day
HBSS	Hank's Balanced Salt Solution
HHV-5	Human Herpesvirus-Five
HIV	Human Immunodeficiency Virus
HMGB1	High Mobility Group Box 1

iCTB	Invasive Cytotrophoblast
IFNAR	Interferon Alpha Receptor
Ifnar1	Interferon Alpha Receptor One
IFN-β	Interferon Beta
IFN-γ	Interferon Gamma
IL	Interleukin
IPAF	Ice Protease-Activating Factor
I.V.	Intravenous
KC	Keratinocyte-Derived Chemokine
LB	Lysogeny Broth
LPS	Lipopolysaccharide
M cell	Microfold Cell
MCP	Monocyte Chemoattractant Protein
MHC	Major Histocompatibility Complex
MLKL	Mixed-Lineage Kinase Domain-Like
MLN	Mesenteric Lymph Node
MNT	Mononuclear Trophoblast
Mo	Monocyte
Mϕ	Macrophage
mRNA	Messenger Ribonucleic Acid
MyD88	Myeloid Differentiation Primary Response Gene 88
N/A	Not applicable
NaCl	Sodium Chloride
NETs	Neutrophil Extracellular Traps
NFκB	Nuclear Factor Kappa-Light Chain Enhancer of Activated B Cells
NIH	National Institutes of Health
NLR	NOD-Like Receptor
NLRC4	NLR Family CARD Domain-Containing Protein 4
NLRP3	NACHT, LRR and PYD Domains-Containing Protein 3
NK	Natural Killer
NOD	Nucleotide-Binding Oligomerization Domain
NPI	Non-Pregnant Infected
NPNI	Non-Pregnant Non-Infected
<i>Nramp1</i>	Natural resistance associated macrophage protein 1 (renamed <i>Slc11a1</i>)
NTS	Non-Typhoidal Salmonellae
OD	Optical Density
PAMPs	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PBS-1%FBS	Phosphate Buffered Saline with 1 Percent Fetal Bovine Serum
PCR	Polymerase Chain Reaction
PE	R-Phycoerythrin
PE-CF594	PE with CF594 (tandem conjugate) with emission max at 612 nm
PE-Cy7	PE with cyanine dye Cy7 (tandem fluorochrome), emission max at 785 nm
PerCP	Peridinin Chlorophyll Protein Complex
PerCP-Cy5.5	PerCP with cyanine dye Cy5.5 (tandem conjugate), emission max at 695 nm
PI	Pregnant Infected

PNI	Pregnant Non-Infected
PRRs	Pattern Recognition Receptors
RIP1	Receptor Interacting Protein 1
RIP3	Receptor Interacting Protein 3
RORγt	Retinoic Acid Receptor-Related Orphan Receptor Gamma Transcription Factor
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
SARS	Severe Acute Respiratory Syndrome
SCV	<i>Salmonella</i> Containing Vacuole
SEM	Standard Error of the Mean
<i>Slc11a1</i>	Solute carrier family 11 member a1 (also known as <i>Nramp1</i>)
SPI1	<i>Salmonella</i> Pathogenicity Island 1
SPI2	<i>Salmonella</i> Pathogenicity Island 2
Spp.	Species
SpT	Spongiotrophoblast
S.Tm	<i>Salmonella enterica</i> serovar Typhimurium
S.TmΔaroA	<i>Salmonella enterica</i> serovar Typhimurium aroA deficient
SynT	Syncytiotrophoblast
TBCs	Trophoblast Cells
TCRβ	T Cell Receptor Beta
TGCs	Trophoblast Giant Cells
TGF-β	Transforming Growth Factor Beta
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TNF-α	Tumour Necrosis Factor Alpha
TNFR1	Tumour Necrosis Factor Receptor 1
TNFR2	Tumour Necrosis Factor Receptor 2
TRADD	TNF Receptor Associated Death Domain
TRAIL	TNF Related Apoptosis-Inducing Ligand
TRAIL-R	TNF Related Apoptosis-Inducing Ligand Receptor
Treg	Regulatory T cell
TTSS	Type Three Secretion System
uDC	Uterine Dendritic Cell
uNK	Uterine Natural Killer
vCTB	Villous Cytotrophoblast
VP+	Vaginal Plug Positive
WHO	World Health Organization
129.B6F1	129X1/SvJ x C57BL/6J F1 hybrid mice

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1.0 Introduction

1.1 Pregnancy

Maternal tolerance towards the semi-allogeneic fetus expressing paternal antigens is required during mammalian pregnancy in order to mitigate rejection¹. However, an effective and robust host immune response to protect the mother and fetus from the threat of infections is also required¹. This poses a unique immunological challenge during pregnancy.

In 1953, Sir Peter Medawar first proposed possible explanations in order to explain the immunological tolerance between the mother and growing fetus: i) anatomical separation between mother and fetus, ii) an antigenically immature fetus lacked antigen expression, and iii) non-specific maternal immune suppression². Medawar's hypothesis formed the basis of investigations for the next four decades, contributing significantly to our current knowledge of placental biology and reproductive immunology. It is now known that the fetus and mother are not anatomically separated in human and murine pregnancies; instead, maternal immune cells and fetal cells (termed trophoblast cells, TBCs) are in close contact with one another³. Furthermore, a crucial feature of the placenta is that the two circulatory systems, both fetal and maternal, circulate independently of one another while at the same time enabling nutrient and gas exchange to occur throughout gestation³. Moreover, maternal lymphocytes are not stimulated by fetal trophoblast cells as they do not express major histocompatibility complex (MHC) Ia antigens which are responsible for rejection of allografts in humans³. Lastly, the maternal immune system at the implantation site is active rather than suppressed although tightly regulated, in order to prevent a maternal immune response towards the semi-allogeneic fetus as well as protect the fetus from deleterious

effects of invading pathogens¹. Therefore, pregnancy encompasses dynamic immunological phases which will be explained in further detail.

1.1.1 Structure and Function of the Placenta

The placenta is a chimeric organ, consisting of both maternal and fetal tissues⁴. It is a highly vascularized tissue which functions to provide nutrients and transfer respiratory gases and waste products between the mother and the developing embryo⁴. It also protects the fetus by acting as a barrier to both potential blood-borne pathogens and maternal immune rejection^{4,5}. The placenta is composed of maternal cells which make up the uterine endometrium (decidua), and specialized fetally derived cells referred to as trophoblast cells.

The progression of placentation involves the proliferation, invasion and differentiation of extraembryonic trophoblastic cells⁶. In humans, there are 3 main types of trophoblast cells which differentiate along the villous or extravillous trophoblast pathways^{6,7}. First, the villous trophoblasts, which form the trophoblastic epithelium, are comprised of cytotrophoblasts (CTB) and syncytiotrophoblasts (SynT)⁶. The cytotrophoblasts compose the fetally derived chorionic villous trees, commonly known as floating villi (FV) (Fig. 1 A and B)^{6,8}. The outer layer of villous cytotrophoblasts (vCTB) differentiates into syncytiotrophoblast cells; a fused, multi-nucleated layer covering the villous trees (Fig. 1 B & C)^{6,9,10}. Together the cytotrophoblasts and syncytiotrophoblasts form the trophoblastic epithelium which covers a core of connective tissue which includes fetal vessels, fibroblasts, and fetal macrophages termed Hofbauer cells (Fig. 1 C)^{6,11}. Furthermore, since the syncytiotrophoblasts line the intervillous space, they are in direct contact with maternal

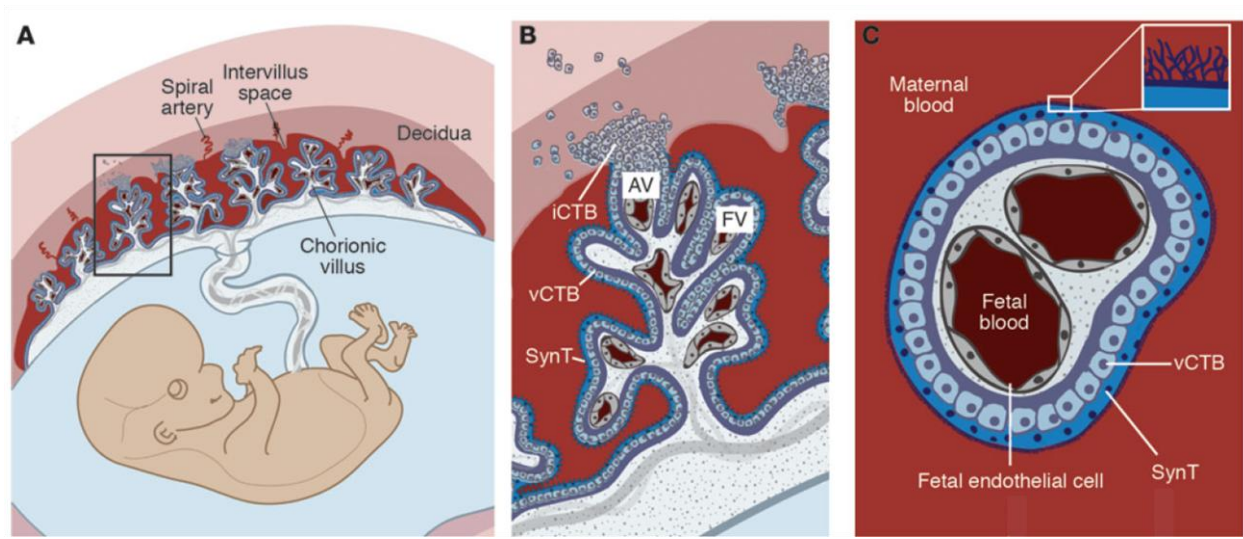


Figure 1

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Maltepe, E., et. al. The placenta: transcriptional, epigenetic, and physiological integration during development. *J. Clin. Invest* **120**, 1016-1025 (2010).

Figure 1. Structures of the human placenta. A) Structure of the human placenta. Fetal derived tissues (depicted in tones of blue) are in close contact with maternal tissues (shown in tones of red). Spiral arteries enable maternal blood to perfuse the chorionic villi in the intervillous space. B) Enlarged view from boxed-in area of panel A. Depiction of a chorionic villous tree known as a floating villi (FV); composed of villous cytotrophoblasts (vCTB) and an outer layer of syncytiotrophoblast (SynT) cells which are in direct contact with maternal blood. The invasive cytotrophoblasts (iCTB), more commonly known as extravillous trophoblasts, differentiate to form anchoring villi (AV), which invade the maternal decidua. C) The cross-sectional anatomy of a floating villous shows syncytiotrophoblasts covered by branched microvilli which function to increase the surface area for nutrient and gas exchange within the placenta.

blood and function to mediate nutrient and gas exchange between mother and fetus (Fig 1 A-C)^{6, 7}.

The third type of trophoblast cell in humans termed extravillous trophoblast (EVT), also known as invasive cytotrophoblasts (iCTB), arises from the tip of the anchoring villi (AV) in which cytotrophoblasts proliferate and differentiate into EVTs (Fig. 1 B)^{6, 7}.

Extravillous trophoblast cells rapidly invade into the uterine wall, anchoring the placenta at the implantation site (decidua), and are juxtaposed to maternal immune cells present within the decidua¹². EVTs also remodel the maternal spiral arteries enabling maternal blood to be routed into the placenta to bathe the fetal villi⁶.

The two main regions within the placenta in which fetal and maternal tissues are in direct contact and necessitate the need for tolerance is at the syncytiotrophoblast-maternal blood interface and the extravillous trophoblast-decidua interface (Fig. 1 B)^{12, 13}.

1.1.2 Stages of Pregnancy

Human gestation is about 280 days long and is divided into three stages: early, mid and late pregnancy¹³. Briefly, early pregnancy is characterized by a strong inflammatory response in which T helper 1 (Th1) inflammatory cytokines play a beneficial role in preparing the uterine bed for implantation of the blastocyst¹³⁻¹⁵. In humans, implantation occurs between 5 and 9 days after ovulation¹³. During implantation, the trophoblast cells break through the epithelial lining of the uterus in order to invade into the endometrial tissue^{13, 15}. The trophoblast cells also erode the endothelial cells of the uterine spiral arteries to enable adequate blood supply into the placenta¹³.

Mid-pregnancy is characterized by rapid fetal growth and development¹³. From mid-pregnancy onwards, there is a transient bias towards a T helper 2 (Th2) humoral (antibody production) response and away from a Th1 cell mediated response to facilitate tolerance towards the semi-allogeneic fetus, as the fetus bears 50% of paternal genes which are viewed as “foreign” by the maternal immune system. This was first proposed by Wegmann and colleagues in the early nineties in order for successful pregnancy to ensue¹⁶. In accordance, it was shown that the fetal-maternal interface readily secreted an abundance of the Th2 cytokines interleukin (IL)-4, IL-5 and IL-10 during all trimesters of gestation¹⁶. Secretion of transforming growth factor beta (TGF- β), and IL-10 play crucial immunosuppressive roles at the fetal-maternal interface by inhibiting the production of inflammatory cytokines such as interferon-gamma (IFN- γ), tumor necrosis factor alpha (TNF- α) and IL-2 which are capable of inducing fetal resorptions when administered to pregnant mice^{16, 17}. Furthermore, administration of recombinant IL-10 via intraperitoneal injection can rescue resorption rates highlighting the importance of a Th2 bias during pregnancy¹⁷.

Such Th2 bias during pregnancy also influences the systemic maternal immune response which is exquisitely illustrated in pregnant women who are diagnosed with certain autoimmune disorders. Rheumatoid arthritis, a cell mediated autoimmune disorder, is ameliorated during pregnancy¹⁸. Contrastingly, the autoimmune disorder systemic lupus erythematosus, which involves excessive antibody production, is exacerbated during pregnancy^{19, 20}.

In spite of a Th2 bias during pregnancy, late pregnancy, specifically parturition, is characterized by a pro-inflammatory Th1 response^{14, 21}. Parturition results in an influx of immune cells and induction of a Th1 response, with increased expression of pro-

inflammatory cytokines within the cervix, myometrium and fetal membranes, to assist in triggering uterine changes which are conducive to labour²¹⁻²⁵. Such responses induce uterine contractions for delivery of the baby and expulsion of the placenta^{21, 22}.

Therefore, the modulation of immunity during pregnancy results in differential responses to a variety of pathogens relative to the non-pregnant state.

1.2 Mouse model of pregnancy

The mouse is a convenient *in vivo* model to not only understand healthy pregnancy, but to study and gain insight into the regulation of immunity to infection during gestation. Many similarities in implantation, placentation and parturition exist between murine and human gestation¹³. Similarities also exist in the immune cell subsets which traverse the placenta during pregnancy in both mice and humans. Furthermore, there are several specialized populations of fetal derived trophoblast cells which are physiologically similar in both mice and humans¹³.

1.2.1 Murine implantation

Murine gestation is 21 days when copulatory/vaginal plug formation is counted as day 0. Between gestational day 4 and 4.5, decidualization occurs in which the luminal epithelium undergoes apoptosis allowing attachment of the blastocyst to the uterine lining on the anti-mesometrial side¹³.

1.2.2 Placentation

Both humans and mice, as well as higher order primates, guinea pigs, and rats have a hemochorial placenta in which fetally derived trophoblast cells are in direct contact with

maternal blood^{12, 13}. Furthermore, the fetus is connected to the placenta by an umbilical cord allowing the umbilical vein to supply the fetus with nutrient-rich blood from the placenta¹³.

The cell types present within the murine placenta serve similar functions in comparison to those found within the human placenta. In the mouse placenta, the fetal and maternal vessels are in close contact with one another to allow nutrient and gas exchange between mother and fetus¹³. This area in which exchange occurs is termed the labyrinth in mice (Fig. 2 D)^{8, 13, 26}. The labyrinth is anchored to the maternal decidua by non-syncytial cells known as spongiotrophoblasts (SpT) (Fig. 2 D & E)^{8, 13}. This spongiotrophoblast region is lined by trophoblast giant cells (TGCs) (akin to extravillous trophoblasts in humans) and functions in anchoring the growing fetus to the maternal decidua and is juxtaposed to maternal immune cells (Fig. 2 E)^{8, 13, 26}.

Within the labyrinth region are the murine fetal villi. Fetal villi within humans and mice have different branching patterns; human villi have a tree-like pattern while mice villi have an interconnected maze-like pattern (Fig 2. A & D)^{5, 26}. These chorionic projections in mice are comprised of stroma, blood vessels and chorionic trophoblasts which differentiate into two layers of multinucleated syncytiotrophoblasts (akin to syncytialized cytotrophoblasts within humans termed syncytiotrophoblasts) and an outermost layer of semi-discontinuous mononuclear trophoblast (MNT) cells (Fig. 2 E)^{5, 8}.

Trophoblast cells erode the endothelial cells of the central spiral artery enabling blood to pass the spongiotrophoblast via blood sinuses and into the tortuous spaces within the murine labyrinth where the fetal villi, specifically the mononuclear trophoblast cells and syncytiotrophoblast cells, are bathed by maternal blood, enabling nutrient and gas exchange (Fig. 2 D & F)^{8, 13, 26}.

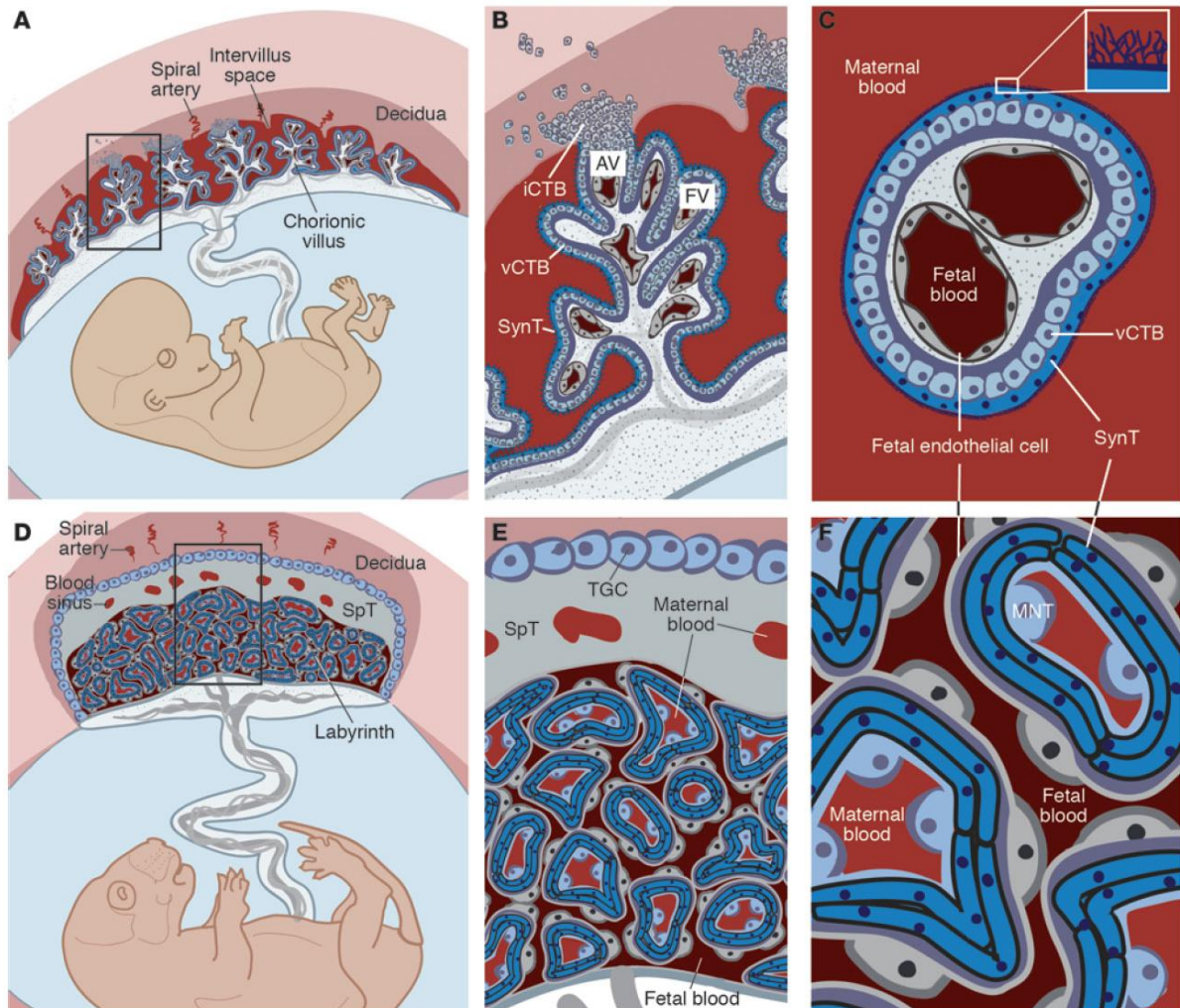


Figure 2

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Maltepe, E., et. al. The placenta: transcriptional, epigenetic, and physiological integration during development. *J. Clin. Invest* **120**, 1016-1025 (2010).

Figure 2. Comparative anatomy of the human and mouse placenta. A-C) Structure of the human placenta and cell types as seen in Figure 1. D) Structure of the mouse placenta. Tissues in blue are of fetal origin while those in red are of maternal origin, both of which are in close contact with one another. Maternal blood passes the spongiotrophoblast (SpT) cells by blood sinuses and enters the labyrinth where nutrient and gas exchange occurs. E) Enlarged view from boxed-in area of panel D. The SpT cells are lined by trophoblast giant cells (TGC), akin to invasive cytotrophoblasts (iCTBs) in B, and function in anchoring the placenta to the uterus. F) Maternal blood is in direct contact with two layers of syncytiotrophoblasts (SynT) (akin to SynT in humans) which are covered by a semi-discontinuous layer of mononuclear trophoblasts (MNT).

During infection, the placenta acts as a “fortress” against various pathogens¹². It is hypothesized that there are two areas in which transmission of pathogens from mother to fetus may occur; the syncytiotrophoblast-blood interface (both humans and mice) and the trophoblast giant cell-decidua interface in mice (extravillous trophoblast-decidua interface in humans)^{12, 13}.

1.3 Immune cell subsets at the feto-maternal interface

Four immune cell subsets are present within the maternal decidua: uterine natural killer (uNK) cells, macrophages, dendritic cells (DCs) and regulatory T cells, play an important immunoregulatory role during successful pregnancy²⁷.

1.3.1 Uterine NK cells

During early pregnancy in humans, the decidua is comprised of roughly 30-40% leukocytes of which 70% are uNK cells^{11, 27}. These cells have a distinct phenotype and function in which they are CD56^{bright}/CD16⁻ in comparison to peripheral blood natural killer (NK) cells which are CD56^{dim}/CD16⁺^{28, 29}. Furthermore, differences in functionality exist between uNK cells and peripheral NK cells. Peripheral NK cells express CD16 which is involved in triggering the lysis of target cells. Uterine NK cells lack the expression of CD16 resulting in a reduced cytotoxic phenotype^{28, 30}.

In both humans and mice, uNK cells are the most abundant cell type during decidualization, an inflammatory process in which the endometrial tissue undergoes cell differentiation, thereby enabling the blastocyst to implant within the uterine bed^{31, 32}. Studies in mice indicate that uNK cells are the dominant cell type representing about 50% of the total leukocyte population by gestational day 5.5, coinciding with implantation³³. These

cells drop in number significantly by gestational day 9.5 in mice, representing 10-15% of all leukocytes (CD45⁺ cells)³³.

During early pregnancy in both humans and mice, uterine NK cells play a central role in mediating angiogenesis and trophoblast invasion through their production of IFN- γ ³⁴⁻³⁷. During this time, vascular changes occur enabling spiral artery remodeling in which the endothelium of the spiral arterioles is eroded by trophoblasts that have migrated from the placenta and invaded the decidua³⁶. This diverts blood flow into the spaces surrounding the villous trees to ensure adequate nutrient exchange between the mother and the growing embryo. Studies indicate that a deficiency of uNKs results in decidual growth defects which include necrosis and edema along with undilated spiral arteries^{30, 38, 39}.

There is little information regarding the percentage and distribution of uNK cells during the second trimester of pregnancy in humans due to difficulty in accessing tissues from healthy normal pregnancies. There are conflicting results as to the number of uterine NK cells present in term placenta as some studies have recorded a reduction in the number of CD56⁺ cells^{40, 41} while others have found a substantial number of uNK cells present within the placenta^{11, 42}. These differences may be attributable to variations in tissue collection, which may alter the decidual cell content¹¹.

1.3.2 Macrophages

Macrophages are the second most abundant cell type in the human decidua, comprising 20-25% of all leukocytes, and are slightly less common in the murine decidua with percentages ranging between 15-20% of all CD45⁺ cells^{4, 33, 43}. In mice, detection of

macrophages consists of F4/80 expression and a lack of CD11c expression⁴⁴. In humans, their detection is characterized by the combined expression of both CD14 and CD68⁴⁴.

The main function of macrophages present within the decidua is to assist with tissue remodelling at the feto-maternal interface, specifically angiogenesis^{4, 44}. The plasticity and functional polarization of macrophages to either M1 (inflammatory) or an M2 (anti-inflammatory) phenotype has been described^{45, 46}. Decidual macrophages produce elevated levels of IL-10 and display a skewed M2 phenotype. Macrophages are polarized to an M2 phenotype through exposure to Th2 cytokines and have been seen during both human and mouse pregnancy⁴⁷. It is postulated that their M2 phenotype during pregnancy is associated with tissue remodeling, scavenging of apoptotic cells, inhibiting inflammation and regulating immune tolerance⁴⁴.

1.3.3 Dendritic cells

Decidual DCs account for roughly 1.7% of all leukocytes present within the human decidua, whereas in mice, these cells account for 5-10% of all decidual leukocytes^{11, 48, 49}. In humans, decidual DCs are characterized based upon their CD83 expression⁴. In mice, decidual DCs can be classified based upon CD11c expression⁴⁹. CD83⁺ DCs are most prevalent within the human decidua during decidualization with a decline in number thereafter⁴. In mice, decidual DCs become most abundant within the mouse decidua during gestational day 5.5, which has been linked to the implantation window^{31, 50}.

Such cells play a pivotal role in implantation with cross-talk occurring with uNK cells⁵⁰. Depletion of DCs during peri-implantation results in early pregnancy failure due to decidualization impairment⁵⁰. Specifically, the absence of DCs resulted in defective stromal

cell proliferation, abnormal cell differentiation, and a reduction in vascularization at the implantation site⁵⁰.

While uterine DCs play a critical role in the development of the decidua, ablation of uterine DCs during syngeneic and T cell deficient pregnancies resulted in embryo resorption, suggesting that uterine DCs are not required for maternal tolerance of the fetus^{50, 51}.

Contrastingly, it was determined that decidual DCs can be further divided into two categories based upon their surface expression of CD11b and CD103⁵². Both CD11c^{high}CD11b^{low}CD103⁺ and CD11c^{high}CD11b^{high}CD103⁻ decidual DCs have lymph node homing capacities within the non-pregnant uterus but such homing capacity is lost following decidualization⁵². Although the reduction in DC density post-implantation may explain the lack of anti-fetal/placental T cell responses, it is more plausible that by restraining DCs within the decidua, immunogenic T cell exposure to fetal/placental antigens is minimized⁵².

1.3.4 Regulatory T cells

Regulatory T cells, Tregs, are central to regulating immune responses elicited throughout the body. They can be characterized based upon their expression of CD4 and CD25 as well as the expression of the transcription factor Forkhead box P3 (Foxp3)⁵³. They are known to inhibit the proliferation and cytokine production of both CD4⁺ and CD8⁺ T cells, immunoglobulin production by B cells, the cytotoxic activity of NK cells, and the maturation of DCs^{54, 55}. They also play an important role in promoting and maintaining immunological tolerance towards the semi-allograft⁵⁶⁻⁵⁸. Although IFN- γ induced inflammation is important in promoting vascular remodeling, and inflammation is required for successful implantation, excessive inflammation can result in embryonic resorption⁵⁹.

Therefore, Tregs may play an important role in regulating such excessive inflammation in order for successful implantation to occur. Studies have shown that Tregs increase in number in both humans and mice during pregnancy, while a reduction may be associated with pregnancy complications⁶⁰⁻⁶³. A reduction in decidual Tregs has been observed in women with pre-eclampsia, sporadic miscarriage, preterm labour and recurrent spontaneous abortion in comparison to normal age-matched controls^{61, 64-69}. Furthermore, depletion of Tregs after murine mating results in the impairment of implantation in allogeneic mice but not syngeneic mice, suggesting that Tregs also play a vital role in inducing tolerance towards paternal antigens^{56, 58, 70, 71}.

1.4 Pregnancy and susceptibility to infection

Bacterial, viral, and parasitic infections acquired during gestation have the potential to pose a significant threat to pregnancy outcome and the health of the fetus. However, only select intracellular infections pose a serious threat to pregnancy, suggesting that pregnancy does not fully compromise immunity to all infections.

There are three major routes in which a pathogen can gain access to the placenta: ascending into the uterus from the lower reproductive tract, trafficking through the maternal circulation, or by descending into the uterus from the peritoneal cavity²².

Infection during pregnancy has the potential to cause a variety of complications, such as pre-term birth, with reports suggesting that roughly 40% of preterm births were a result of infection^{14, 72}. Placental infection can also cause other complications such as spontaneous abortion, still birth, premature birth, chorioamnionitis, fetal infection and severe maternal illness^{73, 74}. Moreover, infection with certain pathogens during pregnancy may cause

maternal death⁷⁵. Aside from the immediate detrimental effects of infection on the outcome of pregnancy, increasing evidence indicates that exposure of the fetus to maternal inflammation due to infection may correlate with increased risk of developing schizophrenia, autism or bipolar disorder later in life⁷⁶⁻⁸².

Human and veterinary case reports, as well as the use of various animal models, have enabled the study of infections during pregnancy and the consequences to both mother and fetus. Below is a summary of some of the pathogens currently known to cause adverse effects to the maternal host and/or fetus during pregnancy in humans or animals, some of which are capable of crossing the placental barrier.

1.4.1 Bacterial infections

Bacteria such as *Listeria monocytogenes*, *Coxiella burnetti* and various *Chlamydia* species have been known agents to cause adverse pregnancy outcomes for many years⁷⁴. As medical care and diagnostics have improved and the research realm has expanded, a variety of other bacteria have been shown to have a predilection for infection at the feto-maternal interface: *Salmonella enterica* Typhimurium, *Brucella abortus*, *Campylobacter rectus* and *Porphyromonas gingivalis*⁸³⁻⁹¹. Most of these bacterial pathogens are implicated in pregnancy complications. All of these bacterial pathogens have intracellular lifecycles and most are able to infect and survive within leukocytes¹².

Listeria monocytogenes is a common foodborne pathogen, while *Campylobacter rectus* and *Porphyromonas gingivalis* are periodontal pathogens, all of which preferentially infect and colonize placental tissues^{89, 90, 92}. *Brucella abortus* and *Coxiella burnetti*, which can be contracted through ingestion, and *Chlamydophila abortus*, a common sexually

transmitted infection, pose a threat to a variety of pregnant animals as infection causes abortion in cattle, sheep and goats, and less commonly, in pigs and horses⁷⁴. Such pathogens cause a variety of detrimental effects during human pregnancy including premature birth, abortion, intrauterine growth restriction or fetal death^{74, 75, 93, 94}.

Murine infection with *Listeria monocytogenes*, *Brucella abortus* or *Coxiella burnetti* resulted in vertical transmission and abortion of murine fetuses^{85, 86, 95}. Furthermore, *L. monocytogenes*, *B. abortus*, *C. burnetti* and *C. abortus* have a predilection for infection and replication within murine trophoblasts compared to other systemic tissues which has been attributed to placental architecture, immune regulatory and physiological conditions at the feto-maternal interface^{85, 86, 95-98}. Histological analysis of murine placentas infected with *L.monocytogenes* reveal that bacteria initially invade the trophoblast giant cells which penetrate the decidua, with progression of infection resulting in massive colonization of syncytiotrophoblasts⁹⁵. The spread of infection may be attributable to cell-to-cell spreading through trophoblastic tissues with subsequent vertical transmission to the fetus⁹⁵. Moreover, some bacterial pathogens are capable of infecting immune cells, such as *Coxiella burnetti*, which is capable of infecting murine macrophages and monocytes⁹⁷.

Murine studies with *L. monocytogenes* and *S. Typhimurium* revealed that fetal demise due to placental infection, as well as increased maternal mortality, can be attributed to high bacterial burden, neutrophil infiltration into the placenta with focal necrosis, and increased production of Th1 inflammatory cytokines, such as IFN- γ , IL-6 and TNF- α , within maternal serum^{84, 95, 99, 100}.

1.4.2 Viral infections

Various acute and chronic viral infections during pregnancy can result in severe maternal illness and a variety of pregnancy complications. Cytomegalovirus (CMV), human immunodeficiency virus (HIV), hepatitis B, varicella zoster, severe acute respiratory syndrome (SARS), influenza virus and the common rhinovirus all pose a risk to pregnant hosts^{13, 75, 101-105}.

Human cytomegalovirus, also known as human herpesvirus-5 (HHV-5) is the leading cause of prenatal viral infection and the most common cause of infection-related congenital disabilities¹⁰⁶. CMV is capable of causing both a primary infection and recurrent infection during pregnancy¹⁰⁶. Primary CMV infections occur in 0.15-2% of all pregnancies and transmission to the fetus occurs in 40% of these pregnancies¹⁰⁶. Roughly 12% of all infected infants succumb to infection within the first month of birth, while most of the remaining infants develop neurological or auditory deficits^{101, 106}. Long term disabilities include mental deficiencies, cerebral palsy, epilepsy, learning disabilities, deafness or hearing impairment or visual deficiencies including blindness¹⁰⁷. Studies indicate that two routes of vertical transmission may occur in humans: across syncytiotrophoblasts with infection in underlying cytotrophoblasts or through extravillous trophoblasts which invade the uterine wall¹⁰¹.

Influenza infection during pregnancy results in increased disease severity and a higher incidence of maternal mortality in comparison to non-pregnant individuals^{75, 108-113}. Furthermore, women in their second or third trimester are at increased risk of developing cardiopulmonary complications, premature delivery or death^{75, 114}. Influenza infection in pregnant women can further be complicated by pneumonia as seen during the 1918 pandemic in which 27% of infected pregnant women died as a result of influenza

infection^{109, 115}. This statistic increased to 50% of infected pregnant women succumbing to infection when complicated with pneumonia^{109, 115}. Reports analyzing the mortality rate of pregnant women amongst women of reproductive age during the 1957 influenza pandemic indicated that the incidence of maternal mortality during pregnancy was 50%^{110, 115}. A systematic review of the recent 2009 H1N1 outbreak indicated that pregnant women were at increased risk for severe disease resulting in hospitalization when compared to non-pregnant women and the general population¹¹⁶. Data from 29 countries indicated that 3110 pregnant women contracted H1N1 of which 1625 (52.3%) were hospitalized with 378 (23.3%) being admitted to the ICU and 130 (8%) succumbing to infection¹¹⁶.

Utilization of pregnant mice to study the effects of influenza infection (via intranasal administration) during pregnancy resulted in increased pulmonary viral titres within infected mothers along with pre-term delivered pups which exhibited significant growth retardation¹¹⁴. There was a reduction in the maternal inflammatory cytokines interferon beta (IFN- β), IFN- γ and IL-1 α and decreased expression of the chemokines monocyte chemoattractant protein-1 (MCP-1) and keratinocyte-derived chemokine (KC). The reduction in cytokine expression was attributed to estrogen levels during pregnancy as non-pregnant mice treated with estrogen pellets beneath the skin displayed increased susceptibility to influenza infection similar to pregnant infected mice¹¹⁴. Furthermore, although there was no difference in immune cell numbers (NK cells, neutrophils or DCs) in pregnant infected compared to non-pregnant infected mice, there was a reduction in CD86 expression on CD11c⁺ cells¹¹⁴. Such results suggested a reduction in DC maturation in infected pregnant mice and estrogen treated mice compared to controls. Moreover, both

pregnant mice and estrogen treated mice displayed reduced splenic cytotoxic T cell responses towards influenza infection¹¹⁴.

1.4.3 Parasitic infections

Several parasitic pathogens, such as *Plasmodium falciparum*, *Toxoplasma gondii*, *Schistosoma* species and *Leishmania major*, all pose a threat during pregnancy and may either be exacerbated during pregnancy or result in congenital parasitic infection¹¹⁷. Maternal infection with such parasitic pathogens can cause abortion, intrauterine growth restriction, fetal infection or the acquisition of congenital malformations such as hearing loss or mental deficiencies¹¹⁸⁻¹²⁰. Congenital transmission mainly occurs due to maternal infection with *Toxoplasma gondii*, *Plasmodium* species and *Leishmania* species^{117, 121}.

Toxoplasma gondii, an intracellular parasite, is contracted mainly through the ingestion of raw or undercooked meat or through exposure to infected cat feces¹¹⁸. *Plasmodium* species such as *Plasmodium falciparum* and *P. vivax*, as well as *Leishmania major*, all of which are intracellular parasites, are vector borne pathogens contracted through mosquito or sand flea bites respectively¹²².

The mechanism by which parasitic placental infection occurs is still poorly understood. During pregnancy, increased pathogen transmission (*Plasmodium* spp., *Leishmania* spp. *Toxoplasma gondii*) from mother to fetus occurs via infected trophoblasts as pregnancy progresses due to the continued decrease in placental thickness¹²⁰.

Use of first trimester human explants revealed that extravillous trophoblasts are more susceptible to *T. gondii* infection and more permissive to parasitic replication in comparison to syncytiotrophoblasts¹²³. Results indicate that the syncytiotrophoblasts are strongly

resistant to *T. gondii* infection and that syncytial damage enables parasite colonization¹²³. Such damage and breach of the syncytiotrophoblasts may be due to physical trauma and inflammation rather than pathogen invasion¹²³. Interestingly, the distribution and localization of *T. gondii* parasites within the placenta were similar to studies conducted with *L. monocytogenes*⁹². Hence, placenta susceptibility to pathogens may not be entirely attributable to the pathogens and the molecular requirements of invasion which they possess, but rather the placenta contains areas (extravillous trophoblast-decidua interface) of vulnerability to pathogen invasion¹²³.

T. gondii infection in pregnant mice resulted in a Treg/Th17 imbalance, in which Tregs were decreased in the spleens and placentas of pregnant infected mice while Th17 cells were increased in both tissues in comparison to pregnant non-infected controls¹²⁴. This was further correlated with increased messenger ribonucleic acid (mRNA) expression of IL-6 and decreased expression of TGF- β , suggestive of Th17 conversion from naive CD4⁺ T cells or Tregs¹²⁴. Furthermore, treatment of human trophoblasts with IL-10 reduced *T. gondii* induced apoptosis¹²⁵. Such data may implicate the reduction of Tregs and associated reduction in secreted IL-10 as a mechanism by which *T. gondii* infection results in trophoblast cell death and adverse pregnancy outcome such as spontaneous abortion or pre-term birth^{124, 126}.

Despite the potential need for sufficient Tregs and the secretion of IL-10 in rescuing pregnancy during *T. gondii* infection, a strong Th1 response is required for successful clearance of *Leishmania major* as seen in studies involving non-pregnant mice¹²⁷. Unfortunately a dichotomy exists in regards to an effective immune response in the clearance of *L. major* during pregnancy; while an effective Th1 response to infection is

detrimental to pregnancy causing implantation failure and fetal loss, the down-modulation of such a response during pregnancy results in a more severe systemic infection¹²⁸. Krishnan and colleagues have shown that pregnancy dampens an effective Th1 response due to diminished IFN- γ and increased Th2 cytokines IL-4, IL-5 and IL-10¹²⁷.

Likewise, malaria in pregnant women, as a result of *Plasmodium falciparum* or *Plasmodium vivax* infection, resulted in increased plasma IL-10 and reduced IFN- γ and IL-17A in comparison to non-pregnant controls¹²⁹. Moreover, increased IL-10 and decreased IFN- γ and IL-17A correlated with increased parasitaemia¹²⁹. Therefore, IL-10 responses may be implicated in the pathogenesis of malaria while IL-17A and IFN- γ may play protective roles in controlling and combating infection. Although such responses may be beneficial in the maternal compartment, a strong Th1 response at the feto-maternal interface may be detrimental, as seen during *Leishmania* infection. Studies investigating placental cytokine expression during *Plasmodium* infection would give insight into the placental immune environment, especially since *Plasmodium falciparum* parasites, which selectively target the placenta, have been recently identified^{130, 131}.

1.5 *Salmonella*

Salmonella is a rod-shaped, gram negative, intracellular, facultative foodborne pathogen belonging to the Enterobacteriaceae family¹³². The genus *Salmonella* is divided into two species, *Salmonella bongori* and *Salmonella enterica*. The latter consists of over 2,500 serological variants (serovars) which are differentiated based on their lipopolysaccharide (O), and flagellar (H) antigens¹³². While all 2,500 serovars are capable of causing disease in humans, some serovars also cause disease in select warm blooded animals

such as *Salmonella* serovar Dublin in cattle or *Salmonella* serovar Typhimurium in mice¹³³⁻¹³⁵.

The clinical manifestations of infection with *Salmonella enterica* species are divided into two groups. *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi A, B, C cause enteric (typhoid) fever in humans while all other *Salmonella enterica* serovars, referred to as non-typhoidal *Salmonella* (NTS), cause gastroenteritis in immunocompetent individuals¹³². Clinical manifestations of typhoid fever in humans include fever, headache, abdominal pain and constipation or diarrhea¹³². Since NTS are usually limited to the gastrointestinal tract, infected individuals present with nausea, vomiting and diarrhea¹³⁶. However, non-typhoidal systemic fever is increasing in prevalence among immunocompromised individuals such as those infected with HIV¹³⁷.

Salmonella infection during pregnancy can result in various complications to both the mother and fetus. Primary complications such as miscarriage, premature and still birth may occur and secondary complications such as osteomyelitis, endocarditis, or kidney dysfunction can affect the mother¹³⁸⁻¹⁴⁰. Vertical transmission to the neonate can occur during gestation as well as post-partum transmission of *Salmonella* species from the mother to the fetus through breast milk^{141, 142}.

A number of typhoidal and NTS related pregnancy complications have been cited in the literature; NTS infections during pregnancy were reported in Germany¹⁴³, Norway¹⁴⁴ and the U.S.¹⁴⁵⁻¹⁴⁷ while typhoidal infections were reported in Cambodia¹⁴⁸ and India^{149, 150}. Case reports have revealed a variety of fetal outcomes; premature birth followed by antibiotic treatment to support recovery, fetal or infant death or septic abortion^{143-146, 148-150}. In some instances, antibiotic-resistant infection in the mother and infant was noted^{146, 150}.

1.5.1 Natural route of infection

Salmonella species are naturally acquired through oral ingestion of contaminated food and water, including, but not limited to, poultry, meat, eggs, processed foods such as peanut butter¹⁵¹, cashew cheese¹⁵² and chia seeds/powder¹⁵³. Infection can also be acquired through contact with a carrier such as pet reptiles, rodents or feeder rodents¹⁴⁶. After ingestion and passage through the stomach, *Salmonella* species are able to colonize the small intestine in one of three ways. *Salmonella* species preferentially adhere and translocate into the microfold cells (M cells) present within the ileum portion of the small intestine as well as the colon^{154, 155}. M cells are specialized phagocytic epithelial cells which sample antigens present within the intestinal lumen, transporting them to the underlying Peyer's patches for antigen presentation¹⁵⁶.

Salmonella species are also capable of infecting the surrounding epithelial cells of the intestine through bacterial mediated endocytosis¹⁵⁷. *Salmonella* encodes two Type III secretion systems (TTSS) within the *Salmonella* pathogenicity island 1 and 2 (SPI1 and SPI2) genes¹⁵⁸. The SPI1-TTSS assembles a needle-like complex which injects effector proteins directly into the cytosol of the host cell¹⁵⁸. These proteins induce host cell cytoskeletal changes resulting in membrane ruffling of the cell, thereby allowing bacterial mediated endocytosis of normally non-phagocytic cells such as the epithelial cells of the intestine¹⁵⁸. Lastly, *Salmonella* can be captured from the intestinal lumen by CD18 expressing phagocytes such as dendritic cells, which extend pseudopods between the epithelial cells of the intestine to sample intestinal antigens¹⁵⁹. *Salmonella*, an intracellular pathogen, resides inside a modified vacuole termed *Salmonella* containing vacuole (SCV)¹⁶⁰. The SPI2-TTSS effector proteins are responsible for such modifications which enable

intracellular growth and replication of *Salmonella* species^{160, 161}. Therefore, *Salmonella* species have various virulence mechanisms which enable them to effectively evade host immune responses, ultimately leading to a chronic infection.

1.5.2 Gut restricted infection

In immunocompetent individuals, NTS infection is usually limited to the gut due to the induction of an early inflammatory response. Infection of the intestinal epithelium results in the secretion of IL-8 and heparin A₃ for the recruitment and migration of neutrophils into the intestinal epithelium respectively¹⁶²⁻¹⁶⁵. The release of cytotoxic granules and stimulation of the innate immune response restricts pathogen dissemination. Mucosal inflammation causes intestinal epithelial damage resulting in fluid secretion and diarrhea¹⁶⁶.

1.5.3 Systemic disseminations of *Salmonella*

Systemic dissemination of *Salmonella* can occur once the intestinal epithelium has been breached and the bacteria reach the blood directly, or infect antigen presenting cells (APCs) such as macrophages and dendritic cells¹⁶⁷. *Salmonella* species capable of reaching the Peyer's patches are able to travel to the mesenteric lymph nodes, gaining access to the bloodstream and disseminating to other organs such as the spleen and liver¹⁶⁷. *Salmonella* species can induce macropinocytosis, thereby allowing the uptake of *Salmonella* into the macrophage and subsequent survival and replication within the *Salmonella* containing vacuole through the help of their SPI2-TTSS effector proteins¹⁶⁸. At times, rather than residing within the SCV in a macrophage, *Salmonella* can induce caspase-1 mediated cell death of macrophages, resulting in the production of pro-inflammatory cytokines, IL-18 and

IL-1 β , which function to recruit additional phagocytes resulting in further infection and dissemination of bacteria^{167, 169}. *Salmonella* species can also be captured by dendritic cells sampling the intestinal lumen^{159, 170, 171}. Both infected macrophages and DCs function as carriers of *Salmonella* allowing for systemic dissemination¹⁷².

1.6 Murine infection models

Salmonella enterica serovar Typhimurium (S.Tm) was identified by Friedrich Loeffler in 1892 as the causative agent of murine typhoid¹³⁴. While it causes a systemic typhoid-like disease in mice, it is the most frequently encountered serological variant by humans along with *Salmonella enterica* serovar Enteritidis, both of which cause gastroenteritis^{134, 173}.

In order to study infection in mice, both intravenous (I.V.) and oral infection methods can be utilized. Intravenous infection allows for the direct study of systemic infection while an oral infection enables study of the natural route of infection and dissemination from the gut to systemic organs¹⁷⁴. Pre-treatment of mice with antibiotics in which the pathogen is resistant to, such as streptomycin prior to *Salmonella* infection, greatly increases pathogen gut colonization leading to gastrointestinal immunopathology^{175, 176}. Such immunopathology of the gut in mice mimics clinical symptoms of enterocolitis in humans, thereby allowing for a more appropriate infection model in order to study the pathogenesis of *Salmonella* and associated host responses¹⁷⁷. Depending on the infection dose in a murine model, the infection is not necessarily limited to the gut, with systemic infection apparent three days post infection confirmed by colony forming units (CFUs) present within the mesenteric lymph node, spleen and liver¹⁷⁸.

1.6.1 Strains and survival kinetics

Dr. Krishnan's lab has utilized two different strains of mice in the study of *Salmonella* pathogenesis during pregnancy: 129X1/SvJ and C57BL/6J mice. 129X1/SvJ mice carry a functional *Slc11a1* gene, more commonly known as *Nramp1*, encoding the natural resistance associated macrophage protein-1, a divalent metal ion transporter which removes iron and manganese from the phagosomes of macrophages and dendritic cells¹⁷⁹⁻¹⁸¹. NRAMP1 functions as an antagonist of bacterial metal acquisition within the phagosome, thereby depleting divalent metal ions required for successful bacterial replication^{182, 183}. Presence of a non-functional *Nramp1* gene has been associated with susceptibility to various intracellular pathogens including various strains of *Salmonella* within mice and humans¹⁸⁴⁻¹⁸⁸. C57BL/6J mice are susceptible to infection due to a single glycine to aspartic acid substitution (G169D) at position 169 of the *Nramp1* gene, causing a single amino acid change from glycine to aspartic acid, rendering the protein non-functional^{180, 189}. Use of a C57BL/6J mouse transgenic for *Nramp1*¹⁹⁰ or a C57BL/6J mouse congenic for *Nramp1*¹⁹¹, both of which carry guanine at position 169, show increased resistance to various pathogens such as *Salmonella* in comparison to wild-type C57BL/6J mice, making them a more desirable strain to study infection during pregnancy^{190, 191}. Furthermore, their C57BL/6J genetic background enables correlations and comparisons to various gene deficient C57BL/6J mice such as different cell death specific strains (*Rip3*^{-/-}, *Caspase-1,11*^{-/-} and *Ifnar1*^{-/-}). Lastly, they are quite eager to mate in comparison to the 129X1/SvJ strain, allowing for pregnancy studies to ensue.

1.6.2 *Salmonella* infection during murine pregnancy

Salmonella Typhimurium infection leads to differential outcomes depending on the strain of mouse studied. Susceptible strains such as C57BL/6J succumb to a low dose I.V. infection of 10^2 colony forming units within 7 days, whereas resistant 129X1/SvJ mice infected with 10^3 CFUs develop a chronic infection lasting 60-90 days, which is eventually cleared⁸³. The increased susceptibility of C57BL/6J mice has been attributed to a mutation in the *Nramp1* gene, which is fully functional in the 129X1/SvJ strain, thereby conferring resistance during infection.

While 129X1/SvJ mice are resistant in a non-pregnant state, mid-pregnant (days 10-12) 129X1/SvJ mice infected with 10^3 CFUs of S.Tm succumb to infection within 7 days⁸³. This was correlated with ~1000 fold increase in bacteria burden in the spleens of pregnant mice compared to non-pregnant controls⁸³. Furthermore, the placenta was highly colonized with more than 10^7 bacteria present within the placentas of infected mice 3 days post-infection, which resulted in greater than 75% fetal loss⁸³. This significant increase in bacterial burden in pregnant hosts compared to resistant non-pregnant hosts indicates a loss of host resistance to S.Tm infection.

Since host adaptive immune responses are delayed during S.Tm infection, a robust innate immune response is pivotal in pathogen control and clearance¹⁹². S.Tm infection in non-pregnant 129X1/SvJ mice resulted in increased production of serum IL-12, coinciding with a significant increase in both NK1.1⁺ and DX5⁺ NK cell populations in the spleen, along with increases in macrophages, dendritic cells and neutrophils⁸³. This increase in NK cell recruitment to the spleen along with increases in macrophages, dendritic cells and

neutrophils was not seen in pregnant infected hosts. Moreover, there was no increase in serum IL-12 in pregnant infected mice⁸³.

Further studies involving C57BL/6Jx129X1/SvJ F1 hybrid mice, referred to as 129.B6F1 which are heterozygous for the *Nramp1* gene, corroborated previous published results with an increase in susceptibility of pregnant mice compared to non-pregnant controls to S.Tm infection⁸⁴. Non-pregnant 129.B6F1 mice developed a chronic infection lasting more than 50 days, while a rapid and fatal systemic infection was seen in pregnant mice which succumbed to infection within 4 days⁸⁴.

Parallel studies involving intravenous infection with a high dose (10^6 CFUs) of an auxotrophic mutant *Salmonella* Typhimurium strain (S.Tm Δ aroA) resulted in 100% of both pregnant and non-pregnant age-matched controls surviving infection (survival conducted for 100 days)⁸⁴. Growth kinetics indicated that while there was an increase in wild-type S.Tm splenic bacterial burden between 24 to 48 hours, little growth was seen after 24 hours with S.Tm Δ aroA infection⁸⁴. This was in stark contrast to placental bacteria burden in which both wild-type S.Tm and S.Tm Δ aroA reached a titre of $\sim 10^8$ bacteria by 72 hours, indicating that S.Tm preferentially replicates within placental tissues compared to other systemic tissues⁸⁴.

Furthermore, wild-type S.Tm infection resulted in overt placental inflammation with a profound increase in the pro-inflammatory cytokines granulocyte-colony stimulating factor (G-CSF), tumour necrosis factor alpha (TNF- α), IL-6 and IFN- γ and IL-18⁸⁴. Despite the profound replication of S.Tm Δ aroA within placental tissues, no inflammatory response was evoked. These results were further corroborated with a massive influx of GR-1 positive neutrophils into the labyrinth trophoblast layer during wild-type S.Tm infection. Tissue

integrity was lost with labyrinth trophoblasts being highly necrotic resulting in fetal loss. Such pathology and fetal loss was not seen during S.Tm Δ aroA infection, with bacteria being limited to the decidua. Such studies indicate that it is not the profound bacterial burden within the placental tissues but rather S.Tm induced placental inflammation which results in severe maternal illness and death, and pathology at the feto-maternal interface⁸⁴.

The culmination of such results indicates that pathology at the fetal-maternal interface due to S.Tm infection occurs in three sequential steps: profound growth of bacteria in the placenta, placental inflammation, and a weak splenic innate immune response^{13, 83, 84}.

Recently, Noto Llana and colleagues studied oral *Salmonella enterica* serovar Enteritidis infection during late stages of gestation (day 15 onwards)¹⁹³. Although all pregnant infected mice survived until delivery, S.Enteritidis infection induced massive maternal infection with substantial bacterial burden in the placenta, as well as vertical transmission to both pre-mature and term pups. Infection also resulted in the induction of inflammatory cytokines in the placenta, amniotic fluid, and maternal serum. Infected placentas and amniotic fluid expressed elevated levels of TNF- α , IL-17 and IFN- γ as well as IL-10¹⁹³. There was also increased production of IFN- γ and IL-10 in maternal circulation. Infection resulted in the induction of apoptosis of placental tissues and fetal growth restriction¹⁹³. Therefore, despite the inflammatory state at the feto-maternal interface, as well as in the maternal systemic compartment, *Salmonella* species may have evolved strategies to thrive in the feto-maternal interface.

In vitro studies further addressed S.Tm infection of human cell types. Profound wild-type S.Tm growth occurred in HeLa (cervical epithelial) cells and JEG-3 (placental choriocarcinoma) cells in comparison to THP-1 (macrophage) cells¹⁹⁴. Infection also

resulted in the production of IL-10 by trophoblast cells whereas there was production of IL-6 by infected macrophages¹⁹⁴. Bacterial localization occurred in early phagosomes within trophoblast cells and late phagosomes within macrophages respectively. Unlike macrophages in which phagolysosome fusion was seen in order to curtail pathogen replication, phagolysosome fusion was hindered in trophoblast cells¹⁹⁴. This was attributed to the production of IL-10 by trophoblast cells as neutralization of IL-10 accelerated phagolysosome fusion, causing a reduction in S.Tm proliferation. These results were further validated *in vivo* through the use of anti-IL-10 treated pregnant mice and IL-10 deficient pregnant mice, both of which displayed a decrease in placental bacterial burden in comparison to controls¹⁹⁴. Thus, the production of IL-10 by trophoblast cells delayed phagosome maturation, enabling S.Tm to replicate profusely¹⁹⁴.

1.7 Inflammation and placental cell death

Inflammation is an important process in which tissues are able to respond to various insults. Inflammation underlies a variety of physiological and pathological processes¹⁹⁵. The classical causes of inflammation are infection and tissue injury, triggering an inflammatory response characterized by the up-regulation of chemokines and inflammatory cytokines for the recruitment of immune cells and plasma proteins to the affected site¹⁹⁵. The inflammatory response is a tightly regulated process; a poor inflammatory response could result in a dampened immune response and inadequate clearance of an invading pathogen, while an overt inflammatory response could result in extensive tissue pathology or sepsis¹⁹⁵.

Inflammation also plays important physiological roles; localized inflammation aids with the establishment and progression of pregnancy as seen during implantation or parturition. However, pathological complications at the feto-maternal interface can arise due to excessive inflammation.

Pre-eclampsia, which affects ~2-8% of all pregnancies, is one such complication as a result of excessive inflammation, consisting of two components: placental and systemic causes^{196, 197}. The primary placental cause of pre-eclampsia is defective spiral artery remodelling leading to placental hypoxia and oxidative stress¹⁹⁷. The secondary systemic cause is postulated to be the result of the stress conditions within the placenta, which cause either the syncytiotrophoblasts to synthesize and secrete the inflammatory cytokine TNF- α , or the dissemination of activated leukocytes, thereby acting as the stimulus to cause a systemic maternal inflammatory response^{198, 199}.

Pre-term labour, a consequence of excessive inflammation which prematurely activates parturition, can be initiated by a variety of mechanisms such as stress or endocrine disorders, cervical disease or utero-placental hemorrhage, but is most commonly caused by intrauterine infection^{22, 200}. In fact, infections are responsible for up to 40% of pre-term labour cases⁷². Thus, overt inflammation, triggered by either infection or endogenous dysregulation, is a major trigger of poor pregnancy outcome.

At the feto-maternal interface, there exists an important cross-talk between trophoblast cells and the maternal immune system in preventing pathogen colonization. Pathogens capable of invading and establishing an infection at the feto-maternal interface can activate the immune system leading to an inflammatory response. In an effort to control,

kill, and clear the invading pathogen, placental cell death may ensue in order to curb the spread of infection.

Trophoblast cells are similar to an innate immune cell due to the expression of pattern recognition receptors (PRRs) on the trophoblast cells which function as environmental sensors¹⁴. Expression of PRRs enables the detection of bacteria and viruses as well as damaged or dying cells. Trophoblasts express scavenger receptors and the mannose binding receptor, but more importantly, the toll-like receptors (TLRs)¹⁴. TLRs are transmembrane proteins capable of recognizing pathogen-associated molecular patterns (PAMPs) that are expressed by bacteria, viruses and parasites²⁰¹. In terms of *Salmonella* infection within the placenta and potentially the trophoblast cells, sensing of bacteria may occur through either TLR4 and/or TLR5 which detect bacterial lipopolysaccharides and flagellin respectively²⁰². TLR4 is also capable of binding endogenous danger-associated molecular patterns (DAMPs) such as heat shock proteins and reactive oxygen species²⁰². The production, release, and secretion of endogenous heat shock proteins and reactive oxygen species are a result of cell stress and/or damage²⁰¹. Activation of TLR4 and TLR5 recruits the intracellular signalling adapter protein myeloid differentiation primary response gene 88 (MyD88), triggering a kinase cascade which activates the nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) pathway and the initiation of an inflammatory response^{201, 202}.

Initiation of an inflammatory response and secretion of cytokines results in the recruitment of immune cells to the site of infection. Depending on the extent of infection and/or cell damage, cell death may be initiated. Cell death of the infected cells eliminates the intracellular niche of the pathogen, thereby exposing the intracellular pathogen to

extracellular immune surveillance²⁰³. Although there are benefits to the host in the initiation of cell death, it is not surprising that pathogens have evolved a variety of strategies to modulate cell death in order to evade detection by the host immune response²⁰⁴. Prevention of cell death enables survival and proliferation of the pathogen within its intracellular niche²⁰⁵. On the contrary, initiation of host cell death allows pathogens to escape from the infected cell with the potential to infect surrounding cells or specific immune cells such as macrophages and dendritic cells^{206, 207}.

1.7.1 Programmed Cell Death

Central to the development, integrity, and homeostasis of a multicellular organism is the genetically regulated process of programmed cell death²⁰⁸. It wasn't until the 1960s, through the use of microscopy and histology, that cell death was beginning to be recognized as a biologically controlled process²⁰⁹. It plays a central role in morphogenesis, tissue remodelling, and the loss of structures: inner cell mass differentiation within the blastocyst, the formation of digits via death of interdigital webs, and deletion of the Wolffian duct (forms the vas deferens, epididymis and seminal vesicles) in males respectively²¹⁰⁻²¹².

To date, a variety of programmed cell death pathways have been identified, the most commonly studied being apoptosis, and in recent years two inflammatory cell death pathways known as necroptosis and pyroptosis.

1.7.2 Apoptosis

Apoptosis is the default programmed cell death pathway and occurs during embryonic development and homeostasis²¹³. It is a “silent” form of cell death, as there is no inflammatory response associated with such cell death²¹⁴. Apoptosis is caspase-8 dependent

and can be triggered by two different pathways: the extrinsic pathway and the intrinsic pathway (Fig. 3)^{215, 216}.

The extrinsic pathway is activated via ligation and stimulation of trans-membrane death receptors belonging to the Tumor Necrosis Factor Receptor (TNFR) family (TNFR, FAS and TRAIL-R) and/or binding of PAMPs to PRRs such as Toll-like receptors^{217, 218}. External signalling via these receptors initiates the formation of the death inducing signalling complex (DISC). The DISC complex consists of the adapter proteins TNF receptor associated death domain (TRADD) and/or Fas-associated death domain (FADD) which serves as a platform for the recruitment and autocatalytic activation of procaspase-8²¹⁷. The initiator caspase-8 cleaves the serine/threonine kinases Receptor Interacting Protein (RIP) 1 and RIP3, preventing their phosphorylation and subsequent activation²¹⁸⁻²²⁰. Caspase-8 also cleaves BH-3 interacting domain death agonist (BID) into its truncated form, which engages the intrinsic mitochondrial pathway to amplify the apoptotic response²¹⁷.

The intrinsic pathway is activated in response to cellular stress such as DNA damage, radiation, growth factor depletion or cytotoxic drugs and is regulated by members of the BCL-2 family of regulator proteins^{207, 220}. The proapoptotic effectors BCL-2-associated X protein (BAX) and BCL-2 antagonist/killer (BAK) induce permeabilization of the mitochondrial outer membrane enabling the release of cytochrome-c into the cytoplasm²¹⁷. The apoptotic protease-activating factor 1 (APAF-1) associates with cytosolic cytochrome-c to form a multimeric complex termed the apoptosome. The apoptosome functions to activate the initiator caspase-9²¹⁷.

Activation of the initiators, caspase-8 and caspase-9 via the extrinsic and intrinsic pathways respectively, enables both pathways to converge with the processing of the

(A) EXTRINSIC PATHWAY

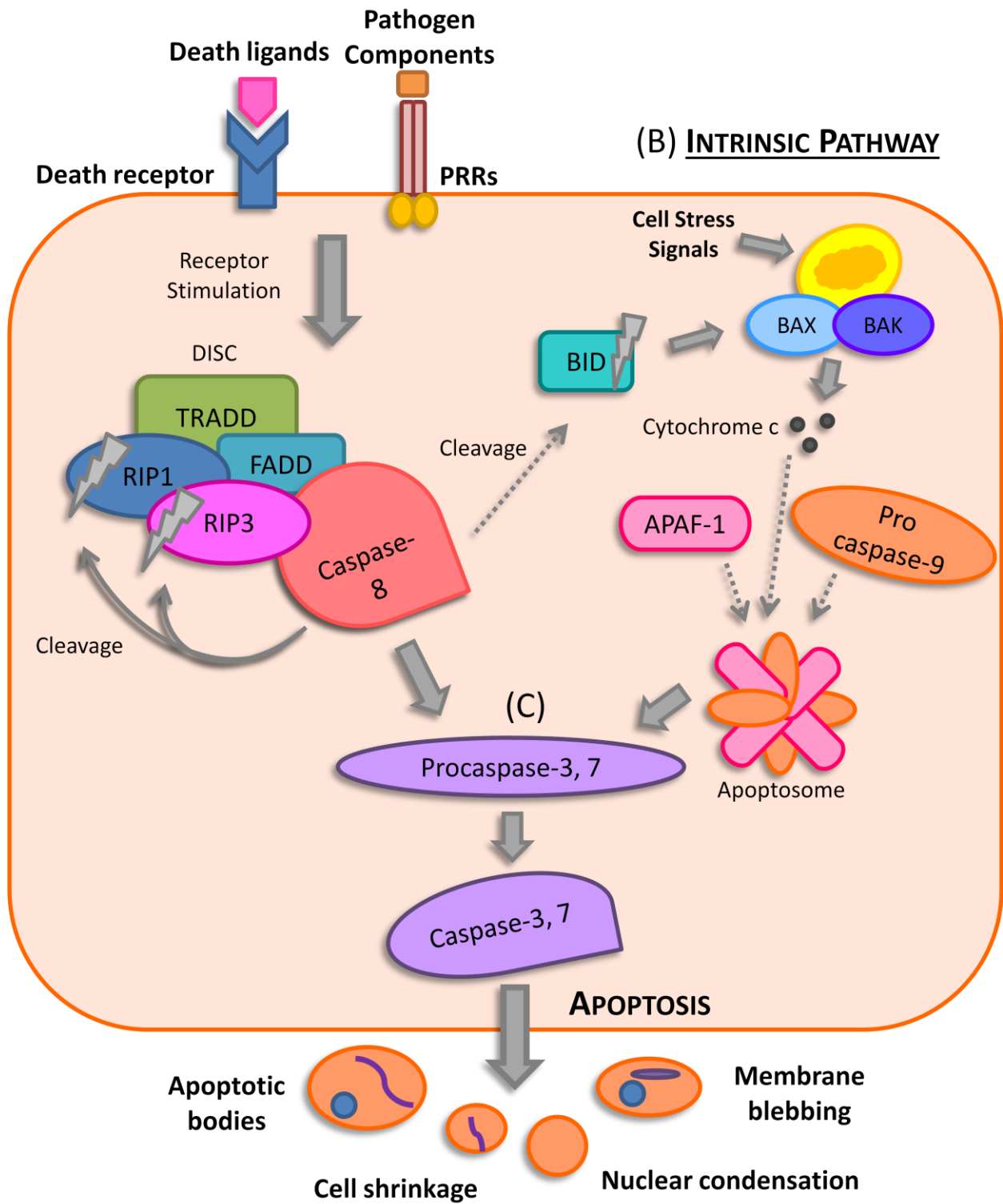


Figure 3

Figure 3. Schematic representation of the extrinsic and intrinsic apoptotic signalling pathways.

A) The extrinsic pathway of apoptosis is activated upon stimulation of death receptors or pattern recognition receptors (PRRs) by their respective ligands, which results in the formation of the death inducing signalling complex (DISC) in which procaspase-8 is recruited and activated. Caspase-8 cleaves RIP1 and RIP3 preventing their phosphorylation and activation. Caspase-8 also cleaves BID, thereby engaging the intrinsic pathway to amplify the apoptotic response. B) The intrinsic pathway of apoptosis is activated upon intracellular stress signals (radiation, toxins, DNA damage, growth-factor depletion). Assembly of pro-apoptotic BAX and BAK oligomers induces the release of cytochrome-c from the mitochondrial intermembrane space. Cytochrome-c associates with APAF-1 to form the apoptosome complex which activates caspase-9. C) Activation of the initiator caspases-8 and/or-9 enables the processing of the executioner caspases-3 and -7, ultimately resulting in the morphological and biochemical characteristic features of apoptosis.

executioner caspases-3 and -7. Activation of caspase-3 and caspase-7 leads to various downstream effects that ultimately result in cytoplasmic and nuclear condensation, chromatin cleavage and the formation of apoptotic bodies through membrane blebbing^{207, 220}. The formation of these bodies enables the plasma membrane to remain intact, preventing the release of any intracellular contents²¹⁴. Such apoptotic bodies are subsequently phagocytosed²²⁰. As a result, such cell death is silent and does not initiate an inflammatory response^{214, 220}.

1.7.3 Necroptosis

Programmed necrosis, termed necroptosis, is a caspase-8 independent cell death pathway which can be observed under a variety of conditions such as exposure to toxins, hypoxia, microbial infection and inflammation (Fig. 4)^{207, 215}.

The serine/threonine kinases RIP1 and RIP3 are key regulators of necroptosis²¹⁶. Necroptosis can be induced by ligation of various death receptors (FAS, TNFR1, TNFR2), toll-like receptors or type I interferon receptor (IFNAR) signalling^{216, 220, 221}. The TNF signalling pathway has been most studied and best exemplifies the induction of necroptotic cell death. Following Tumour Necrosis Factor (TNF) binding to its receptor TNFR1, recruitment of TNF receptor-associated death domain (TRADD) occurs followed by RIP1 recruitment. In the absence of caspase-8, due to its inactivity, RIP1 associates with RIP3, resulting in auto and trans-phosphorylation leading to the formation of the necrosome^{216, 220}. Phosphorylation of RIP3 initiates necroptosis^{216, 220}.

A variety of downstream effectors induce necroptosis through the induction of increased reactive oxygen species (ROS) production, and lysosomal destabilization²⁰⁷.

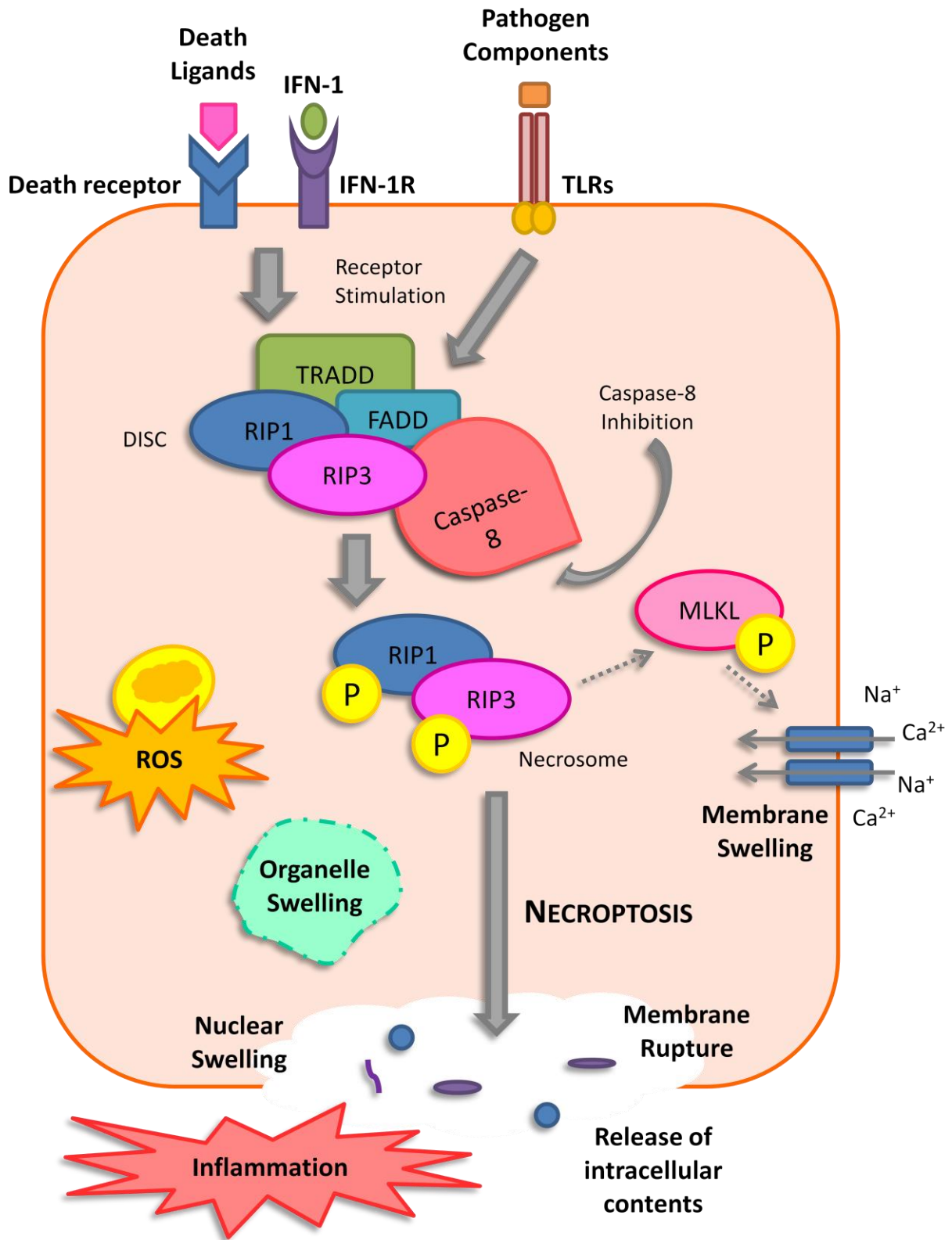


Figure 4

Figure 4. Schematic representation of the necroptotic signalling pathway. Necroptosis can be triggered upon stimulation of death receptors or toll-like receptors when caspase-8 is inhibited. Binding of TNF to TNFR1 results in the recruitment of TRADD, FADD, RIP1 and RIP3. The inactivation of caspase-8 enables RIP1 and RIP3 to autophosphorylate and transphosphorylate forming the necrosome. RIP3 phosphorylates various downstream effectors, such as MLKL, leading to necroptotic cell death. Phosphorylated MLKL enables it to oligomerize, bind phosphatidylinositol phosphates and form membrane disrupting pores which enable the influx of sodium and calcium ions. Cell swelling ensues with membrane rupture, releasing intracellular contents into the extracellular milieu.

Mixed-lineage kinase domain-like protein (MLKL) is one downstream effector which is phosphorylated by RIP3^{216, 220, 222}. Phosphorylated MLKL translocates to the plasma membrane, binds to phosphatidylinositol phosphates and forms a pore-forming complex^{216, 220}. The formation of disrupting pores enables the influx of both sodium and calcium into the cell, leading to a rise in osmotic pressure and cell swelling²¹⁶. The end result is membrane rupture with massive DAMP release; a strong inducer of inflammation²⁰⁷.

1.7.4 Pyroptosis

Lastly, a second type of inflammatory cell death pathway known as pyroptosis is a caspase-1 dependent pathway (Fig. 5)²⁰⁷. Intracellular pathogens can activate the innate immune system when PAMPs or DAMPS are recognized by cytoplasmic PRRs known as NLRs (nucleotide-binding domain and leucine-rich repeat containing, or NOD-like receptor)^{207, 223}.

NLRs function in a similar manner to cell surface TLRs, acting as sensors within the cell²²⁴. Studies involving S.Tm infection of macrophages and DCs has resulted in pyroptotic cell death²²⁵⁻²²⁷. Thus, pathogen invasion or the induction of pathogen cytotoxins into cells such as macrophages, DCs or potentially trophoblast cells, can be detected by NLRs followed by pyroptotic cell death²²⁸.

The NLRs form large cytosolic protein complexes termed “inflammasomes”²²⁹. Bacterial LPS is detected by NOD-, LRR- and pyrin domain containing 3 (NLRP3) inflammasome while the Ice protease-activating factor (IPAF) inflammasome (also known as NLRC4) detects bacterial flagellin components of gram negative bacteria^{207, 230}. Upon stimulation, the NLR undergoes oligomerization and associates with the adapter protein

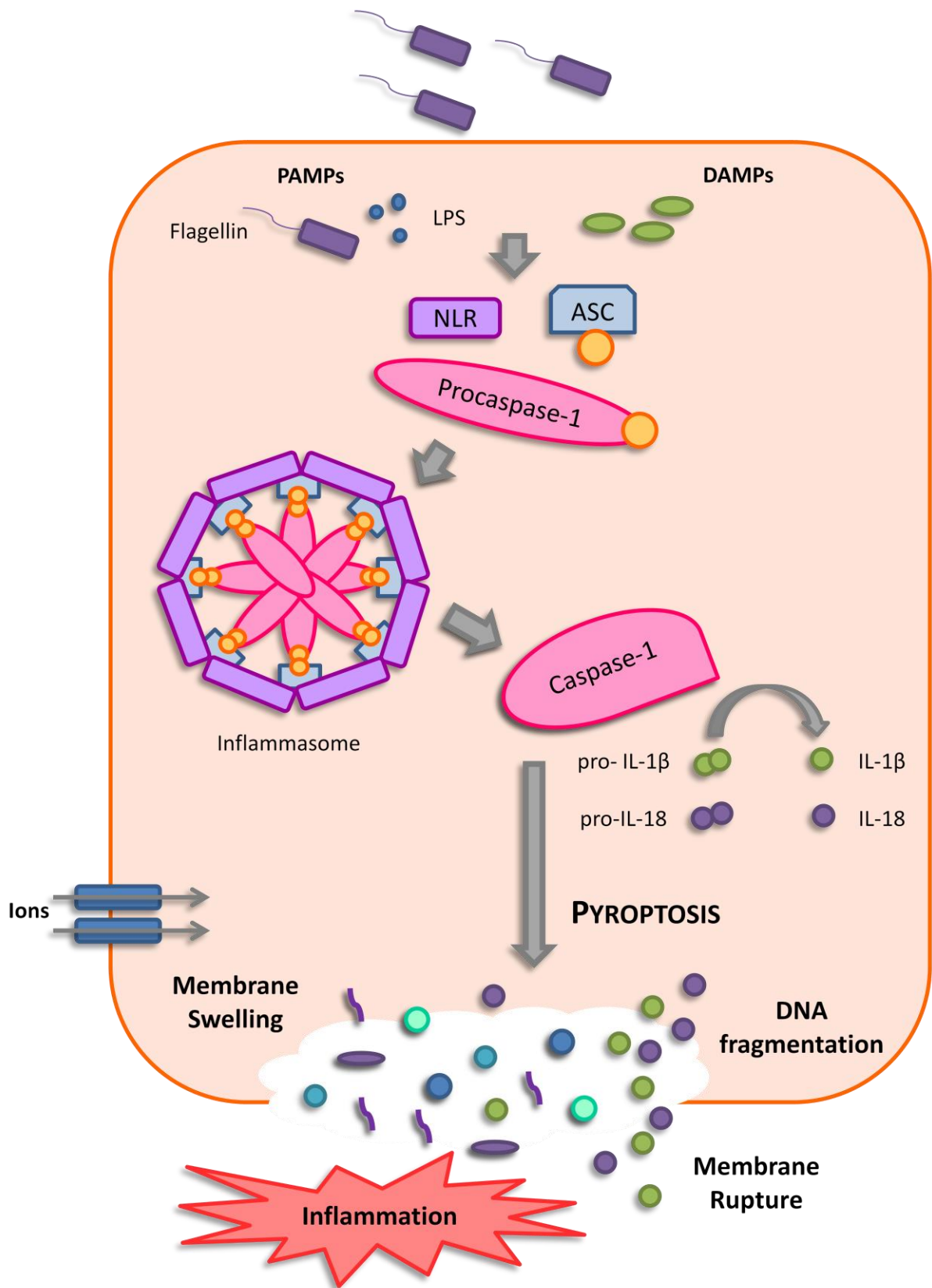


Figure 5

Figure 5. Schematic representation of the pyroptotic signalling pathway. Pyroptosis can be triggered upon detection and stimulation of NLRs by PAMPs and DAMPs within the cell cytoplasm. Assembly of the inflammasome enables the activation of caspase-1. Active caspase-1 leads to membrane pore formation with an influx of ions and subsequent membrane rupture. Caspase-1 mediated maturation of pro-IL-1 β and pro-IL-18 into their mature secretory forms results in the induction of an inflammatory response.

apoptosis-associated speck protein containing a CARD (ASC)^{207, 231, 232}. The caspase activation and recruitment domain (CARD) on the ASC molecule enables oligomerization through CARD-CARD interactions with subsequent recruitment and proximity-induced auto-activation of caspase-1^{207, 231, 232}. The downstream effects are pore formation within the plasma membrane of the cell which promotes cell swelling and membrane rupture, and release of intracellular contents into the extracellular milieu²⁰⁷. Furthermore, unlike necroptosis but similar to apoptosis, nuclear condensation and DNA fragmentation occurs²⁰⁷. In addition, caspase-1 cleaves pro-IL-1 β and pro-IL-18 into their mature, biologically active and secretory forms IL-1 β and IL-18 leading to the induction of an inflammatory response^{207, 232}.

Thus, programmed cell death is either tolerogenic or immunogenic, depending on the type of cell death which is initiated, and necroptosis and/or pyroptosis is postulated to be a mechanism of overt inflammation at the feto-maternal interface during *Salmonella* Typhimurium infection.

2.0 Rationale and Hypothesis

2.1 Rationale

Salmonella is a prominent foodborne pathogen causing illness in both developed and developing nations. It is estimated that 1.3 billion cases of salmonellosis occurs each year worldwide with roughly 3 million deaths caused by NTS²³³. According to the 2013 April Morbidity and Mortality report from the CDC, of the 19,056 culture confirmed foodborne pathogen cases in the U.S., *Salmonella* had the highest incidence with 7,277 cases resulting in 2,003 hospitalizations and 27 deaths. The top *Salmonella* serotypes were Enteritidis (19%), Typhimurium (14%) and Newport (10%).

In the developing world, non-typhoidal *Salmonella* infections are the leading cause of bloodstream infection with a case fatality rate of 20-25% in both children and adults¹³⁷. Furthermore, NTS infection is the main cause of meningitis with a case fatality rate of 52% in children and 80% in adults¹³⁷. Severe invasive NTS infection is a growing concern in sub-Saharan Africa where comorbidities such as HIV²³⁴, malaria²³⁵, sickle cell disease²³⁶ or schistosoma²³⁷ infection are prevalent. Environmental risk factors also contribute to a higher prevalence of invasive NTS in sub-Saharan Africa, as disease prevalence is increased during the rainy season when fecal organisms are highly prevalent in drinking water sources^{238, 239}.

According to the World Health Organization (WHO) Essential Drugs Monitor, roughly 500,000 pregnant women die each year, of which more than 99% are in developing countries²⁴⁰. Although various causes/complications such as unsafe abortion and haemorrhage result in maternal death, infections are considered a major cause of pregnancy complication²⁴⁰.

The various case reports within the literature highlight the need to study *Salmonella* infection during pregnancy in order to understand the host-pathogen interplay with the hopes of developing new and effective therapies. While *Salmonella* Typhimurium infection in pregnant mice causes rapid and severe fetal loss and maternal death due to massive placental TBC proliferation, there is a gap in our knowledge regarding the mechanism(s) of increased susceptibility to *Salmonella* infection during pregnancy.

- Previous published studies have shown that mice which lack a functional *Nramp1* gene succumb to low doses of *Salmonella* in a non-pregnant state. Considering pregnant C57BL/6J mice rapidly succumb to S.Tm infection within 2-3 days, pregnant congenic *Nramp1*^{+/+} mice may exhibit reduced susceptibility to S.Tm infection, enabling a greater time frame to study the adverse effects of S.Tm infection during murine pregnancy.
- Murine pregnancy studies have highlighted that S.Tm replicate profoundly in placental tissue compared to systemic tissues. Such studies investigated bacterial burden amongst pooled placentas of individual mice. However, there are multiple fetal-placental units during murine pregnancy. Thus, are all placentas within an individual mouse infected equally, or is a focus of infection established, resulting in the subsequent spread of infection to surrounding placentas as time progresses? Furthermore, host immune responses may differ at each fetal-placental unit based on the degree of infection, highlighting the importance of examining the extent of infection amongst individual placental tissues.
- Immunophenotyping of spleens during S.Tm infection has revealed differences in immune cell subsets between pregnant infected and non-pregnant infected hosts. Thus, does differential immune modulation exist at the feto-maternal interface compared to the

systemic compartment in pregnant infected mice compared to non-pregnant infected mice and pregnant non-infected mice? Considering that modulation of host immune responses to intracellular infections in the placenta is not well understood, does an ineffective immune response exist, thereby resulting in placental cell death?

- Many intracellular pathogens evoke cellular inflammation and programmed cell death. Trophoblast cell death may be distinct from that encountered in other infected cells, thereby contributing to overt inflammation. The mechanism of *Salmonella* induced placental inflammation and trophoblast cell death is not understood and may be a result of either the necroptotic or pyroptotic inflammatory signaling pathway. These studies are designed to bridge this lacuna in our knowledge of the *in vivo* interactions of infectious agents and the placenta.

2.2 Hypothesis

We hypothesize that profound *in vivo* placental *Salmonella enterica* serovar Typhimurium infection first triggers a local inflammatory response resulting in trophoblast cell death, which promotes bacterial dissemination, evoking overt inflammation and precipitating a cascade of systemic responses resulting in severe maternal illness, fetal and/or maternal death.

2.3 Objectives

The main objective is to address the kinetics and consequence of placental S.Tm infection in pregnant mice to elucidate the mechanism(s) of increased susceptibility of pregnant hosts to foodborne infections. This objective will be reached through the completion of three aims:

1. Elucidate the kinetics of placental S.Tm infection in *Nramp1*^{+/+} mice
2. Profile the differentially regulated immune cell populations at the feto-maternal interface during S.Tm infection
3. Delineate the *in vivo* modulation of cell death pathways influencing the health of the placenta through the use of cell death gene-deficient mouse strains

3.0 Methods

3.1 Mice

Mice were housed in the animal facility at the National Research Council Canada (Human Health Therapeutics, Ottawa, Ontario, Canada) and maintained in accordance with the guidelines of the Canadian Council on Animal Care. All protocols/procedures were approved by the institutional Animal Care Committee. C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). All mice which were ordered, were acclimated for 1 week prior to any matings or experiments. *Rip3*^{-/-} mice were provided by Dr. V. Dixit (Genentech, San Francisco, USA)²⁴¹. *Ifnar1*^{-/-} mice were from Dr. K. Murali-Krishna (Emory University, Atlanta, Georgia, USA)^{242, 243}. *Caspase-1,11*^{-/-} double knock-out mice were a kind gift from Dr. R. Flavell (Yale University, New Haven, Connecticut, USA)²⁴⁴. *Nramp1*^{+/+} mice were provided by Dr. G. Barton (University of California, Berkeley, Berkeley, California, USA)¹⁹¹. *Rip3*^{-/-}, *Ifnar1*^{-/-}, *Caspase-1,11*^{-/-} and *Nramp1*^{+/+} were bred in-house. All mice were bred on the C57BL/6 genetic background.

Rip3^{-/-} mice and *Caspase-1,11*^{-/-} mice were independently crossed with *Nramp1*^{+/+} mice. F2 progeny were genotyped to determine the lack of specific cell death genes *Rip3* or *Caspase-1,11* and the presence of the *Nramp1* gene. F2 progeny of suitable genotypes were then paired and bred. This process was continued until the 4th filial generation, generating two new knock-out strains on the *Nramp1*^{+/+} background, referred to as *Rip3*^{-/-}*Nramp1*^{+/+} and *Caspase-1/11*^{-/-}*Nramp1*^{+/+}.

3.2 Genotyping

Rip3 or *Caspase-1,11* deficiency was validated by Polymerase Chain Reaction (PCR) of genomic DNA from mouse ear punches. Briefly, genomic DNA was isolated using the Proteinase K (Sigma-Aldrich Canada Co., Oakville, ON) method followed by amplification via PCR. The amplified DNA product was separated on a 1 to 1.5% agarose gel and visualized with ethidium bromide. To determine the presence of a functional *Nramp1* gene, genomic DNA isolated from ear punches was purified and the *Nramp1* gene was amplified by PCR. The purified PCR product was then screened by sequencing for a single point mutation (guanine to alanine) at position 169, resulting in a glycine to aspartic acid substitution within NRAMP1. The primer pairs utilized are listed in Table 1.

3.3 Mating and pregnancy

For mating, two female mice, age-matched, eight to twelve weeks old, were placed into a cage overnight with one male. The following morning, detection of a copulatory plug, referred to as being vaginal plug positive (VP+), was deemed as day 0 of pregnancy. For all experiments, female mice were placed into mating over a span of two nights, with detection of copulatory plugs occurring each successive morning.

3.4 Bacteria and *in vivo* infections

Salmonella enterica serotype Typhimurium strain SL1344 was used for all experiments (abbreviated as S.Tm). Wild-type S.Tm were grown in liquid culture in brain heart infusion (BHI) medium (Difco Laboratories, BD Biosciences, Franklin Lakes, NJ) under constant shaking at 37°C. At mid log phase ($OD_{600} = 0.8$), S.Tm were harvested and

Table 1

Primers utilized for determining the presence of the *Nramp1* gene or lack of the cell death genes *Rip3* or *Caspase-1*

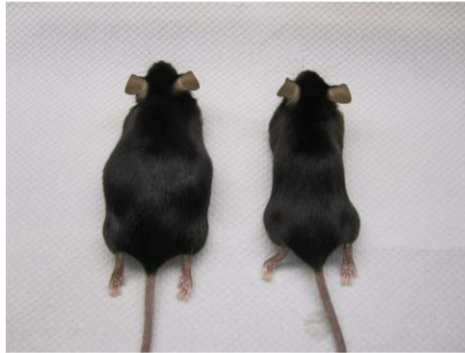
Mouse Strain	Primers utilized for genotyping	
<i>Nramp1</i> ^{+/+}	<i>Nramp1</i> forward	5' – TCATCGGGACGGCTATCTCCTTCAA – 3'
	<i>Nramp1</i> reverse	5' – TTGCGCAAACCTAGGGGTACAGGGA – 3'
<i>Rip3</i> ^{-/-}	<i>Rip3</i> forward common	5' – AATCGTTCCTGGATGGTGAG – 3'
	<i>Rip3</i> reverse wild-type	5' – GGAGCCATTCTCCATGAATC – 3'
	<i>Rip3</i> reverse mutant	5' - GATCCTGATCCTGACCCTGA – 3'
	<i>Rip3</i> neomycin	5' – ATCGACAAGACCGGCTTCCATCCGA – 3'
<i>Caspase-1</i> ,11 ^{-/-}	<i>Caspase-1</i> forward wild-type	5' – GAGACATATAAGGGAGAAGGG – 3'
	<i>Caspase-1</i> forward mutant	5' – TGCTAAAGCGCATGCTCCAGACTG – 3'
	<i>Caspase-1</i> reverse common	5' – ATGGCACACCACAGATATCGG – 3'

frozen at -80°C in 20% glycerol. Serial dilutions in 0.9% saline were spread on BHI agar plates containing streptomycin (Sigma-Aldrich) to determine bacterial concentration.

For *in vivo* intravenous (I.V.) infections, a frozen bacterial stock of S.Tm was thawed and diluted in 0.9% saline to a final concentration of 1×10^3 bacteria/200 μ L 0.9% saline as determined by retrospective bacterial plate counts. Pregnant mice (day 11 and 12 of vaginal positivity) along with non-pregnant controls (Fig. 6 A & B), were inoculated with 1×10^3 S.Tm suspended in 200 μ L of 0.9% saline via the lateral tail vein. Post-inoculation colony forming unit assays were carried out to verify mouse inoculation concentrations for each experiment.

For *in vivo* oral gavage infections, pregnant mice (day 8 and 9 of vaginal positivity) along with non-pregnant controls were deprived of food and water for 3 hours (at 7 a.m.) prior to infection. At 10 a.m., fasting mice were treated orally with 20 mg streptomycin sulfate salt dissolved within 100 μ L of Type 1 ultrapure water (Millipore Canada Ltd. Etobicoke, ON). Two hours after antibiotic treatment (at 12 noon) food and water were returned ad libitum. Prior to initiating infection, wild-type Salmonella Typhimurium strain SL1344 were grown overnight in Lysogeny Broth (LB) broth containing 50 μ g/mL of streptomycin at 37°C with shaking at 200 rpm in a shaking incubator. The following morning at 7 a.m. pregnant mice (day 9 and 10 of vaginal positivity) and non-pregnant controls were fasted of food and water. Early that same morning, S.Tm was subcultured in fresh LB broth containing 50 μ g/mL of streptomycin at a ratio of 1:40 (500 μ L overnight culture in 20 mL LB broth containing streptomycin). The bacterial culture was grown to a standardized OD₆₀₀ of 0.8 within 2.5 to 3 hours. The appropriate amount of culture was spun down at 12,000 rpm for 5 minutes and re-suspended in the appropriate volume of 0.9%

(A)



(B)



(C)



Figure 6

Figure 6. Images of pregnant versus non-pregnant mice and tissues. A) A gestational day 15 mouse on the left versus an age-matched non-pregnant female on the right. B) Uterine horns with embryos (gd 15) in a pregnant non-infected mouse. Left and right horns are designated by experimenter's left and right and not the mouse's left and right. Four implants on left horn, 3 implants on right horn. C) Uterine horns of a non-pregnant non-infected mouse.

saline for a final concentration of 1×10^8 S.Tm/100 μ L 0.9% saline. Initiation of the subculture was started at an appropriate time in order to reach the appropriate OD₆₀₀ of 0.8 at ~9:30 a.m. enabling mice to be inoculated at 10 a.m., 24 hours after oral streptomycin treatment. Mice were inoculated by oral gavage with 1×10^8 S.Tm/100 μ L of 0.9% saline at 10 am. Food and water were returned ad libitum at 12 noon that same day.

3.5 Monitoring the clinical course of infection

Pregnant mice along with age-matched non-pregnant controls were monitored twice per day throughout the study. Animals were ranked on a daily basis according to appearance. The rankings were as follows: A – healthy; B – ruffled fur, lively; C – ruffled fur, activity slowing, sick; D – ruffled fur, hunched, very little activity, eyes squeezed shut, very sick; E – moribund; F – deceased. Animals were humanely euthanized when the experimental endpoint of C or D was reached.

3.6 Assessment of bacterial burden in organs

For intravenous infection experiments, mice were euthanized by isofluroane inhalation followed by cervical dislocation between 24 and 96 hours post infection. For intragastric infection experiments, mice were euthanized 5 days post oral challenge. Spleen, liver, mesenteric lymph nodes (MLN) and placentas were aseptically removed and placed in various amounts of RPMI 1640 medium (Gibco, Life Technologies, Burlington, ON) (spleen, liver, pooled placenta – 10mL/tissue, MLN – 3mL, individual placentas – 1mL). For the spleen and MLN, single cell suspensions were obtained by homogenizing the organ between frosted ends of two glass slides. Liver and placentas (pooled or individual) were placed in glass tubes and homogenized using a motorized homogenizer. In cases where it

was difficult to discern a resorbed placenta from a resorbed fetus, the entire uteroplacental unit was collected and homogenized. Ten-fold serial dilutions of the tissue homogenates, in 100µL volume of 0.9% saline, were carried out and plated on LB agar plates. In order to assess bacterial burden at early time points, 1 mL of homogenate was plated on LB agar. In order to assess total bacterial burden in individual placentas, the remaining homogenate (800 µL) was plated between two plates (400 µL each). Plates were placed in an incubator overnight at 37°C and colony forming units were counted 24 hours later.

3.7 Assessment of pregnancy outcome

Placental/fetal resorptions were identified by the necrotic/hemorrhagic appearance of the placenta as well as the notably smaller size and necrotic appearance of the fetus when compared with normal viable fetuses and placentas (Fig. 7 A-D). The percent resorption rate was calculated using the formula $R/(R+V) \times 100$, where R is the number of resorbing/resorbed placentas/fetuses and V is the number of viable placentas/fetuses per mother.

3.8 Flow cytometric analysis of immune cell subsets

Single cell suspensions of spleens from pregnant-infected (PI), non-pregnant infected (NPI), pregnant non-infected (PNI) and non-pregnant non-infected (NPNI) mice, as well as placentas from PI and PNI mice, were analyzed to determine the various proportions of immune cell types based on the expression of specific cell surface markers. Spleens were collected in 10 mL of RPMI containing 8% fetal bovine serum (FBS) (North Bio Inc., Toronto, ON). Single cell suspensions of spleens were obtained by homogenizing the organ between the frosted ends of two glass slides. Spleen homogenate was passed

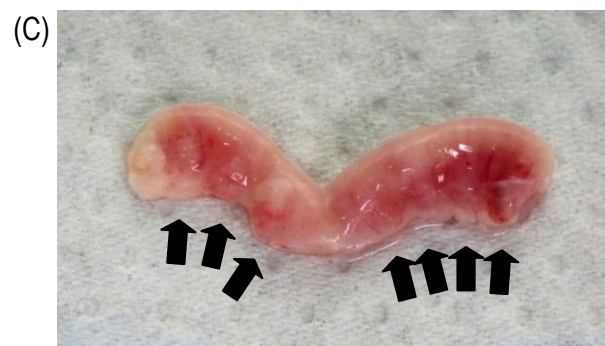


Figure 7

Figure 7. Images of pregnancy outcome in a naive versus infected state. A) Uterine horns containing gestational day 15 fetuses from a pregnant non-infected mouse with no resorptions. Four implants on left horn, 5 implants on right horn. B) Uterine horns containing gestational day 15 fetuses from a pregnant infected mouse with 4 resorptions indicated by black arrows. Two healthy implants and 2 resorbed implants on left horn, 2 resorbed implants and 2 healthy implants on right horn. C) Uterine horns containing gestational day 15 fetuses from a pregnant infected mouse with 100% resorptions. Three resorbed implants on left horn and 4 resorbed implants on right horn indicated by black arrows. D) Healthy gestational day 15 fetus from a pregnant non-infected mother. Attachment of the umbilical cord from the fetus to the placenta is visible.

through a 100 μ m cell strainer (Falcon, BD Biosciences, Mississauga, ON) and counted using a hemocytometer.

For pooled placental analysis, tissue was collected in 10mL of RPMI 1640 medium containing 8% FBS. For individual placental analysis, each placenta was collected in 1mL RPMI 1640 medium containing 8% FBS. Single cell suspensions of placental tissue (pooled or individual) were obtained by lightly homogenizing the tissue in 0.2% collagenase type IV (Worthington Biochemical Corporation, Lakewood, NJ) in Hank's balanced salt solution (HBSS) and incubated with light shaking (100 rpm) for 45 minutes at 37°C. In order to determine which individual placentas were infected in pregnant-infected hosts, 100 μ L of homogenate was plated onto LB plates prior to the 45 minute incubation. Plates were incubated for 24 hours at 37°C and counted the following day. Samples were then filtered (100 μ m mesh cell strainer) to obtain a single cell suspension. Cells were then subjected to 40%/70% Percoll Plus (GE Healthcare Biosciences) gradient centrifugation (2000 rpm, 25 minutes, no brake). The lymphocyte interface was collected, washed and counted.

For staining procedures, 1×10^6 cells of each tissue sample were first incubated for 10 minutes at 4°C with anti-mouse CD32/CD16 (Fc γ II/III receptor) suspended in phosphate buffered saline (PBS) (Sigma-Aldrich) supplemented with 1% bovine serum albumin (Sigma-Aldrich) (PBS-1%BSA) to prevent non-specific antibody binding to Fc receptors. Samples were then incubated with the following primary antibodies (0.5 μ g/mL) for an additional 30 minutes at 4°C: PerCP-Cy5.5-conjugated anti-mouse CD45 (BD Biosciences; cat # 561869), FITC-conjugated anti-mouse Cytokeratin-7 (Santa Cruz Biotechnology; cat # sc-23876 FITC), APC-conjugated anti-mouse CD11b (BD Biosciences; cat # 561690), PE-CF594-conjugated anti-mouse CD11c (BD Biosciences; cat # 562454), BV421-conjugated

anti-mouse Ly-6G (BD Biosciences; cat # 562737), PE-Cy7-conjugated anti-mouse CD49b (eBioscience; cat # 25-5971-82), APC-Cy7-conjugated anti-mouse TCR β (BD Biosciences; cat # 560656), BV605-conjugated anti-mouse F4/80 (BioLegend; cat # 123133), BUV395-conjugated anti-mouse CD19 (BD Biosciences; cat# 563557). Cells were washed with PBS-1%BSA and re-suspended in 400 μ L PBS. Data acquisition was performed on the BD LSR Fortessa® using BD FACSDiva® software (BD) and data analysis was performed using FlowJo® software (Tree Star Inc.).

3.9 Statistical Analysis

The non-parametric, two-tailed Mann Whitney U test and analysis of variance (ANOVA) followed by the appropriate post-hoc tests, were used as appropriate and stated within the figure legends of the corresponding figure, in order to determine the statistical significance of the experimental data.

4.0 Results

4.1 S.Tm infection survival kinetics in C57BL/6J and *Nramp1*^{+/+} strains

The clinical course of systemic S.Tm infection was examined in congenic *Nramp1*^{+/+} and C57BL/6J mouse strains during pregnant and non-pregnant state. Systemic infection was initiated with 10³ CFUs of S.Tm during mid-pregnancy (days 11-12) along with age-matched non-pregnant controls. Non-pregnant *Nramp1*^{+/+} mice survived significantly longer than non-pregnant C57BL/6J mice with median days of survival being 29 and 6 days respectively (Fig. 8). However, following initiation of infection during pregnancy, ~71% of *Nramp1*^{+/+} mice succumbed to infection within 6 to 11 days, which was significantly longer compared to C57BL/6J mice which succumbed to infection within 2 to 3 days. Thus, while pregnancy increased the susceptibility of both strains to S.Tm infection, *Nramp1*^{+/+} mice lived significantly longer than C57BL/6J mice.

4.2 Exacerbation of placental S.Tm infection compared to systemic tissues in *Nramp1*^{+/+} hosts

Pregnant *Nramp1*^{+/+} mice infected systemically with 10³ S.Tm exhibited a rapid increase in bacterial burden within the pooled placental tissue between 24 and 72 hours (Fig. 9 A-C). Systemic organs, such as spleen and liver showed similar bacterial titres in comparison to non-pregnant controls at each time point (Fig. 9 A-C). At 24 hours, mean bacterial burden in pooled placentas was dramatically higher (~10⁶ CFUs) compared to spleen and liver of the same host (~10³ CFUs) (Fig. 9 A). By 72 hours, ~10⁹ bacteria could be recovered from pooled placentas of individual mice. While the systemic organs also

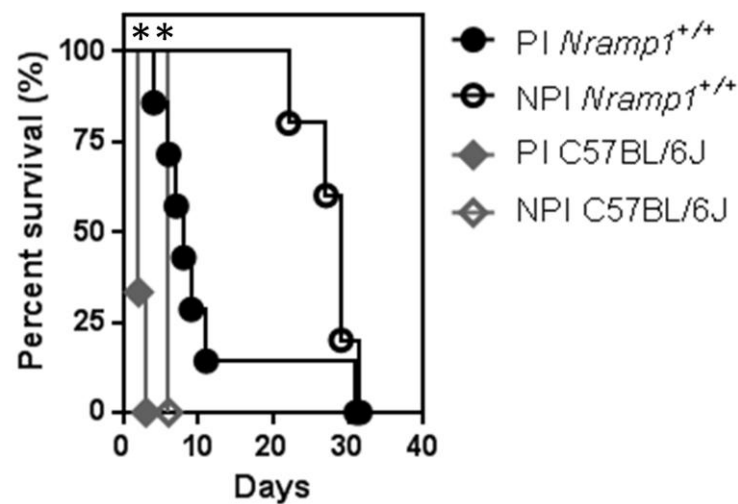


Figure 8

Figure 8. Survival kinetics of pregnant infected and non-pregnant infected *Nramp1*^{+/+} and C57BL/6J mice. Pregnant mice were infected mid-pregnancy with 10³ S.Tm along with age-matched non-pregnant controls; 3-7 mice per category. Median days of survival are listed as follows: PI *Nramp1*^{+/+} = 8, NPI *Nramp1*^{+/+} = 29, PI C57BL/6J = 2, NPI C57BL/6J = 6. Pregnant infected *Nramp1*^{+/+} mice survived significantly longer (**p<0.001) than pregnant infected C57BL/6J mice based on the log rank (Mantel-Cox) test.

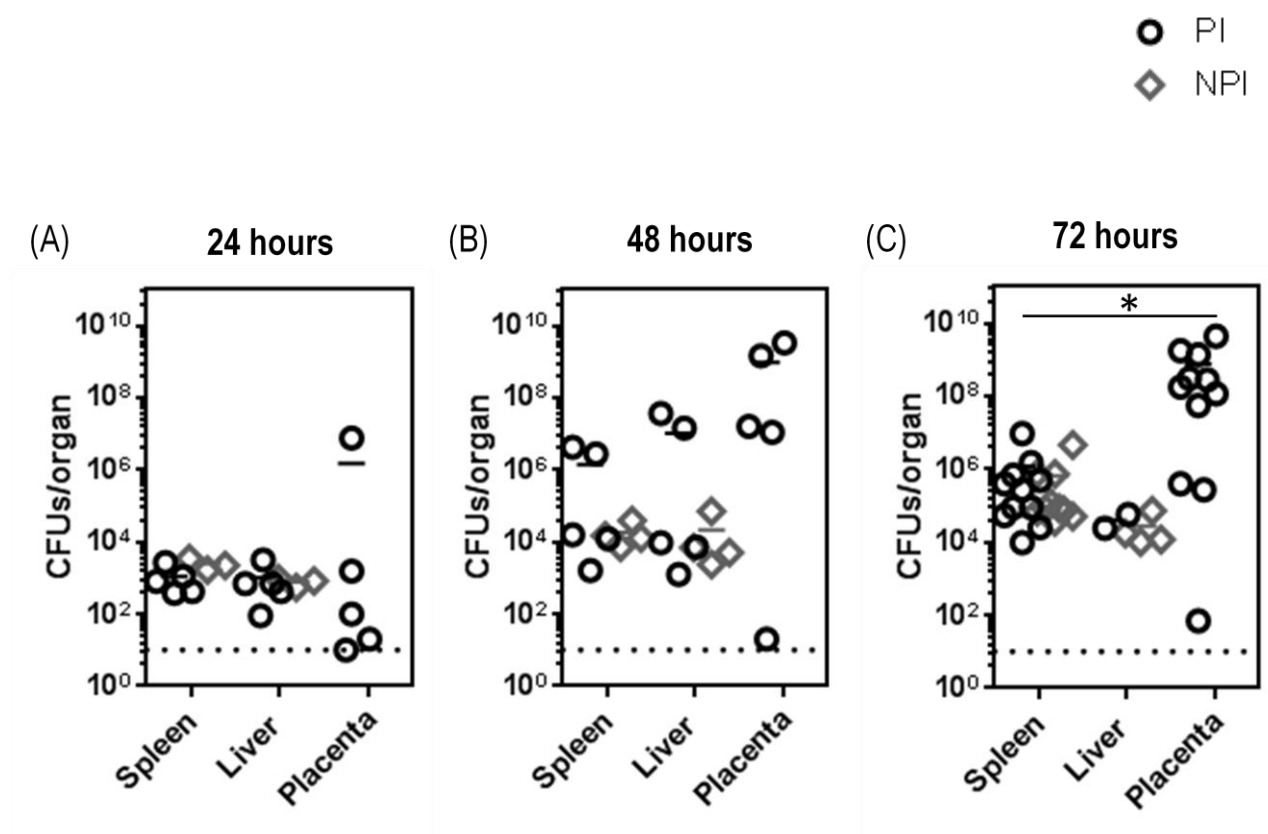


Figure 9

Figure 9. Kinetics of S.Tm infection in pregnant *Nramp1*^{+/+} mice. A) Bacterial burden in spleen, liver and pooled placentas 24 hours after 10³ S.Tm infection. B) Bacterial burden 48 hours post-infection. C) Bacterial burden 72 hours post-infection. Pooled placental bacterial burden was significantly higher (*p<0.05) than spleen bacterial burden by 72 hours; based on the two-tailed Mann Whitney *U* test. Each data point represents an individual mouse. Experiment was repeated three times; 2-11 mice per category depending on time point. Mean (horizontal line) and detection limit (dotted line) are shown.

showed a steady increase in bacterial burden, there was no significant difference between the non-pregnant and pregnant groups (Fig. 9 C). Thus, S.Tm bacteria showed a preference to survive and proliferate at the placental interface.

4.3 Kinetics of bacterial burden in individual placentas within a mouse

We next wanted to determine the manner in which infection within individual concepti sites was occurring. Individual placentas were carefully dissected out from each mother and processed for determination of bacterial burden. Twenty-four hours after systemic infection, a few select placentas per mother were infected with $\sim 10^2$ - 10^5 CFUs (Fig. 10 A & B). Furthermore, CFUs from individual placental tissue were similar in trend to the CFUs of pooled tissue (Fig. 10 B and Fig. 9 A). By 48 hours, there was a steady increase (66%) in the number of infected placentas, correlating to a higher mean bacterial burden ($\sim 10^6$ S.Tm per placenta) (Fig. 10 A & B). By 72 hours $\sim 80\%$ of the individual placentas were infected with a further increase in mean bacterial burden reaching $\sim 10^9$ S.Tm (Fig. 10 A & B).

Overall, these data suggest that infection of pregnant hosts with 10^3 S.Tm during mid-pregnancy resulted in a sequential increase in the number of infected placentas. This correlated with progressive resorptions with $>50\%$ fetal loss by 72 hours (Fig. 10 C).

A schematic representation of the implantation sites for 24 (Fig. 11), 48 (Fig. 12) and 72 (Fig. 13) hours post-infection suggest that a focus of infection is established at an individual implantation site, with an increase in bacterial burden in surrounding placentas as time progresses leading to resorptions.

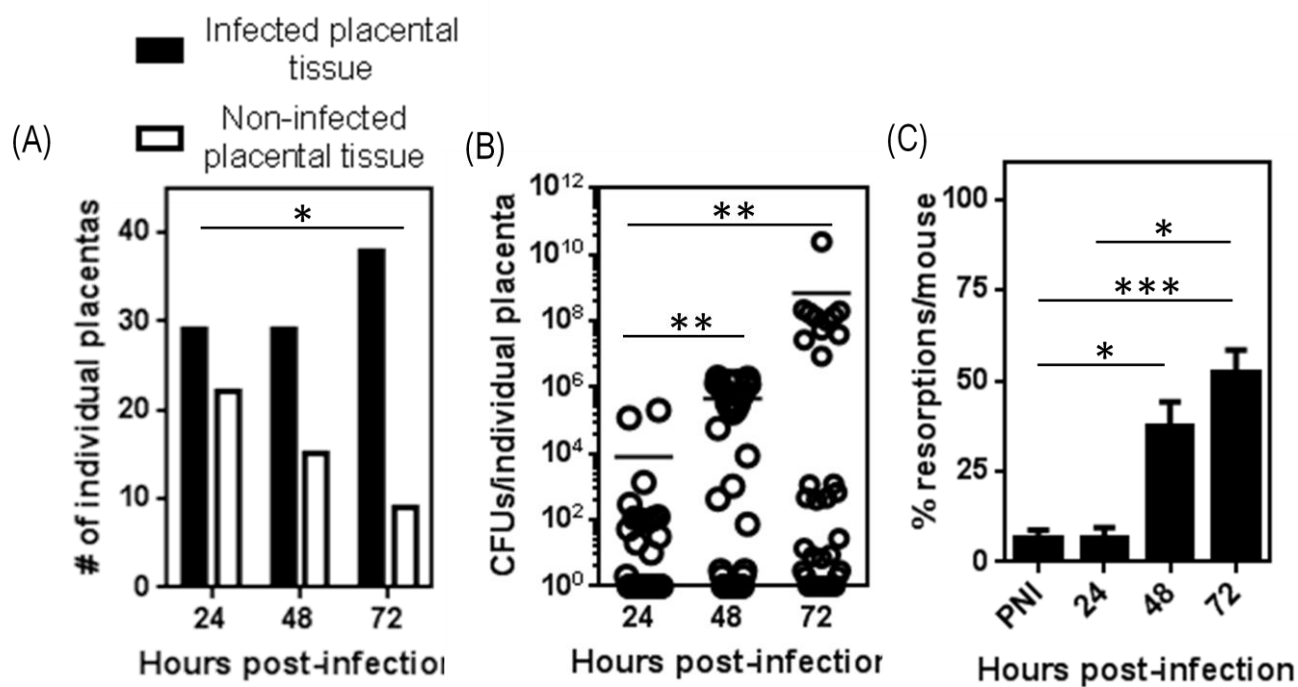


Figure 10

Figure 10. Kinetics of infection amongst individual placentas. A) Proportion of the number of individual infected versus non-infected placentas at 24, 48 and 72 hours post-infection. N numbers are as follows: 24 hours: 29 infected, 22 non-infected, 51 total; 48 hours: 29 infected, 15 non-infected, 44 total; 72 hours: 38 infected, 9 non-infected, 47 total. There is a significant ($*p \leq 0.05$) association/relationship in the number of individual infected placentas as time progresses; based on a chi-square test of independence. B) Kinetics of infection in individual placentas at 24, 48 and 72 hours after infection. Each data point represents the bacterial burden of a single placenta. Experiment was repeated 6 times. Mean indicated by horizontal line. Detection limit is equal to one. Individual placentas were collected from 6-7 pregnant mice at each time point with individual placenta n numbers as follows: 24 hours = 51 placentas, 48 hours = 44 placentas, 72 hours = 47 placentas. Bacterial burden is significantly higher ($**p \leq 0.001$) in individual placentas at 72 hours and at 48 hours in comparison to 24 hours; based on a one-way ANOVA nonparametric Kruskal-Wallis test. C) Percentage of resorbed placentas per mouse at 24, 48 and 72 hours post-infection. Resorption rates per mouse are based on all experiments conducted with *Nramp1*^{+/+} mice with n numbers per time point as follows: PNI = 34 mice, 24 hours = 11 mice, 48 hours = 37 mice, 72 hours = 52 mice. Data represent mean $\pm$ SEM. Resorption rates are significantly higher at 48 hours ($*p \leq 0.05$) and 72 hours ($***p \leq 0.001$) post-infection in comparison to PNI mice and significantly higher at 72 hours ($*p \leq 0.05$) post-infection in comparison to 24 hours; based on a one-way ANOVA nonparametric Kruskal-Wallis test.

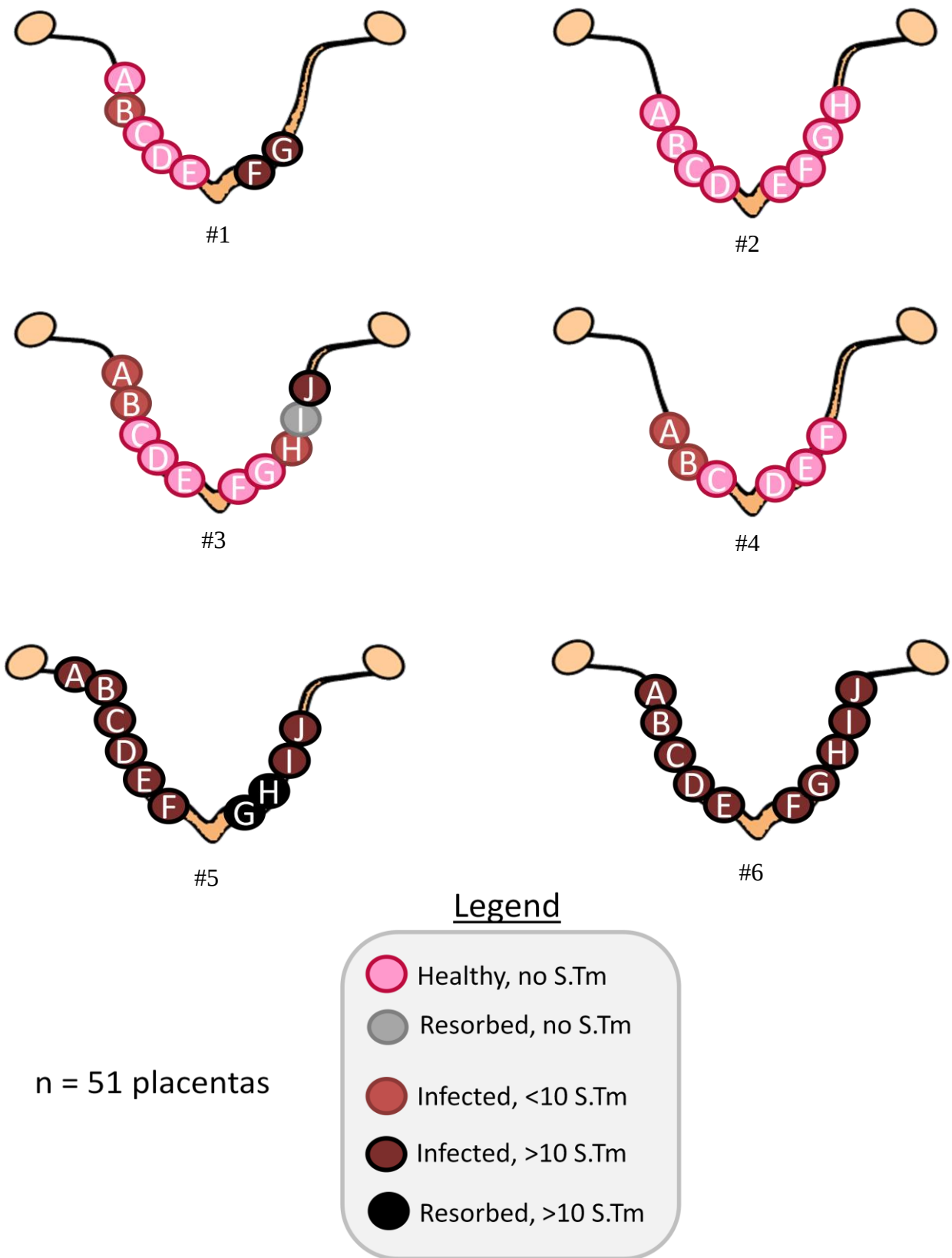
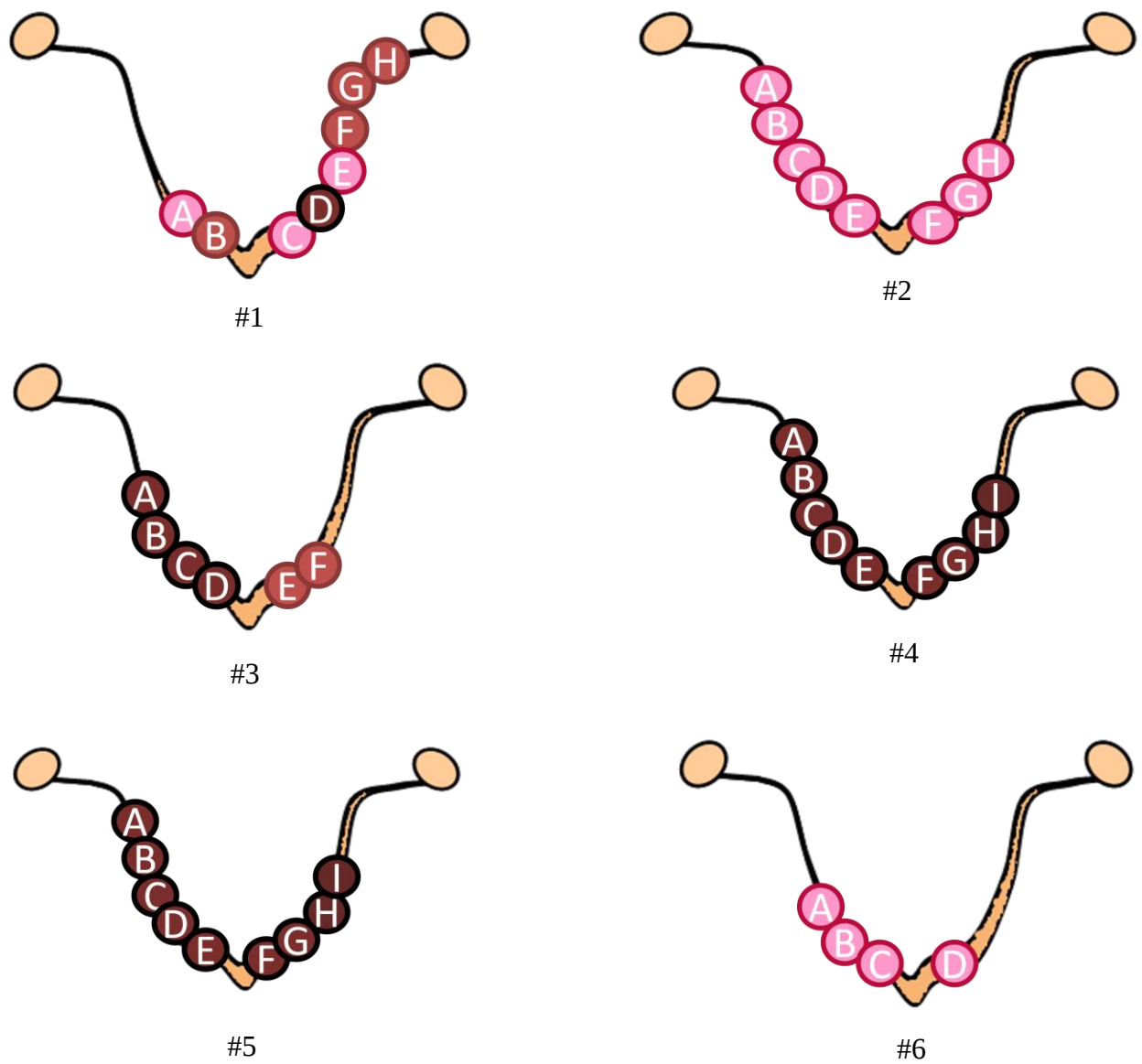


Figure 11

Figure 11. Schematic representation of the implant layout and outcome 24 hours after 10^3 S.Tm infection. Outcome of individual implants and location on uterine horns are illustrated. Layouts are representative of experimenter's view with mouse lying supine. Left and right horns are designated by experimenter's left and right and not mouse's left and right. Number beneath each depiction corresponds to mouse number.



n = 44 placentas

Legend

- Healthy, no S.Tm
- Resorbed, no S.Tm
- Infected, <10 S.Tm
- Infected, >10 S.Tm
- Resorbed, >10 S.Tm

Figure 12

Figure 12. Schematic representation of the implant layout and outcome 48 hours after 10^3 S.Tm infection. Outcome of individual implants and location on uterine horns are illustrated. Layouts are representative of experimenter's view with mouse lying supine. Left and right horns are designated by experimenter's left and right and not mouse's left and right. Number beneath each depiction corresponds to mouse number.

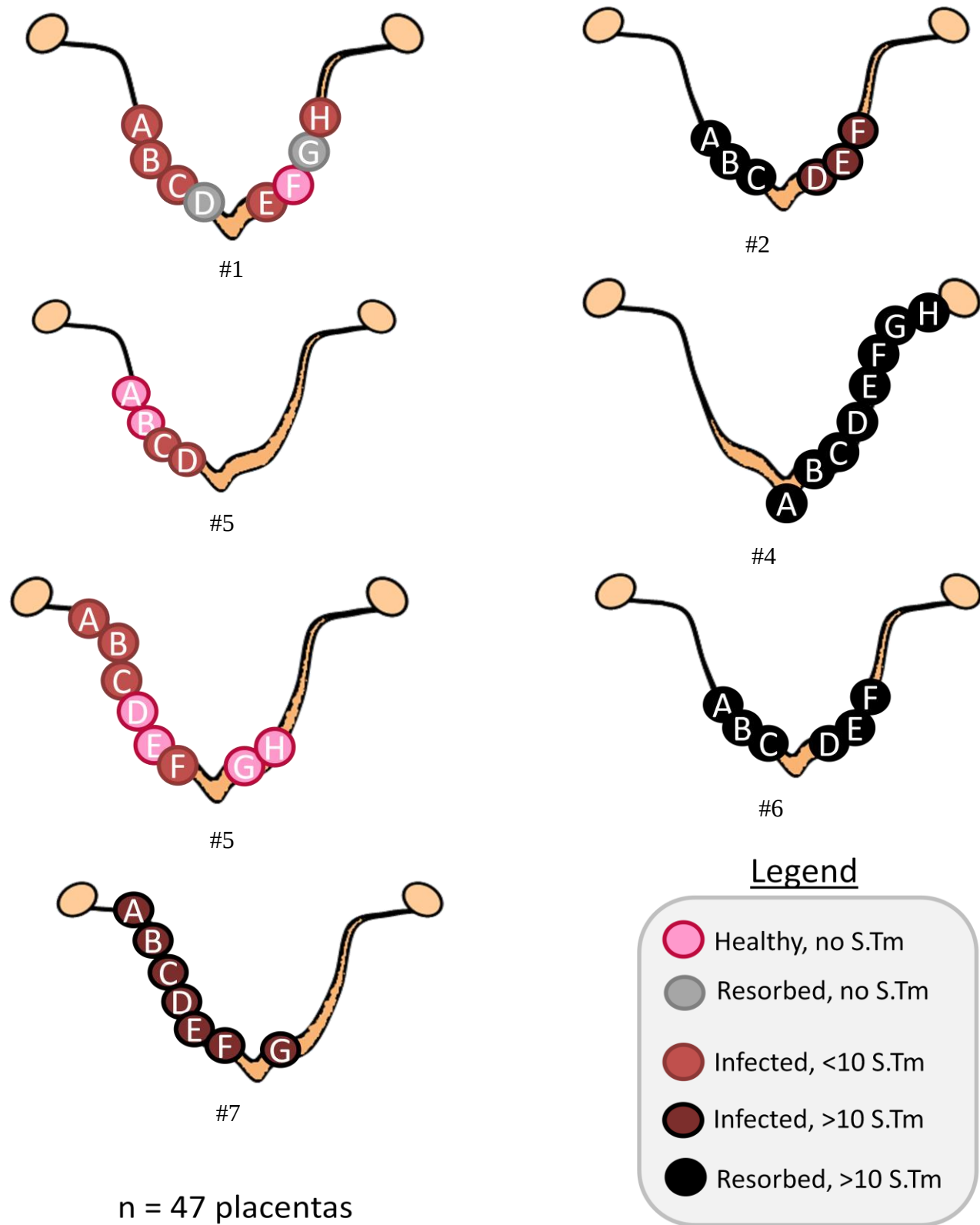


Figure 13

Figure 13. Schematic representation of the implant layout and outcome 72 hours after 10^3 S.Tm infection. Outcome of individual implants and location on uterine horns are illustrated. Layouts are representative of experimenter's view with dissected mouse lying on its back. Left and right horns are designated by experimenter's left and right and not mouse's left and right. Number beneath each depiction corresponds to mouse number.

4.4 Immune cell populations at the feto-maternal interface following systemic S.Tm infection

In order to elucidate the mechanism(s) of increased susceptibility to S.Tm infection during pregnancy, we sought to investigate the splenic and decidual immune cell populations by flow cytometry. Four categories of *Nramp1*^{+/+} mice were examined (non-pregnant non-infected, non-pregnant infected, pregnant non-infected, pregnant infected) for comparative analysis of potential immune cell differences during pregnancy and during infection. Pooled placentas from two mice were combined in order to yield a large enough leukocyte cell number ($\sim 1 \times 10^6$ cells) for acquisition and analysis.

Figure 14 highlights the gating strategy utilized to examine the leukocyte populations of the spleen and figure 15 depicts the gating strategy used for placental immunophenotyping.

At 48 hours post-infection, splenic neutrophil, macrophage/monocyte, DC, B, T and NK cell numbers of PI mice were significantly higher compared to NPI mice (Fig. 16). PNI mice also had significantly higher splenic cell numbers compared to NPNI mice (Fig. 16). A similar trend was seen when comparing neutrophil and macrophage/monocyte cell percentages (Fig. 17 A & B). However, there were no differences in cell number or percentage between PI and PNI mice suggesting that changes in the various splenic immune cell populations correlate to pregnancy rather than ongoing infection (Fig. 16 & Fig. 17 A-G). Overall, pregnant mice showed a significant increase in several immune cell subsets in the spleen compared to non-pregnant mice (Fig. 16).

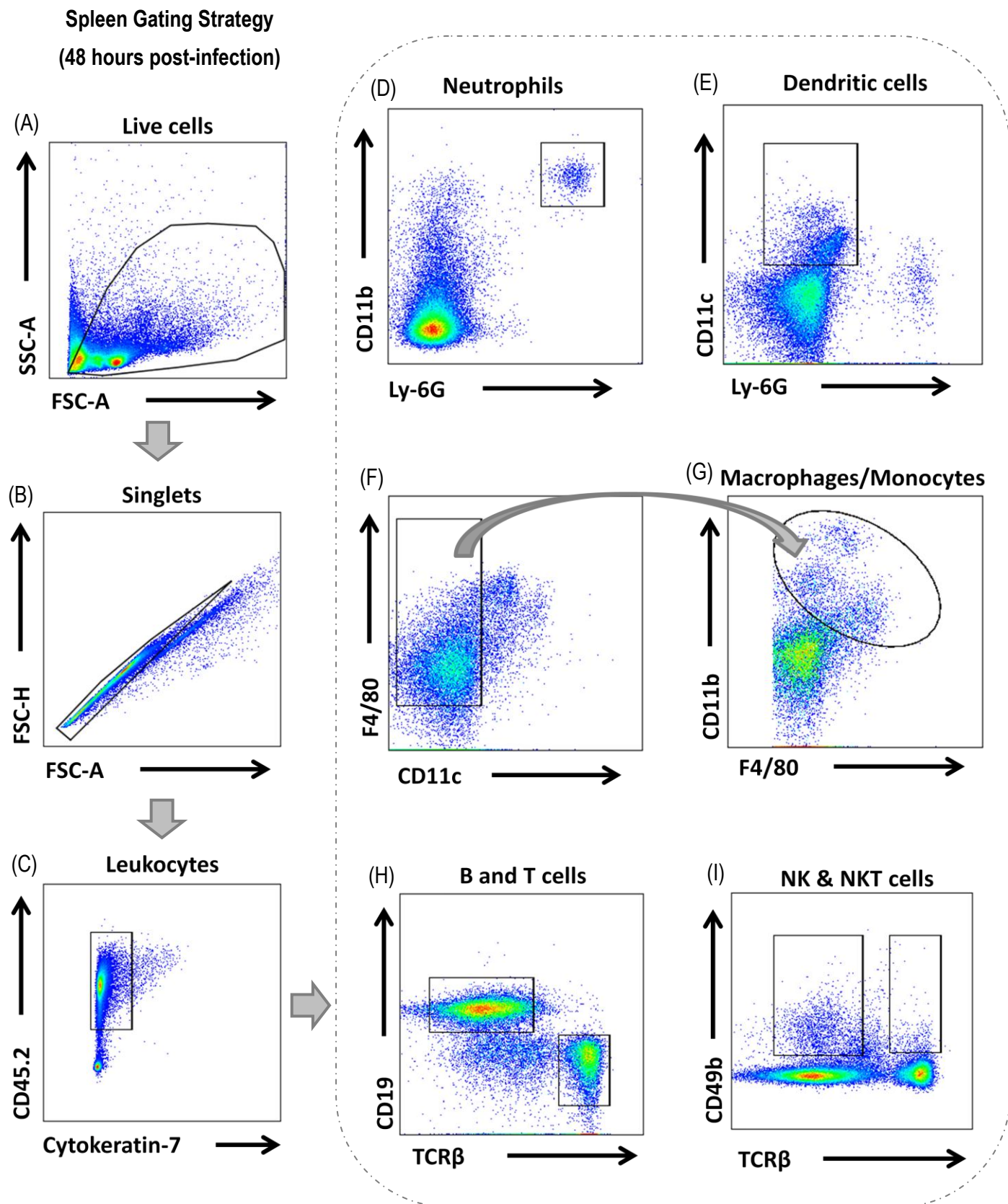


Figure 14

Figure 14. Gating strategy for splenic immune cell populations 48 hours post-infection.

A) Live cells B) Singlets C) Leukocytes [CD45.2⁺] D) Neutrophils [Ly-6G⁺CD11b⁺] E) Dendritic cells [Ly-6G⁻CD11c⁺] F) Gated CD11c⁻F4/80 expressing population to yield Macrophages/Monocytes in G. G) Macrophages/Monocytes with varying expression levels of F4/80 [F4/80^{bright, intermediate, dim}CD11b⁺] H) B lymphocytes [TCRβ⁻CD19⁺] and T lymphocytes [TCRβ⁺CD19⁻] I) NK cells [TCRβ⁻CD49b⁺] and NKT cells [TCRβ⁺CD49b⁺]. NK: Natural Killer, NKT: Natural Killer T

Pooled Placenta Gating Strategy
(48 hours post-infection)

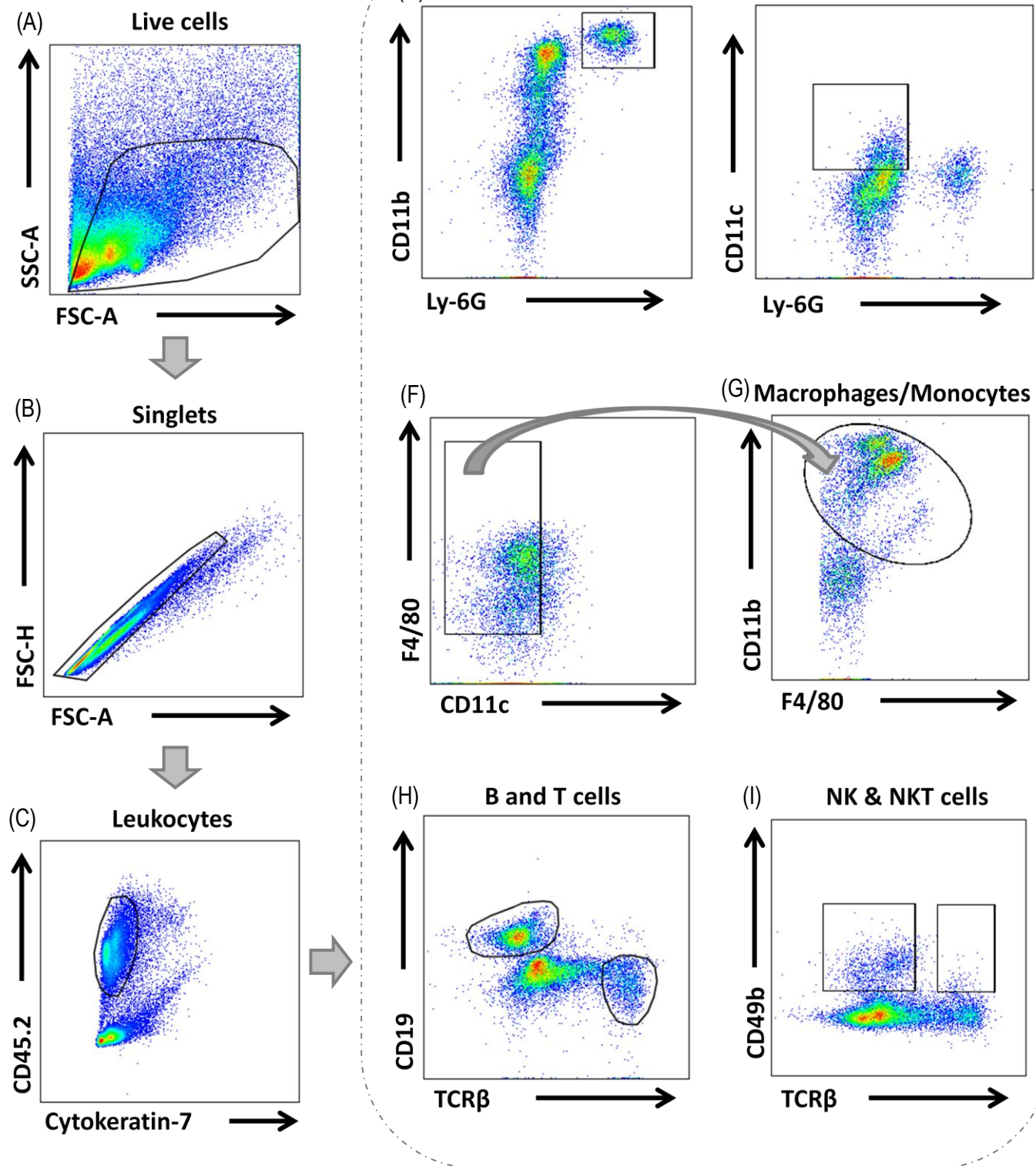


Figure 15

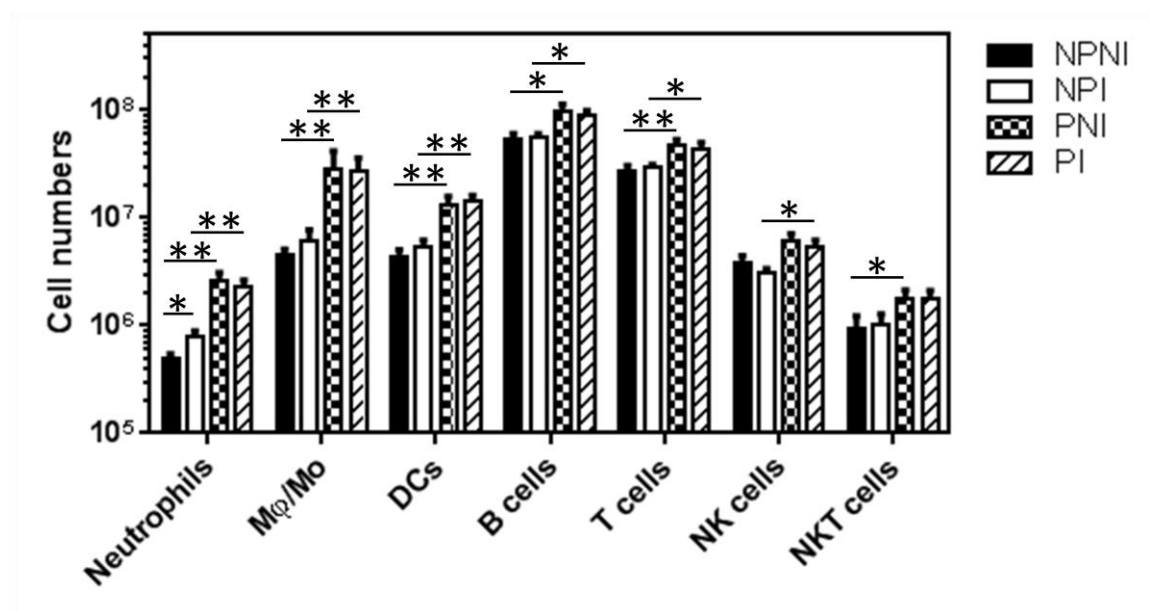


Figure 16

Figure 16. Splenic immune cell numbers 48 hours post-infection. Splenic immune cell numbers amongst the various immune cell populations analyzed. Pregnant mice were infected with 10^3 CFUs of S.Tm during mid-pregnancy (days 11-12) along with non-pregnant controls. Spleens and placentas were collected 48 hours later from PI and NPI mice and from age and gestational day matched PNI mice and NPNI mice. The majority of splenic numbers are significantly higher in the spleens of PNI mice compared to NPNI mice and in PI mice compared to NPI mice; based on the two-tailed Mann Whitney *U* test, comparing the following groups: NPNI vs. NPI, PNI vs. PI, NPNI vs. PNI, NPI vs. PI. Experiment was repeated 3 times with a total of 6 mice per category. Data represent mean $\pm$ SEM. M ϕ : Macrophages, Mo: Monocytes, DCs: Dendritic cells, NK: Natural Killer, NKT: Natural Killer T. * $p \leq 0.05$, ** $p \leq 0.01$

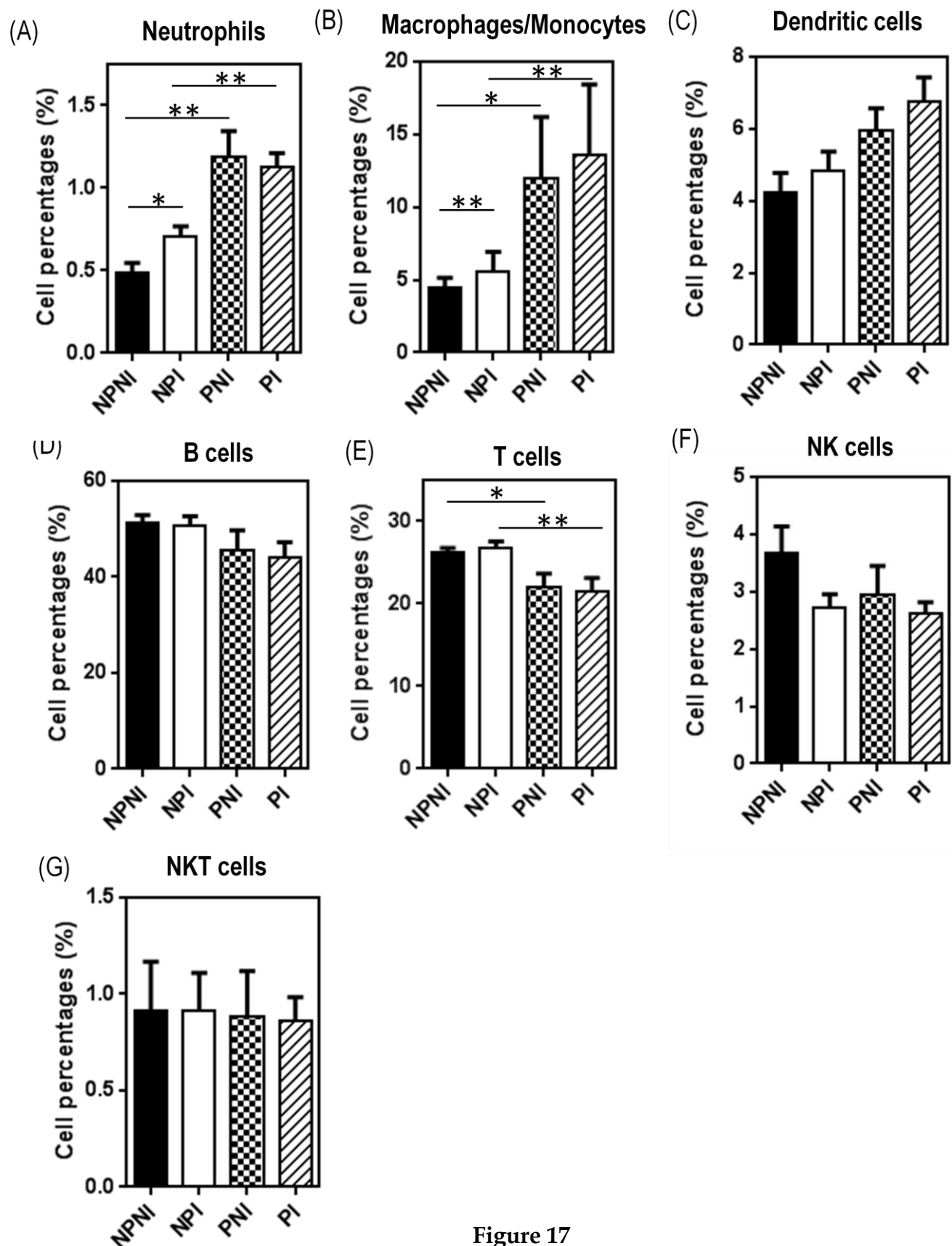


Figure 17

Figure 17. Splenic immune cell percentages 48 hours post-infection. A) Neutrophil cell percentages. NPI mice have significantly higher ($*p \leq 0.05$) neutrophil cell percentages in their spleen compared to NPNI mice. PNI mice have significantly higher ($**p \leq 0.01$) neutrophil cell percentages in their spleens compared to NPNI mice. PI mice have significantly higher ($**p \leq 0.01$) neutrophil cell percentages in their spleen compared to NPI mice; based on the two-tailed Mann Whitney *U* test. B) Macrophage/Monocyte cell percentages. NPI mice have significantly higher ($**p \leq 0.01$) M ϕ /Mo cell percentages in their spleen compared to NPNI mice. PNI mice have significantly higher ($*p \leq 0.05$) M ϕ /Mo cell percentages in their spleens compared to NPNI mice. PI mice have significantly higher ($**p \leq 0.01$) M ϕ /Mo cell percentages in their spleen compared to NPI mice; based on the two-tailed Mann Whitney *U* test. C) Dendritic cell percentages D) B cell percentages E) T cell percentages. NPNI mice have significantly higher ($*p \leq 0.05$) T cell percentages in their spleen compared to PNI mice. NPI mice have significantly higher ($**p \leq 0.01$) T cell percentages in their spleen compared to PI mice; based on the two-tailed Mann Whitney *U* test. F) Natural killer cell percentages G) Natural killer T cell percentages. Experiment was repeated 3 times with a total of 6 mice per category. Data represent mean $\pm$ SEM. M ϕ : Macrophages, Mo: Monocytes.

In contrast, analysis of placental immune cells revealed no significant difference between PI and PNI mice 48 hours after infection (Fig. 18 A-G). As stated above, at 48 hours post-infection just over 50% of the individual placentas were found to be infected. Therefore, we surmised that studying pooled placentas from a pregnant mother may counterbalance any differences seen in infected versus non-infected concepti.

We next assessed immune cell subsets in spleen and individual placentas at 72 hours post-infection to assess the effect of the progress of infection. Individual placentas were carefully dissected from each mouse and 100 μ L of homogenate was plated in order to determine status of infection. In parallel, the cells from individual placentas were processed for flow cytometry and a revised 9 colour panel was devised to allow for analysis of immune cell subsets in a single tube. Figure 19 highlights the updated gating strategy for spleen cells while figure 20 depicts the gating strategy for individual placentas. Experiments were repeated a total of 5 times to allow for a sufficient number of viable placentas for collection.

4.4.1 Pregnant infected hosts exhibit a reduction in splenic neutrophil recruitment 72 hours post-infection

Firstly, similar to data for 48 hours post-infection, most splenic immune cell numbers were higher in the pregnant compared to the non-pregnant state (Fig. 21 B). In non-pregnant mice, splenocyte numbers increased significantly upon infection, which was attributed to an increase in neutrophil numbers, whereas there were no significant differences between PI and PNI mice (Fig. 21 A & B). The increase in neutrophils upon infection in non-pregnant mice was also evident, although not significant, when data were analyzed as percentages, whereas no significant changes were seen in most other immune cell populations amongst

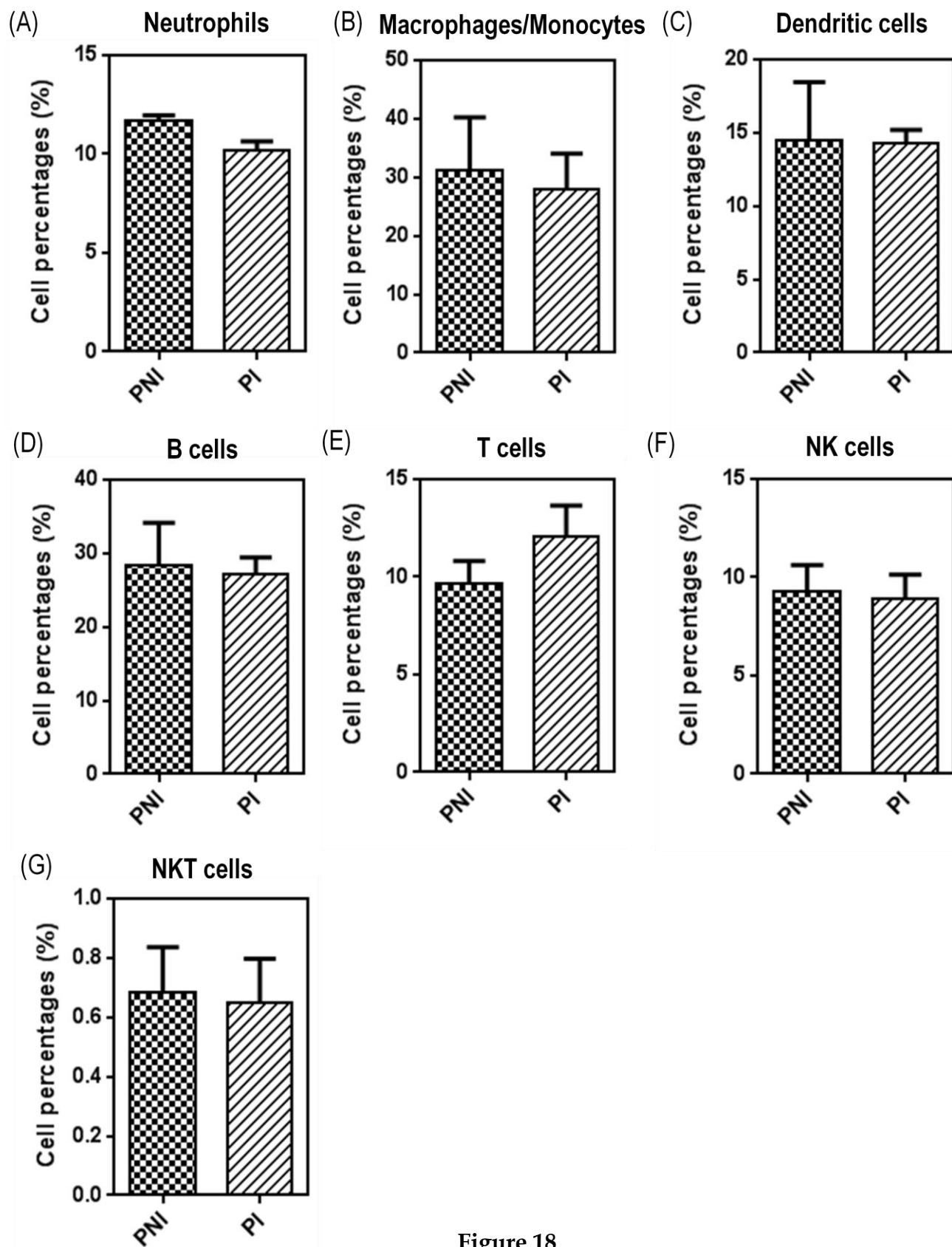


Figure 18

Figure 18. Pooled placental immune cell percentages 48 hours post-infection. A) Neutrophils B) Macrophages/Monocytes C) Dendritic cells D) B cells E) T cells F) Natural killer cells G) Natural killer T cells. Pregnant mice were infected with 10^3 CFUs of S.Tm during mid-pregnancy (days 11-12). Spleens and placentas were collected 48 hours later from PI mice and from age and gestational day matched PNI mice. Experiment was repeated 3 times with a total of 6 PI and 6 PNI mice. Placentas from two pregnant mice, either PI or PNI, were pooled for sufficient immune cell analysis; therefore 3 data sets were analyzed. Data represent mean $\pm$ SEM.

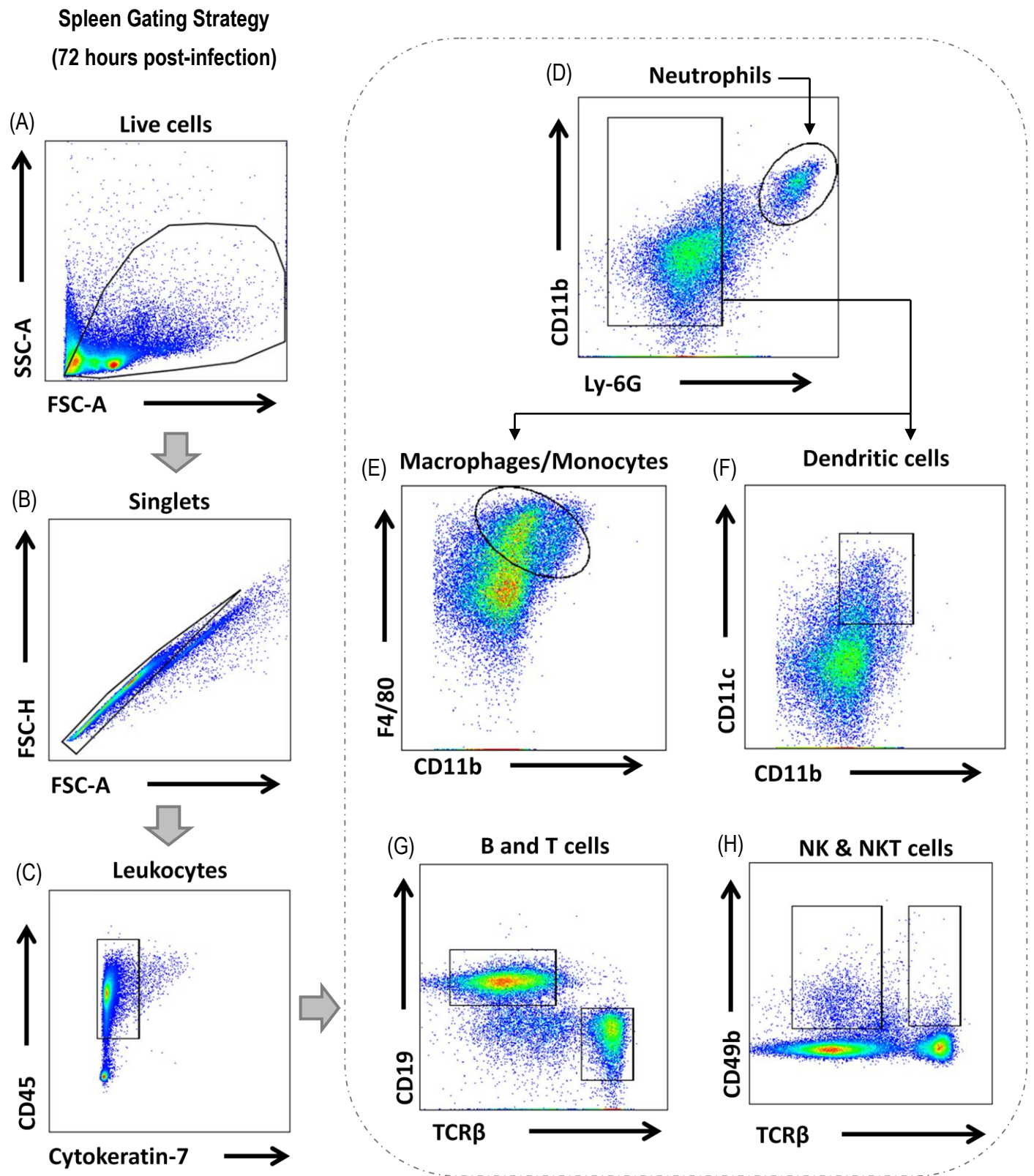


Figure 19

Figure 19. Gating strategy for splenic immune cell populations 72 hours post-infection.

A) Live cells B) Singlets C) Leukocytes [CD45⁺] D) Neutrophils [Ly-6G⁺CD11b⁺]. Gate on Ly-6G⁻CD11b^{-/+} population yields populations in E and F. E) Macrophages/Monocytes [CD11b⁺F4/80⁺] F) Dendritic cells [CD11b⁺CD11c⁺] G) B lymphocytes [TCRβ⁻CD19⁺] and T lymphocytes [TCRβ⁺CD19⁻] H) Natural killer cells [TCRβ⁻CD49b⁺] and Natural killer T cells [TCRβ⁺CD49b⁺].

Individual Placenta Gating Strategy
(72 hours post-infection)

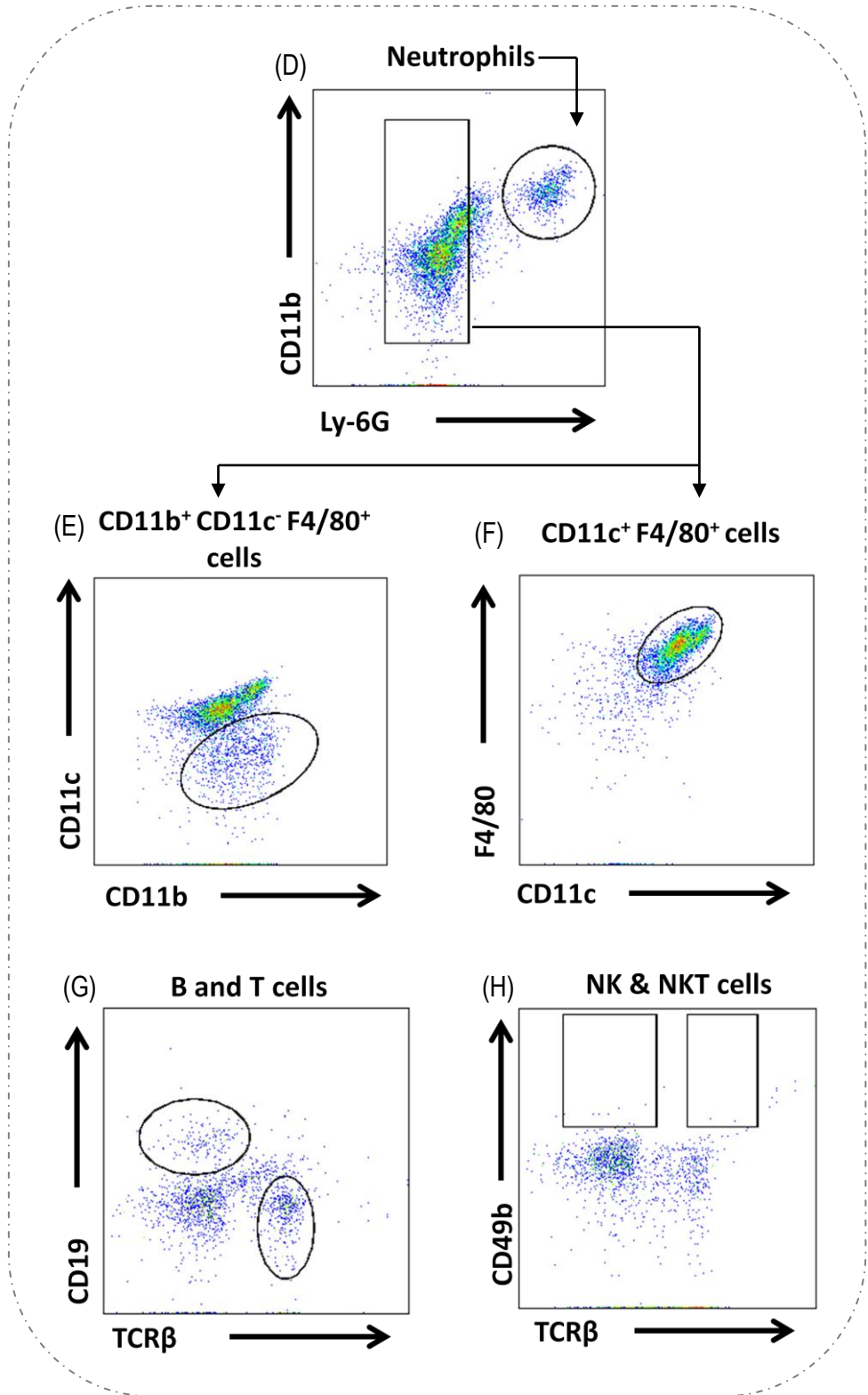
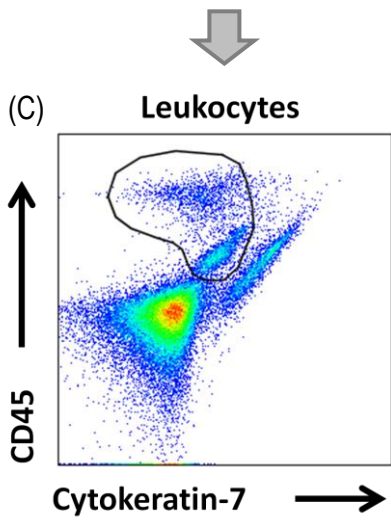
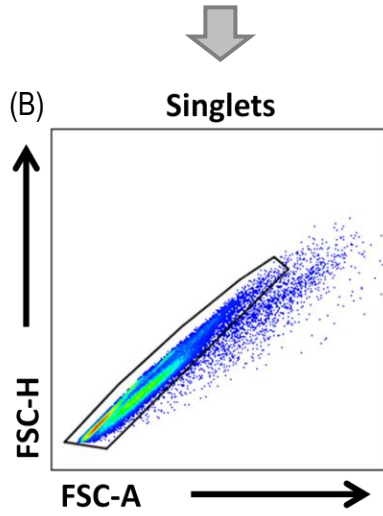
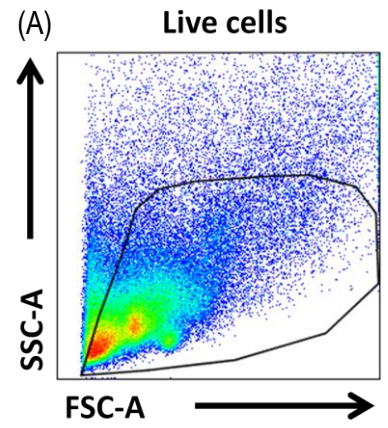


Figure 20

Figure 20. Gating strategy for individual placental immune cell populations 72 hours post-infection. A) Live cells B) Singlets C) Leukocytes [CD45⁺Cytokeratin-7⁻] D) Neutrophils [Ly-6G⁺CD11b⁺]. Gate on Ly-6G⁻CD11b^{-/+} population yields populations in E and F. E) CD11b⁺CD11c⁻F4/80⁺ cells (Macrophages) F) CD11c⁺F4/80⁺ cells (Dendritic cells) G) B lymphocytes [TCRβ⁻ CD19⁺] and T lymphocytes [TCRβ⁺CD19⁻] H) Natural killer cells [TCRβ⁻CD49b⁺] and Natural killer T cells [TCRβ⁺CD49b⁺].

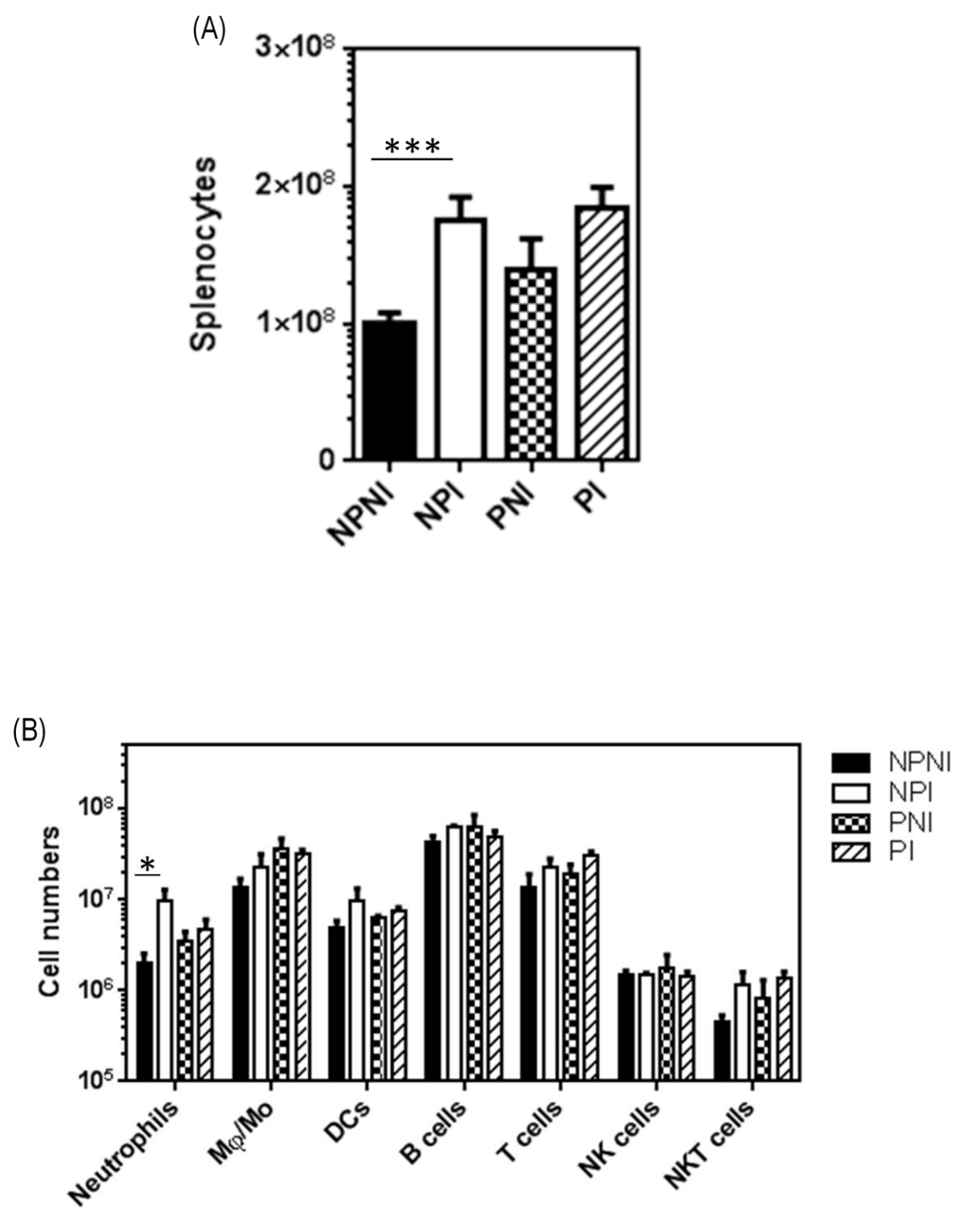


Figure 21

Figure 21. Splenic immune cell numbers 72 hours post-infection. A) Splenocyte count. Splenocytes in NPI mice are significantly higher ($***p \leq 0.001$) compared to NPNI mice; based on a two-tailed Mann Whitney *U* test comparing the following groups: NPNI vs. NPI, PNI vs. PI, NPNI vs. PNI and NPI vs. PI. B) Splenic immune cell numbers amongst the various immune cell populations analyzed. Splenic neutrophil numbers are significantly higher ($*p \leq 0.05$) in the spleens of NPI mice compared to naive NPNI mice; based on the two-tailed Mann Whitney *U* test comparing the following groups: NPNI vs. NPI, PNI vs. PI, NPNI vs. PNI, NPI vs. PI. Pregnant mice were infected with 10^3 CFUs of *S.Tm* during mid-pregnancy (days 11-12) along with non-pregnant controls. Spleens and placentas were collected 72 hours later from PI and NPI mice and from age and gestational day matched PNI mice and NPNI mice. Experiment was repeated 5 times with a total of 5 NPNI mice, 3 NPI mice, 3 PNI mice, and 9 PI mice per category. Data represent mean $\pm$ SEM. M ϕ : Macrophages, Mo: Monocytes, DCs: Dendritic cells, NK: Natural Killer, NKT: Natural Killer T.

the various groups (Fig. 21 B & 22 A, C-G). Although PI mice had an increase in splenic macrophage/monocyte percentage compared to NPI mice, there was no difference when compared to PNI controls (Fig. 22 B). Overall, the systemic immune cell profile reveals that while pregnancy may confer a baseline increase in certain cell types, infection does not evoke a further proliferative response.

4.4.2 Pregnant hosts display increased recruitment of immune cell types to the placenta 72 hours post-infection

Figure 20 depicts the placental gating strategy and demonstrates that the CD45 population seen amongst individual placentas includes both CD45^{bright} and CD45^{dim} populations.

Placental analysis revealed an increase in the percentage of neutrophils (Fig. 23 A), CD11b⁺CD11c⁻F4/80⁺ cells (Fig. 23 B), T lymphocytes (Fig. 23 E) and natural killer cells (Fig. 23 F) in infected placenta compared to pregnant non-infected tissue. There was also a significant reduction in the percentage of CD11c⁺F4/80⁺ double positive cells in comparison to placentas from naive pregnant mice (Fig. 23 C). The CD11c⁺F4/80⁺ was consistently a very bright population amongst individual placentas and were also noted to be CD45^{dim}.

In summary, by analysis of individual placentas we could discern the differential modulation of several immune types in response to local infection.

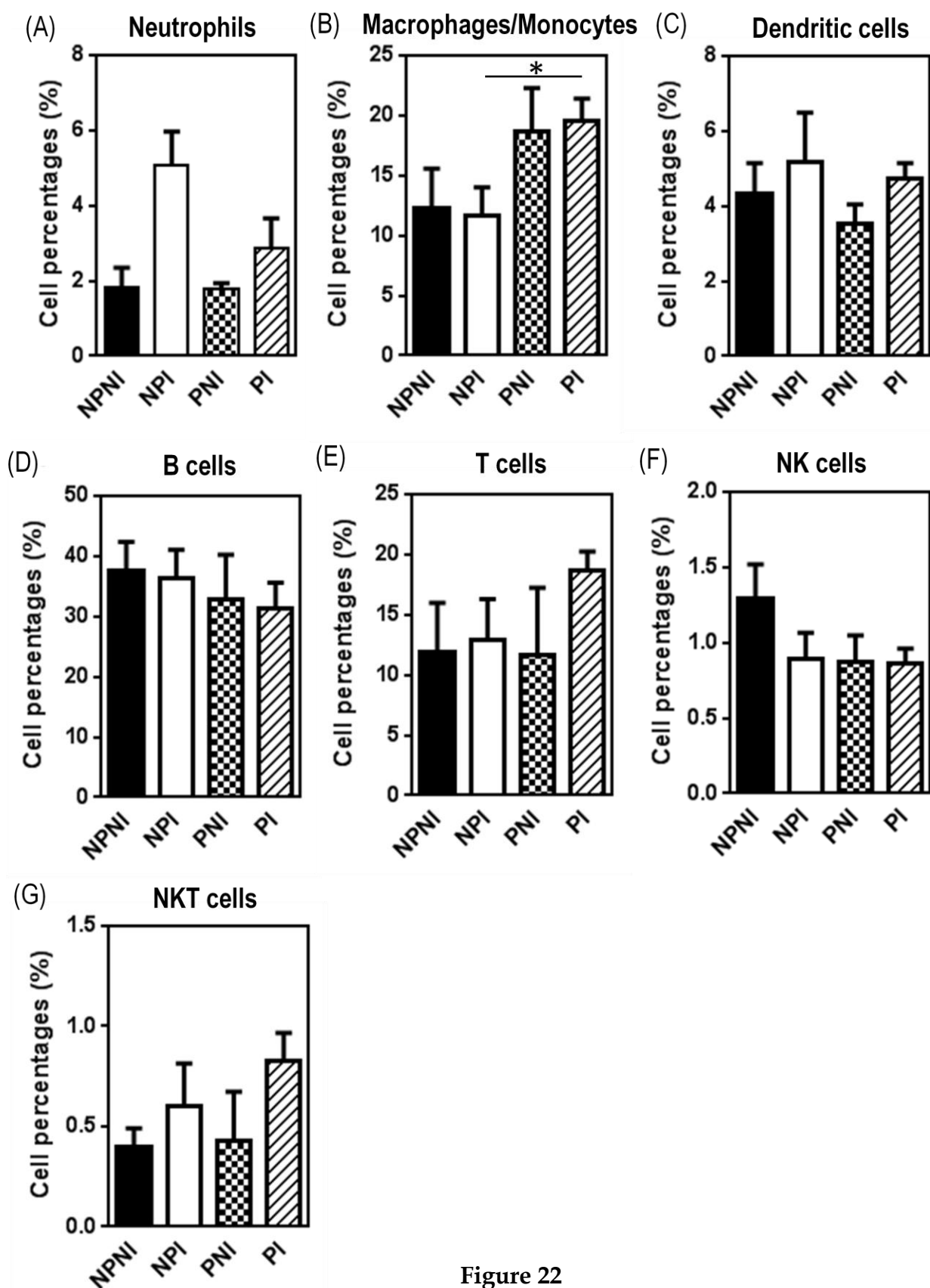


Figure 22

Figure 22. Splenic immune cell percentages 72 hours post-infection. A) Neutrophil cell percentages. B) Macrophage/Monocyte cell percentages. PI mice have significantly higher ($*p \leq 0.05$) Macrophage/Monocyte cell percentages in their spleen compared to NPI mice; based on a two-tailed Mann Whitney *U* test comparing the following groups: NPNI vs. NPI, PNI vs. PI, NPNI vs. PNI and NPI vs. PI. C) Dendritic cell percentages. D) B cell percentages. E) T cell percentages. F) Natural killer cell percentages. G) Natural killer T cell percentages. Experiment was repeated 5 times with a total of 5 NPNI mice, 3 NPI mice, 3 PNI mice, and 9 PI mice per category. Data represent mean $\pm$ SEM.

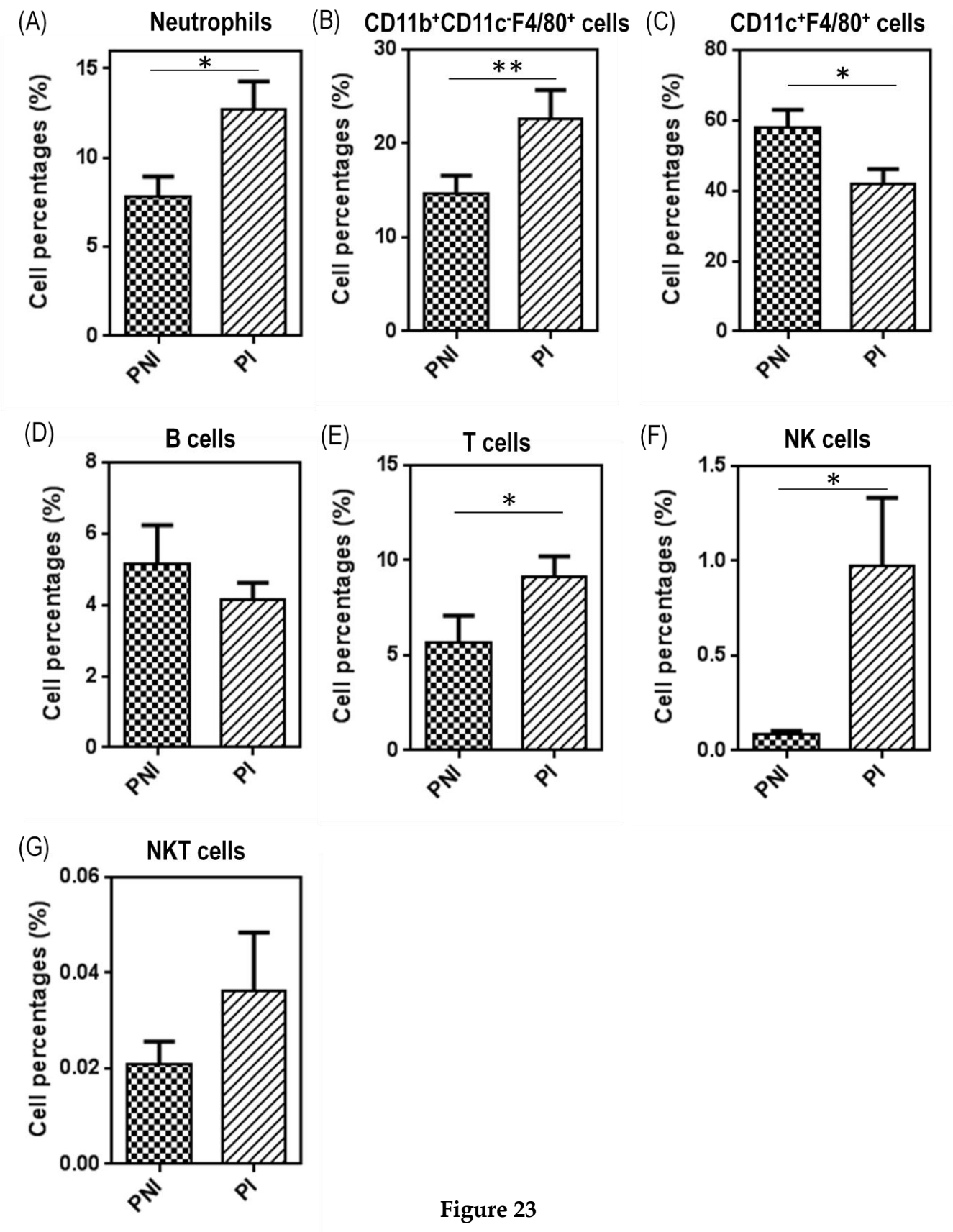


Figure 23

Figure 23. Analysis of individual placental immune cell percentages 72 hours post-infection. A) Neutrophils B) CD11b⁺CD11c⁻F4/80⁺ cells (Macrophages) C) CD11c⁺F4/80⁺ cells (Dendritic cells) D) B cells E) T cells F) Natural killer cells G) Natural killer T cells. Pregnant mice were infected with 10³ CFUs of S.Tm during mid-pregnancy (days 11-12). Spleens and individual placentas were collected 72 hours after infection from PI mice and from age and gestational day matched PNI mice. Experiment was repeated 5 times with a total of 3 PNI mice and 9 PI mice resulting in a total number of individual placentas as follows: PNI = 24, PI = 11. PI mice have a significant increase in the percentage of neutrophils (*p≤0.05), CD11b⁺CD11c⁻ cells (**p≤0.01), T lymphocytes (*p≤0.05) and NK cells (*p≤0.05) within an individual placenta during infection compared to PNI mice. PI mice have significantly less CD11c⁺F4/80⁺ cells (*p≤0.05) in an individual placenta compared to PNI mice. Statistics based on a two-tailed Mann Whitney *U* test. Data represent mean ± SEM.

4.5 Intravenous model of infection: effect of S.Tm infection and host survival in pregnant *Nramp1*^{+/+} cell death deficient strains

As the wild-type C57BL/6J mice rapidly succumb to intravenous S.Tm infection, the *Nramp1* background strain is more suitable for studying systemic effects of infection during pregnancy. Cell death deficient strains (*Caspase-1,11*^{-/-} and *Rip3*^{-/-}) on the C57BL/6J background were crossed with *Nramp1*^{+/+} mice to generate cell death deficient strains expressing the functional *Nramp1* gene.

Survivals involving PI and NPI age-matched *Caspase-1,11*^{-/-}*Nramp1*^{+/+}, *Rip3*^{-/-}*Nramp1*^{+/+} along with *Nramp1*^{+/+} controls, all resulted in PI mice succumbing to S.Tm infection significantly sooner compared to their NPI counterparts (Fig. 24 A-C). NPI *Nramp1*^{+/+} mice lived significantly longer compared to PI mice (Fig 24 A) which succumb to infection within 7 days. When comparing PI *Caspase-1,11*^{-/-}*Nramp1*^{+/+} to NPI *Caspase-1,11*^{-/-}*Nramp1*^{+/+}, although survival curves are significantly different, this cell death deficient strain has a low survival rate following S.Tm infection even in the non-pregnant state relative to wild-type *Nramp1*^{+/+} mice (Fig. 24 B). Lastly, *Rip3*^{-/-}*Nramp1*^{+/+} mice show a similar trend to *Nramp1*^{+/+} controls, where PI mice succumb to infection significantly sooner than NPI mice (Fig. 24 C).

4.6 Intravenous infection outcome in *Nramp1*^{+/+} cell death deficient strains

Bacterial burden in systemic and placental tissues as well as the qualitative extent of inflammation was then examined amongst the various *Nramp1*^{+/+} cell death deficient strains compared to *Nramp1*^{+/+} controls at various time points.

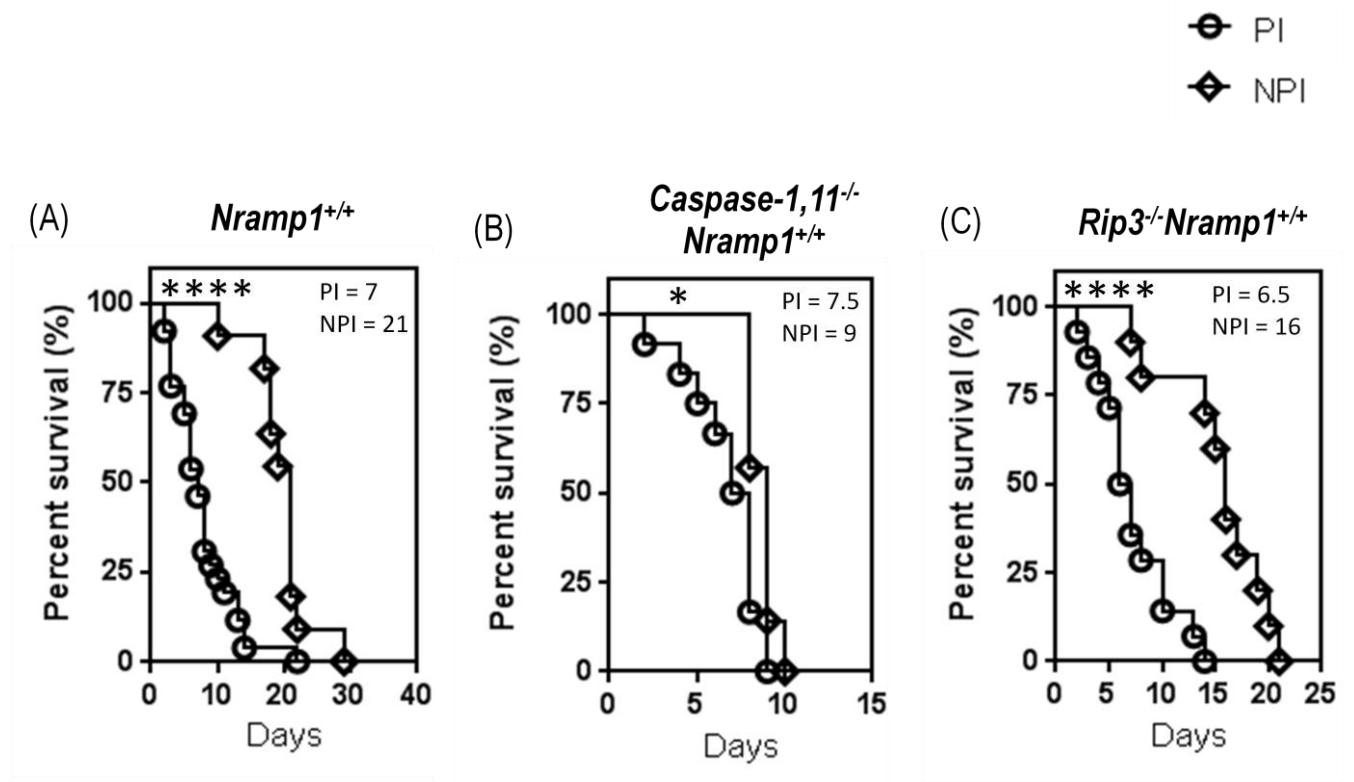


Figure 24

Figure 24. Survival kinetics of pregnant infected and non-pregnant infected cell death deficient strains on the *Nramp1* background versus *Nramp1*^{+/+} control mice. A) PI *Nramp1*^{+/+} mice versus NPI *Nramp1*^{+/+} mice. Non-pregnant infected *Nramp1*^{+/+} mice live significantly longer (****p≤0.0001) than pregnant infected *Nramp1*^{+/+}. B) PI *Caspase-1,11*^{-/-} *Nramp1*^{+/+} mice versus NPI *Caspase-1,11*^{-/-} *Nramp1*^{+/+}. Non-pregnant infected *Caspase-1,11*^{-/-} *Nramp1*^{+/+} live significantly longer (*p ≤0.05) than pregnant infected *Caspase-1,11*^{-/-} *Nramp1*^{+/+} mice. C) PI *Rip3*^{-/-} *Nramp1*^{+/+} versus NPI *Rip3*^{-/-} *Nramp1*^{+/+}. Non-pregnant infected *Rip3*^{-/-} *Nramp1*^{+/+} live significantly longer (****p ≤0.0001) than pregnant infected *Rip3*^{-/-} *Nramp1*^{+/+} mice. Pregnant mice along with age-matched non-pregnant mice were infected with 10³CFUs of S.Tm via intravenous route. Median days of survival are listed in the top right corner of each graph. Survivals were repeated at least twice to achieve 7 to 26 mice per category. Significance based on the log rank (Mantel-Cox) test.

Pyroptosis is a caspase-1 dependent cell death pathway, therefore, studies involving *Caspase-1,11* deficient mice enable the study of S.Tm infection in pregnant and non-pregnant hosts and its correlation to this cell death pathway.

Intravenous challenge with 10^3 S.Tm in PI *Caspase-1,11*^{-/-}*Nramp1*^{+/+} mice with age-matched NPI *Caspase-1,11*^{-/-}*Nramp1*^{+/+}, PI and NPI *Nramp1*^{+/+} controls resulted in no significant differences in bacterial burden within the spleen, liver or pooled placentas of *Caspase-1,11*^{-/-}*Nramp1*^{+/+} compared to *Nramp1*^{+/+} controls both in a pregnant or non-pregnant state (Fig 25 A & B). Furthermore, there was no difference in the resorption rate at 72 hours post-infection (Fig 26).

Necroptosis, the second type of inflammatory cell death pathway, is a RIP3 dependent pathway. Pregnant and non-pregnant *Rip3*^{-/-}*Nramp1*^{+/+} mice along with age-matched *Nramp1*^{+/+} controls were challenged with 10^3 S.Tm. Bacterial burden 48 hours post infection revealed no differences in titres amongst spleen, liver or pooled placentas compared to controls regardless of pregnant/non-pregnant state (Fig 27 A & B).

Although there was a steady increase in bacterial burden by 72 hours, there were no differences in bacterial burden in spleen, liver or pooled placentas between *Rip3*^{-/-}*Nramp1*^{+/+} and *Nramp1*^{+/+} controls (Fig 28 A & B).

Preliminary studies conducted at 96 hours post-challenge further revealed no differences in bacterial titres of spleen, liver and pooled placentas between *Rip3* cell death deficient mice and *Nramp1*^{+/+} controls (Fig 29 A & B).

Although there were no differences in bacterial burden between *Rip3*^{-/-}*Nramp1*^{+/+} and *Nramp1*^{+/+} mice, the rate of resorptions between pregnant strains differed at certain time

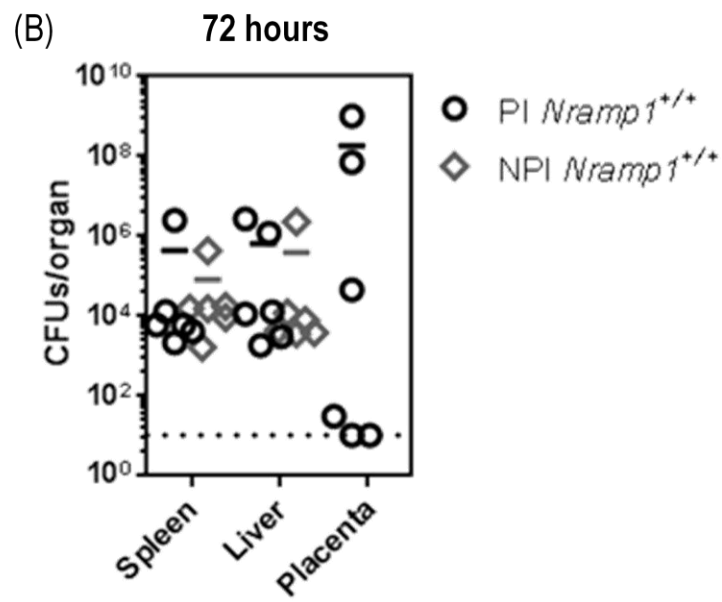
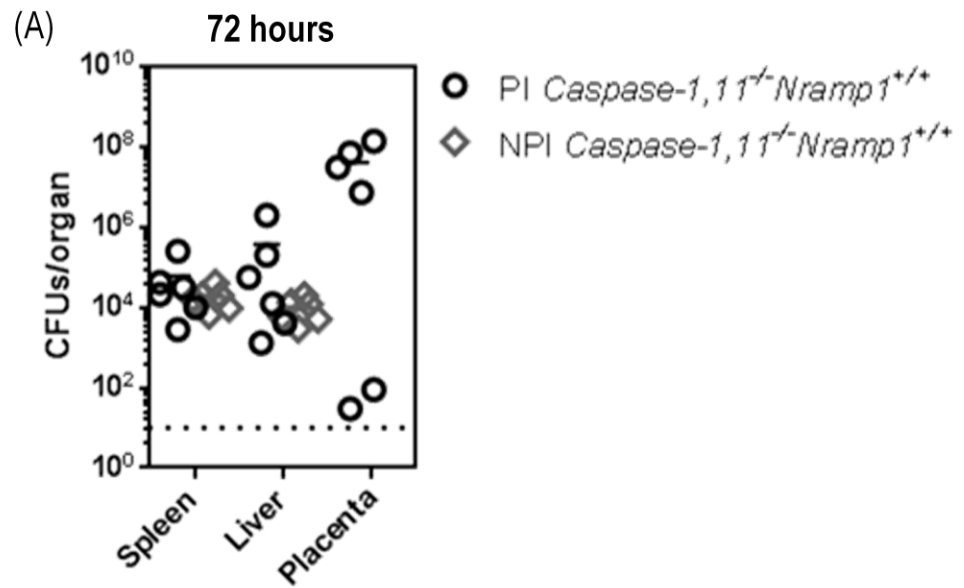


Figure 25

Figure 25. Infection outcome in *Caspase-1,11*^{-/-}*Nramp1*^{+/+} mice compared to *Nramp1*^{+/+} controls. A) Bacterial burden in pregnant infected and non-pregnant infected age-matched *Caspase-1,11*^{-/-}*Nramp1*^{+/+} mice. B) Bacterial burden in pregnant infected and non-pregnant infected age-matched *Nramp1*^{+/+} controls. Pregnant mice (*Caspase-1,11*^{-/-}*Nramp1*^{+/+} and *Nramp1*^{+/+}) along with non-pregnant age-matched controls were infected intravenously with 10³ S.Tm. Mice were euthanized 72 hours after infection with collection of spleen, liver and pooled placenta tissue. Each data point is indicative of bacterial burden within a single mouse. Mean (horizontal line) and detection limit (dotted line) are shown. Experiment was repeated twice; 6 mice per category.

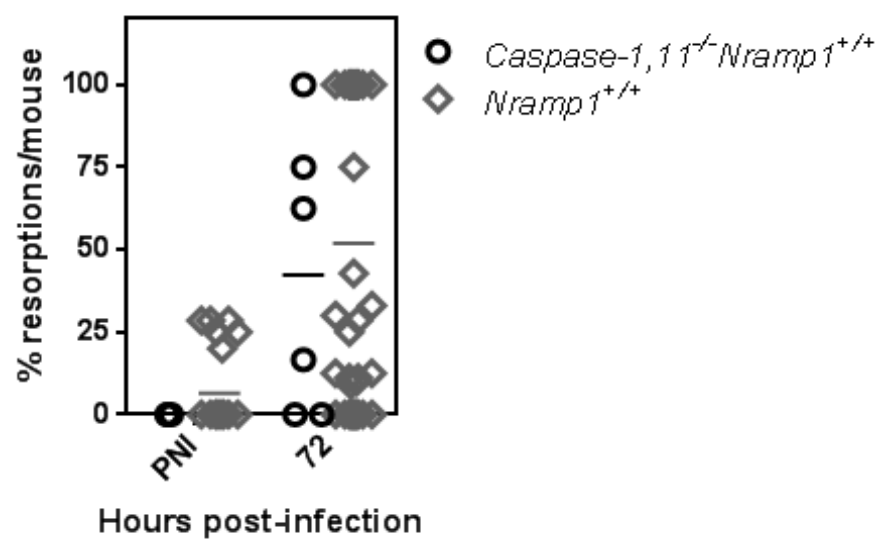


Figure 26

Figure 26. Resorption rate in *Caspase-1,11*^{-/-}*Nramp1*^{+/+} mice compared to *Nramp1*^{+/+} controls 72 hours after infection. Percent resorptions in *Caspase-1,11*^{-/-}*Nramp1*^{+/+} compared to *Nramp1*^{+/+} mice. Pregnant mice (*Caspase-1,11*^{-/-}*Nramp1*^{+/+} and *Nramp1*^{+/+}) were infected intravenously with 10³ S.Tm.. Mice were euthanized 72 hours after infection and resorption rates were recorded. PNI resorption rates refer to baseline resorptions in pregnant naive mice. Each data point is indicative of the resorption rate of a single mouse. Mean is depicted by a horizontal line. Experiment was repeated twice; 6 mice per category.

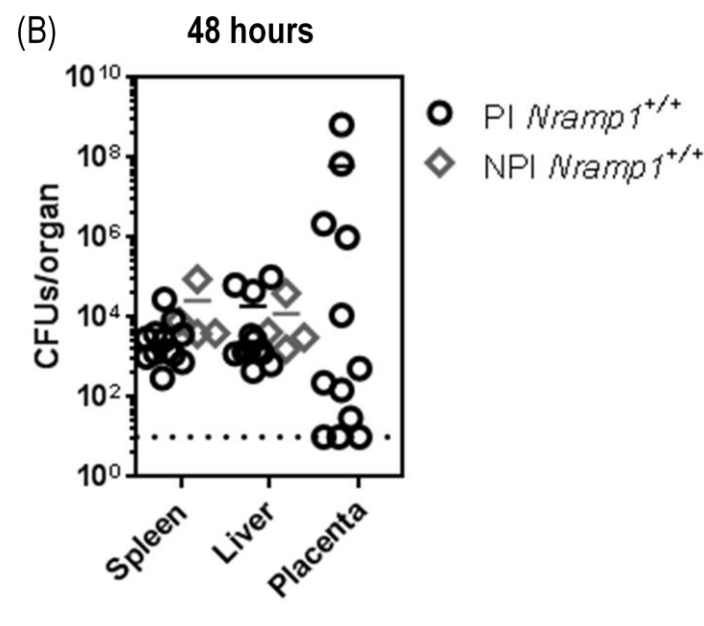
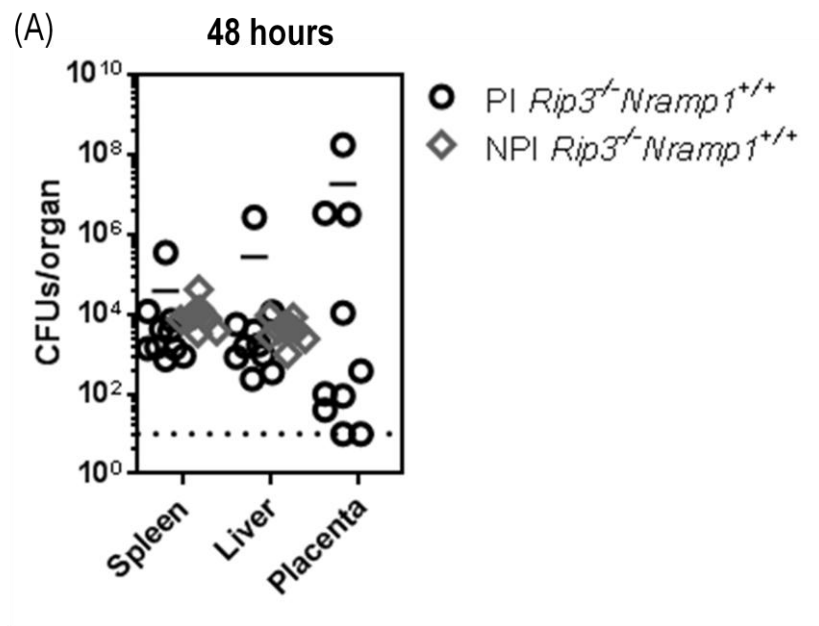


Figure 27

Figure 27. Bacterial burden in *Rip3*^{-/-}*Nramp1*^{+/+} mice compared to *Nramp1*^{+/+} controls 48 hours post-infection. A) Bacterial burden in pregnant infected and non-pregnant infected age-matched *Rip3*^{-/-}*Nramp1*^{+/+} mice. B) Bacterial burden in pregnant infected and non-pregnant infected *Nramp1*^{+/+} control mice. Pregnant mice (*Rip3*^{-/-}*Nramp1*^{+/+} and *Nramp1*^{+/+}) along with non-pregnant age-matched controls were infected intravenously with 10³ S.Tm. Mice were euthanized 48 hours after infection with collection of spleen, liver and pooled placenta tissue. Each data point is indicative of bacterial burden within a single mouse. Mean (horizontal line) and detection limit (dotted line) are shown. Experiment was repeated a total of four times; 4-12 mice per category.

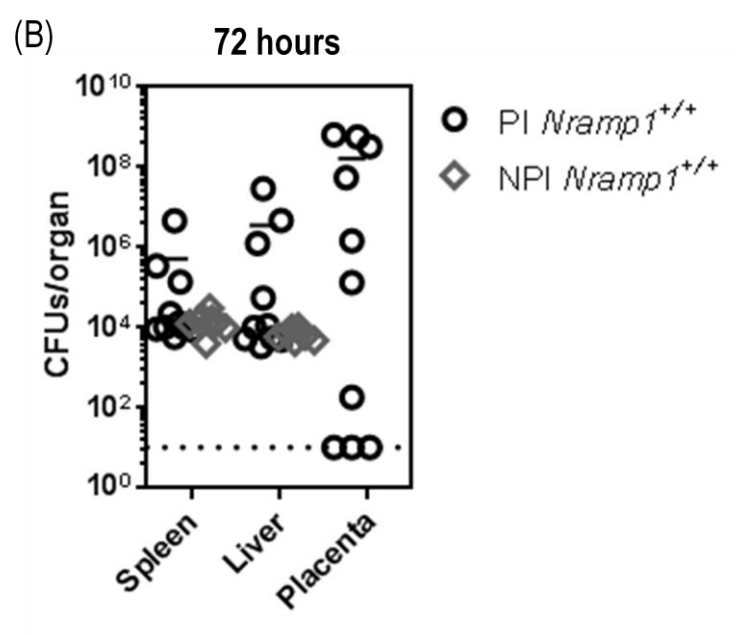
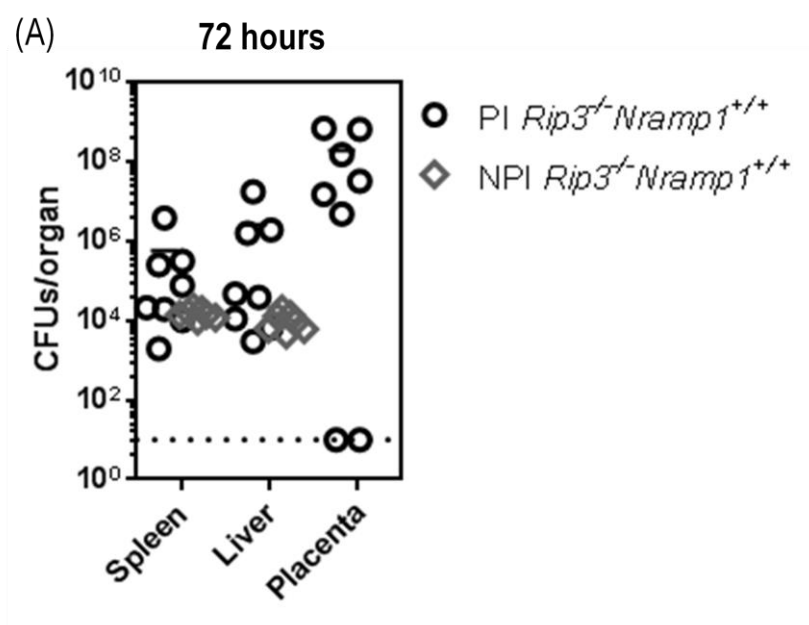


Figure 28

Figure 28. Bacterial burden in *Rip3*^{-/-}*Nramp1*^{+/+} mice compared to *Nramp1*^{+/+} controls 72 hours post-infection. A) Bacterial burden in pregnant infected and non-pregnant infected age-matched *Rip3*^{-/-}*Nramp1*^{+/+} mice. B) Bacterial burden in pregnant infected and non-pregnant infected *Nramp1*^{+/+} control mice. Pregnant mice (*Rip3*^{-/-}*Nramp1*^{+/+} and *Nramp1*^{+/+}) along with non-pregnant age-matched controls were infected intravenously with 10³ S.Tm. Mice were euthanized 72 hours after infection with collection of spleen, liver and pooled placenta tissue. Each data point is indicative of bacterial burden in a single mouse. Mean (horizontal line) and detection limit (dotted line) are shown. Experiment was repeated a total of three times; 7-10 mice per category.

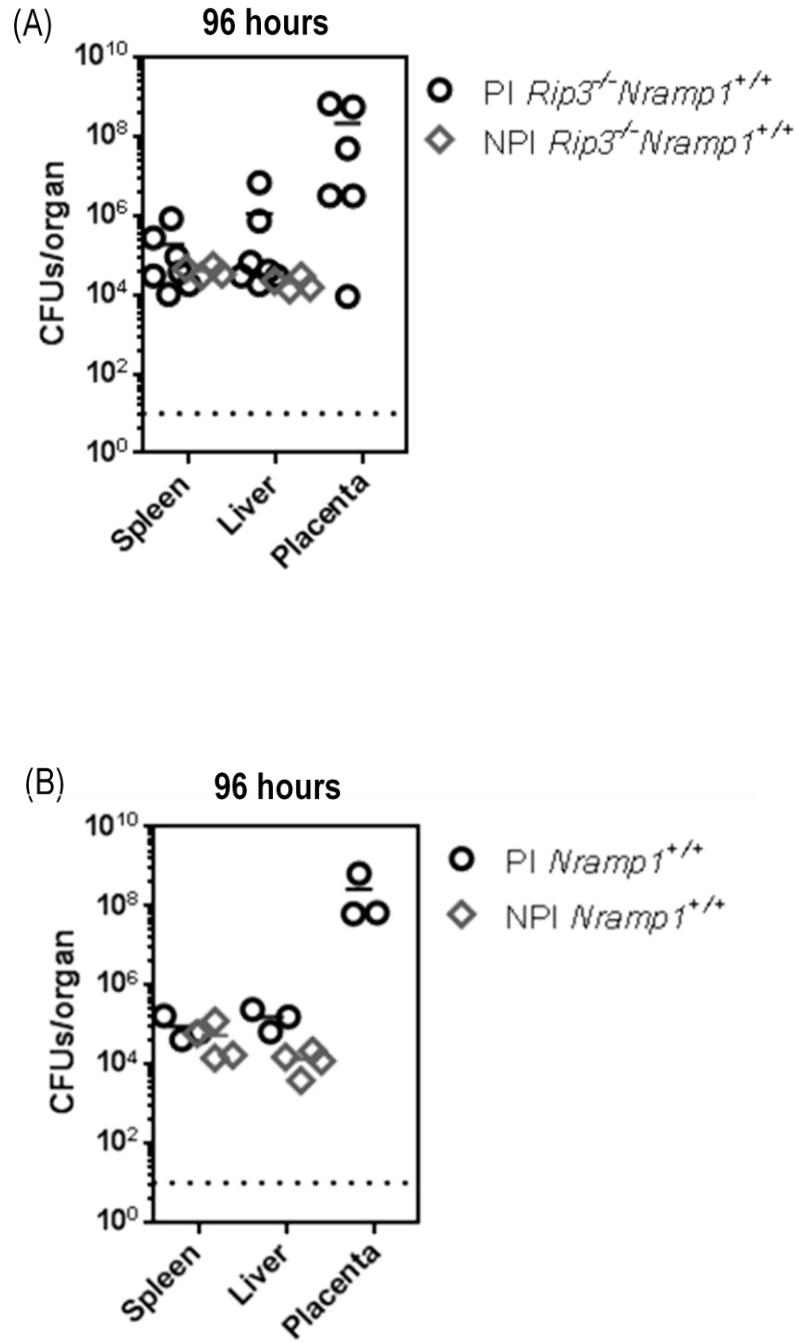


Figure 29

Figure 29. Bacterial burden in *Rip3*^{-/-}*Nramp1*^{+/+} mice compared to *Nramp1*^{+/+} controls 96 hours after infection. A) Bacterial burden in pregnant infected and non-pregnant infected age-matched *Rip3*^{-/-}*Nramp1*^{+/+} mice. B) Bacterial burden in pregnant infected and non-pregnant infected age-matched *Nramp1*^{+/+} control mice. Pregnant mice (*Rip3*^{-/-}*Nramp1*^{+/+} and *Nramp1*^{+/+}) along with non-pregnant age-matched controls were infected intravenously with 10³ S.Tm. Mice were euthanized 96 hours post-infection with collection of spleen, liver and pooled placenta tissue. Each data point is indicative of bacterial burden within a single mouse. Mean (horizontal line) and detection limit (dotted line) are shown. Experiment was repeated twice; 3-6 mice per category.

points (Fig 30 A). Forty-eight and 72 hours after challenge, there was a slight reduction in the mean percent resorption rate of *Rip3^{-/-}Nramp1^{+/+}* mice compared to *Nramp1^{+/+}* controls (Fig 30 A & B). However, by 96 hours, *Rip3^{-/-}Nramp1^{+/+}* mice had a significantly lower mean resorption rate compared to *Nramp1^{+/+}* controls (Fig. 30 A & B). This was in contrast to the resorption rates of *Caspase-1,11^{-/-}* mice at 72 hours, in which the average resorption rates remained similar to wild-type *Nramp1^{+/+}* mice (Table 1).

In summary, although S.Tm are able to successfully infect, colonize, and replicate to the same magnitude within the placentas of both *Rip3^{-/-}Nramp1^{+/+}* and *Nramp1^{+/+}* mice, inhibition of necroptosis resulted in the reduction of damage to the placental –fetal unit suggesting reduced inflammation-induced pathology.

The results obtained through the use of cell death deficient strains during intravenous infection in pregnancy suggest that inflammation and necroptotic cell death may be the cause for placental loss, rather than absolute bacterial burden.

4.7 Standardization of an oral gavage protocol

Considering the fact that *Salmonella* species are foodborne pathogens and must be ingested in order to naturally cause illness in humans and animals, we next studied oral infection in pregnant and non-pregnant mice.

An oral gavage protocol was standardized, in order to obtain consistent gut colonization of S.Tm. Since S.Tm strain SL1344 is resistant to streptomycin, 24 hours prior to infection, C57BL/6J mice were pre-treated with 20 mg streptomycin in 100 μ L of deionized water compared to control mice (receiving only 100 μ L of deionized water) in order to deplete the competing gut microbiota which hinder infection and colonization by

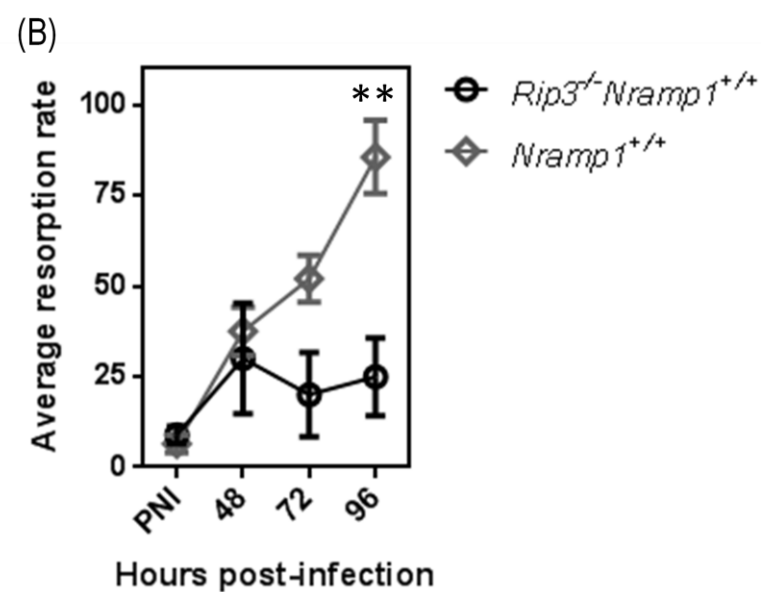
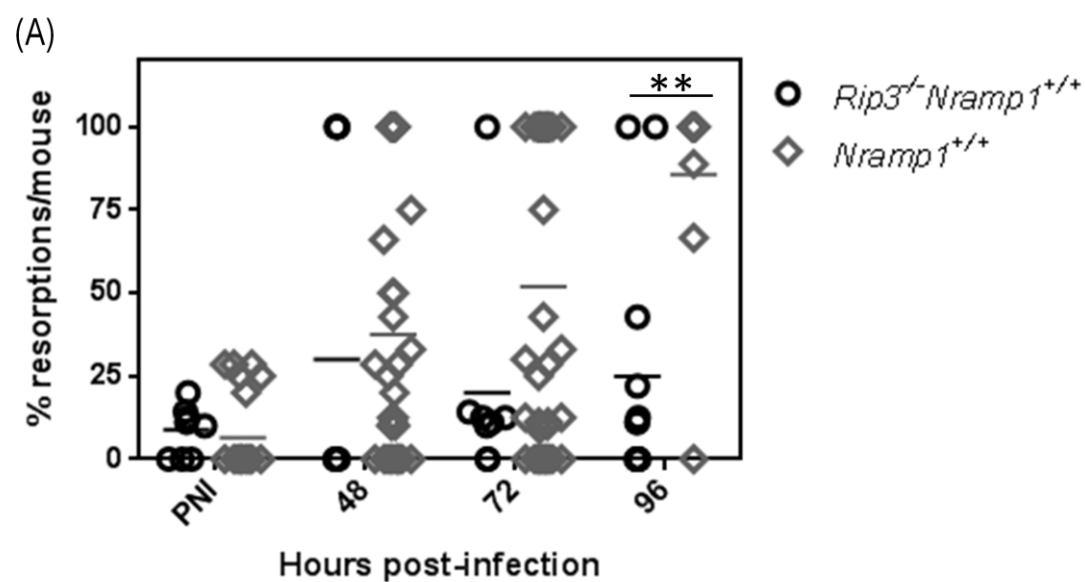


Figure 30

Figure 30. Resorption rate in *Rip3*^{-/-}*Nramp1*^{+/+} compared to *Nramp1*^{+/+} controls 48 to 96 hours post-infection. A) Percent resorptions per mouse (*Rip3*^{-/-}*Nramp1*^{+/+} versus *Nramp1*^{+/+} controls) in a pregnant non-infected state (baseline) compared to 48-96 hours post-infection; PI *Rip3*^{-/-}*Nramp1*^{+/+} mice have a significant reduction in resorption rate compared to *Nramp1*^{+/+} controls as time progresses; based on a two-way ANOVA and post-hoc (Sidak's multiple comparisons) test. B) Average resorption rate of *Rip3*^{-/-}*Nramp1*^{+/+} mice versus *Nramp1*^{+/+} mice per time point. *Rip3*^{-/-}*Nramp1*^{+/+} mice have a significant reduction in the average resorption rate compared to *Nramp1*^{+/+} controls as time progresses; based on a two-way ANOVA and post-hoc (Sidak's multiple comparisons) test. Pregnant mice (*Rip3*^{-/-}*Nramp1*^{+/+} and *Nramp1*^{+/+}) along with non-pregnant age-matched controls were infected intravenously with 10³ S.Tm. Mice were euthanized 48, 72 and 96 hours after infection and resorption rates were recorded. Each data point is indicative of the resorption rate of a single mouse in A, while average resorption rates per time point are shown in B. Experiment was repeated a two-six times depending on time point; 8-12 *Rip3*^{-/-}*Nramp1*^{+/+} mice per category, 10-52 *Nramp1*^{+/+} mice per category depending on time point.

Table 2

Average resorption rates amongst cell death deficient strains compared to *Nramp1*^{+/+} controls at various time points after systemic infection (Mean ± SEM)

Strain	Time Point of Infection			
	PNI	48 hours	72 hours	96 hours
<i>Nramp1</i> ^{+/+}	6.49 ± 2.36	37.50 ± 6.67	51.94 ± 6.48	85.54 ± 10.08
<i>Caspase-1,11</i> ^{-/-} <i>Nramp1</i> ^{+/+}	0.00 ± 0	N/A	42.35 ± 17.36	N/A
<i>Rip3</i> ^{-/-} <i>Nramp1</i> ^{+/+}	8.93 ± 2.42	30.00 ± 15.28	*20.05 ± 11.59	**24.96 ± 10.75

*p≤0.05: *Rip3*^{-/-}*Nramp1*^{+/+} mice have a significantly lower resorption rate compared to *Nramp1*^{+/+} controls at 72 hours post-infection; based on a one-way ANOVA and post-hoc (Sidak's multiple comparisons) test.

** p≤0.01 - *Rip3*^{-/-}*Nramp1*^{+/+} mice have a significantly lower resorption rate compared to *Nramp1*^{+/+} controls at 96 hours post-infection; based on a one-way ANOVA and post-hoc (Sidak's multiple comparisons) test.

N/A: not available

pathogens. Untreated mice showed variability in bacterial burden within systemic tissues compared to treated mice (Fig. 31 A). It was therefore concluded that streptomycin pre-treatment is necessary to establish a consistent and replicable infection. Infection of pre-treated pregnant mice receiving 10^7 CFUs of S.Tm showed few bacteria in the mesenteric lymph nodes and spleen and no bacteria within the placentas by day 3 post challenge (Fig. 31 B). Five days after challenge showed similar trends with bacteria present within the spleen and mesenteric lymph nodes but high variability within the placentas of C57BL/6J mice (Fig. 31 C). Increase in the infectious dose to 10^8 S.Tm resulted in significant and more consistent infection in systemic tissues and placental tissues 5 days post-oral infection (Fig. 31 D).

Standardization of an oral infection model was then attempted in pregnant *Nramp1*^{+/+} mice. Oral challenge with 10^8 S.Tm resulted in no bacteria within the placentas of infected *Nramp1*^{+/+} mice (Fig. 32 A). An increase in infectious dose to 5×10^8 CFUs resulted in variable placental infection (Fig. 32 B). These results suggest that the natural route of S.Tm infection during pregnancy can promote systemic dissemination and placental colonization. Nevertheless, *Nramp1*^{+/+} mice exhibit reduced and variable placental bacterial burden following oral infection which may be attributed to the resistant phenotype. Therefore, for the study of intravenous infection the *Nramp1* strain was used whereas for oral infection kinetics the C57BL/6J strain was studied (Fig. 33). Both models of infection enabled the study of infection during mid-pregnancy as experimental end-points were set for gestational day 15 regardless of the route of infection (Fig. 33).

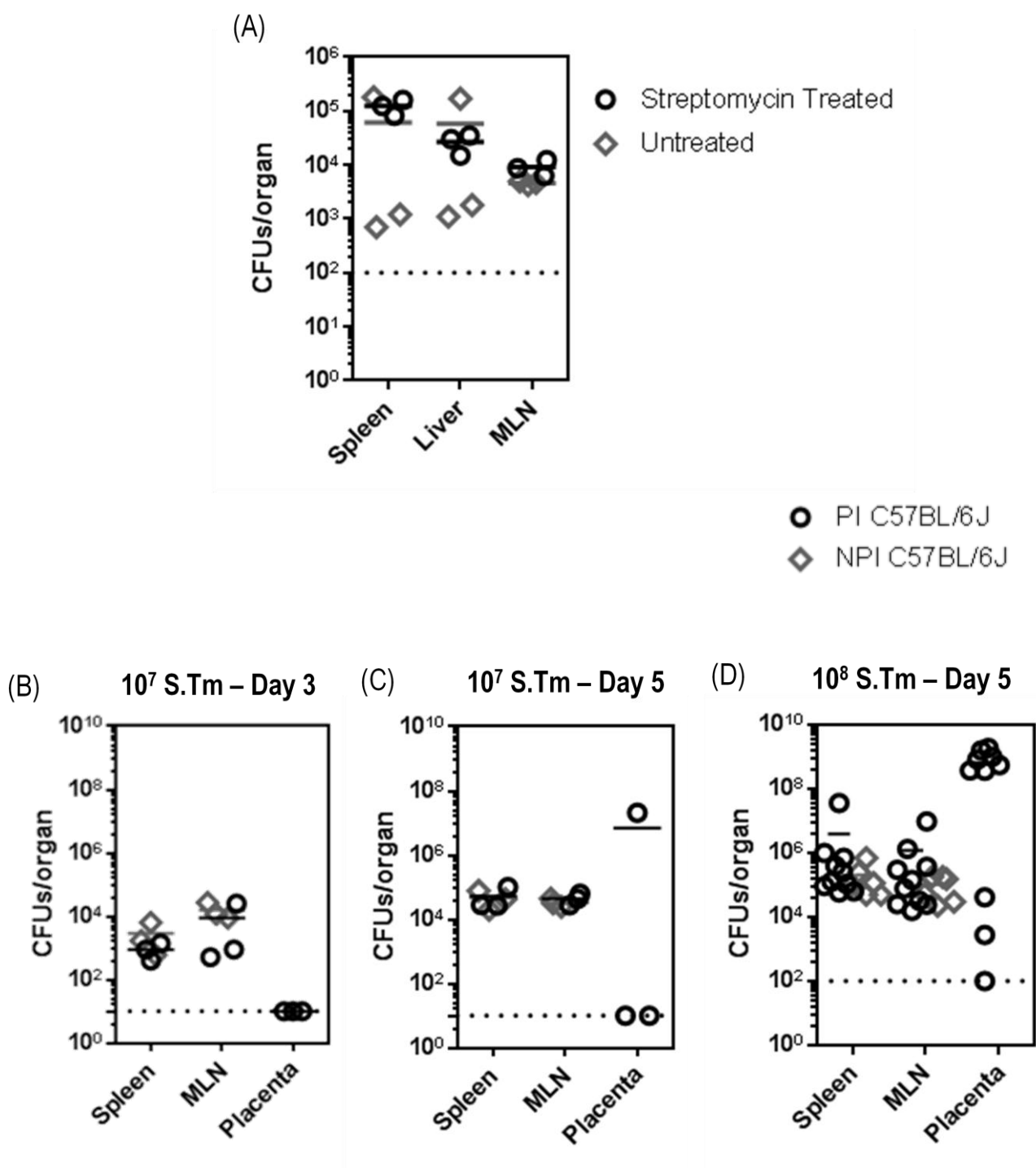


Figure 31

Figure 31. Standardization of an oral gavage protocol with streptomycin pre-treatment in C57BL/6J mice. A) Bacterial burden amongst systemic organs in non-pregnant C57BL/6J mice 5 days after oral infection with 10^7 S.Tm, with or without streptomycin pre-treatment (20 mg streptomycin/100 μ L H₂O). B) Bacterial burden in pregnant and non-pregnant C57BL/6J mice 3 days after oral infection with 10^7 S.Tm and streptomycin pre-treatment. C) Bacterial burden in pregnant and non-pregnant C57BL/6J mice 5 days after oral infection with 10^7 S.Tm and streptomycin pre-treatment. D) Bacterial burden in pregnant and non-pregnant C57BL/6J mice 5 days after oral infection with 10^8 S.Tm and streptomycin pre-treatment. Experiment was conducted twice. Mean (horizontal line) and detection limit (dotted line) are shown. All experiments were conducted once unless otherwise stated; 3-10 mice per category. MLN: mesenteric lymph nodes

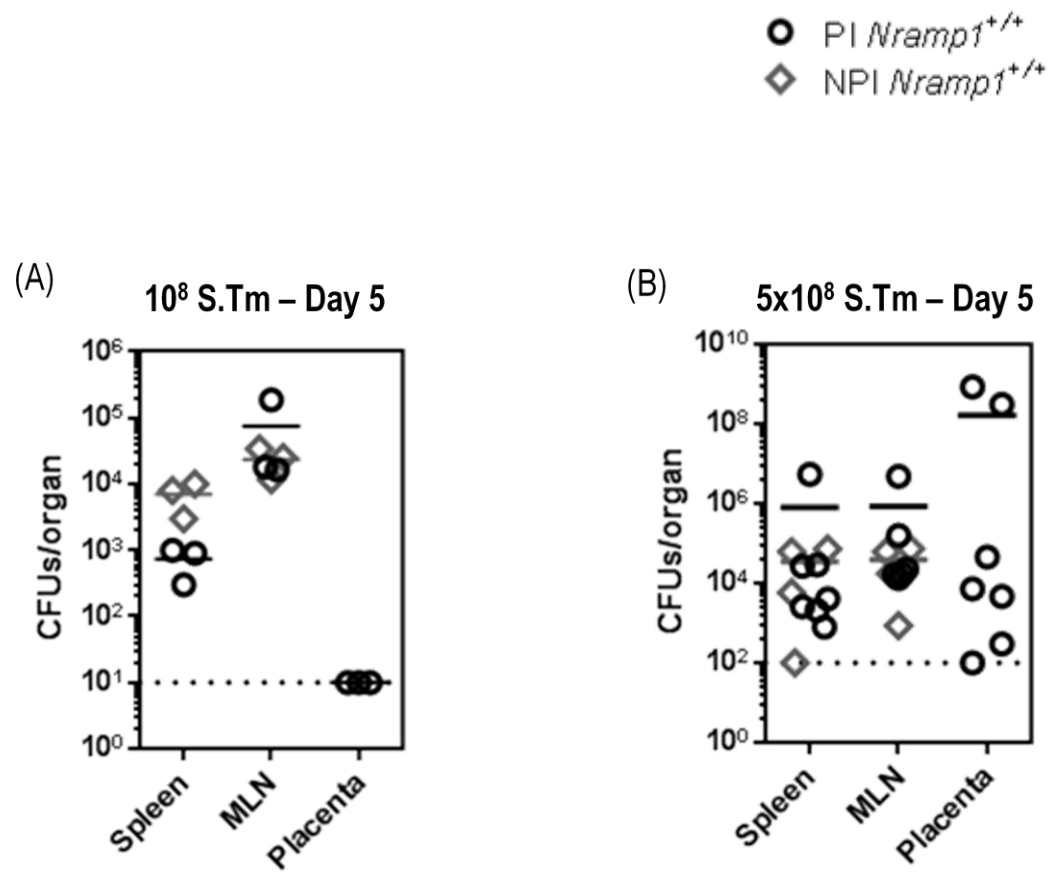
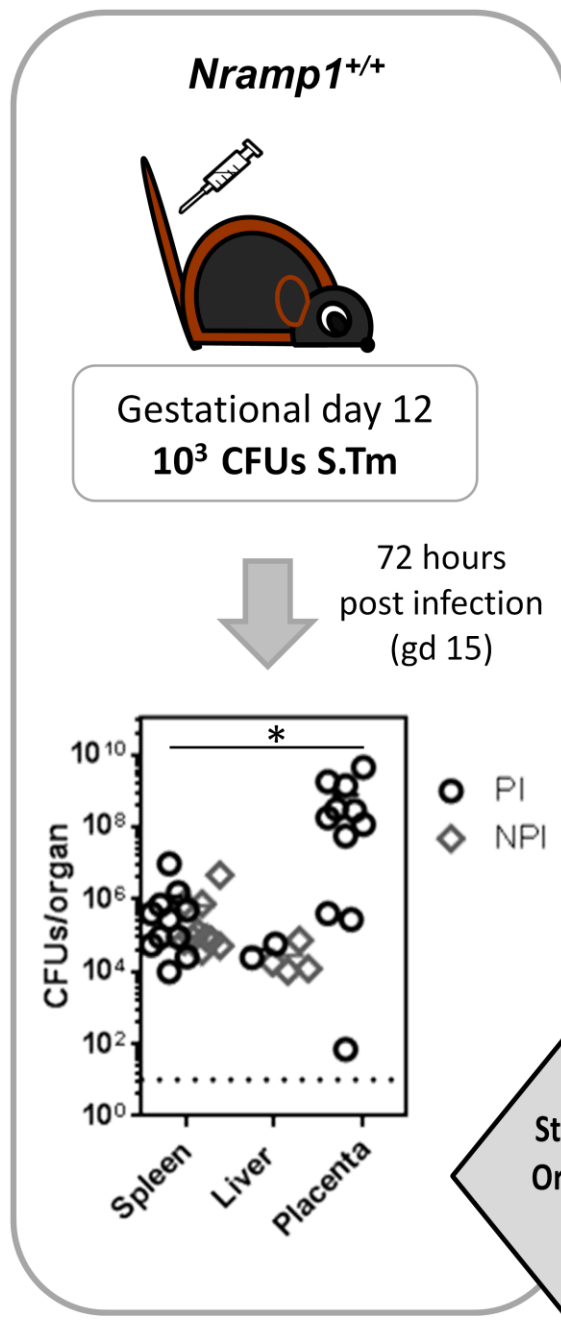


Figure 32

Figure 32. Attempted standardization of an oral gavage protocol with streptomycin pre-treatment in *Nramp1*^{+/+} mice. A) Bacterial burden in pregnant and non-pregnant *Nramp1*^{+/+} mice 5 days after oral infection with 10⁸ S.Tm and streptomycin pre-treatment. B) Bacterial burden in pregnant and non-pregnant *Nramp1*^{+/+} mice 5 days after oral infection with 5x10⁸ S.Tm and streptomycin pre-treatment. Experiment was conducted twice. Mean (horizontal line) and detection limit (dotted line) are shown. All experiments were conducted once unless otherwise stated; 3-7 mice per category.

A) Intravenous Infection Model



B) Oral Infection Model

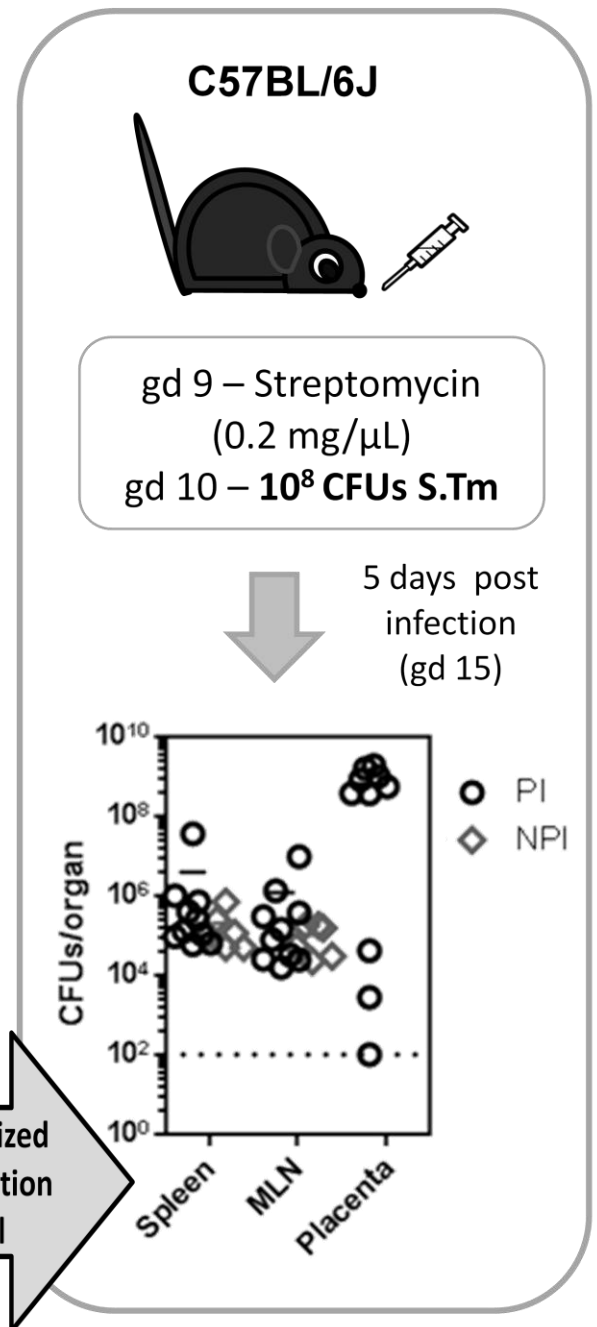


Figure 33

Figure 33. Infection models used to study *in vivo* *Salmonella enterica* serovar Typhimurium infection during pregnancy. A) *Nramp1*^{+/+} mice and associated cell death deficient mice on the *Nramp1*^{+/+} background were utilized to study systemic infection during pregnancy. B) C57BL/6J mice and associated cell death deficient mice on the C57BL/6J background were utilized to study oral infection during pregnancy. Oral infection was standardized to achieve a consistent and replicable infection in pooled placental tissue. Furthermore, bacterial burden during oral infection (5 days post-oral challenge) in C57BL/6J mice reaches similar titres in pooled placental tissue when compared to pooled placental tissue in *Nramp1*^{+/+} mice during systemic infection (3 days post-infection).

4.8 Oral model of infection: effect of S.Tm infection and host survival in pregnant C57BL/6J cell death deficient strains

A variety of cell death gene deficient strains on the C57BL/6J background were studied to determine oral infection outcome in pregnant hosts.

Survival curves of pregnant infected and non-pregnant infected cell death deficient strains revealed differences in host survival compared to C57BL/6J controls (Fig 34 A-F). *Caspase-1,11*^{-/-} mice both in a pregnant and non-pregnant state are as susceptible to infection as C57BL/6J controls (Fig. 34 A & B). Double knockout *Caspase-1,11*^{-/-}*Ifnar1*^{-/-} mice, both pregnant and non-pregnant, also show susceptibility to S.Tm infection similar to *Caspase-1,11*^{-/-} or wild-type C57BL/6J controls (Fig. 34 C). A similar trend was seen in pregnant and non-pregnant double knock-out *Caspase-1,11*^{-/-}*Rip3*^{-/-} mice (Fig. 34 D). Oral infection of *Rip3*^{-/-} mice resulted in pregnant *Rip3*^{-/-} mice living longer than non-pregnant *Rip3*^{-/-} mice with a median day of survival of 6 and 5 respectively (Fig. 34 E). Lastly, S.Tm infected *Ifnar1*^{-/-} showed increased survival relative to wild-type C57BL/6J mice. Furthermore, pregnancy did not increase the susceptibility to S.Tm infection in the absence of IFNAR (Fig. 34 F).

Comparison of PI cell death deficient survival curves to PI C57BL/6J controls reveal that *Caspase-1,11*^{-/-} mice are as susceptible as C57BL/6J controls (Fig. 35 A). On the other hand, PI *Rip3*^{-/-} mice live significantly longer ($p \leq 0.001$) than PI C57BL/6J (Fig. 35 A). Similarly, PI *Ifnar1*^{-/-} mice live significantly longer ($p \leq 0.0001$) than PI C57BL/6J mice (Fig. 35 A). Such survival results prompted us to further investigate *Caspase-1,11*^{-/-}, *Rip3*^{-/-} and

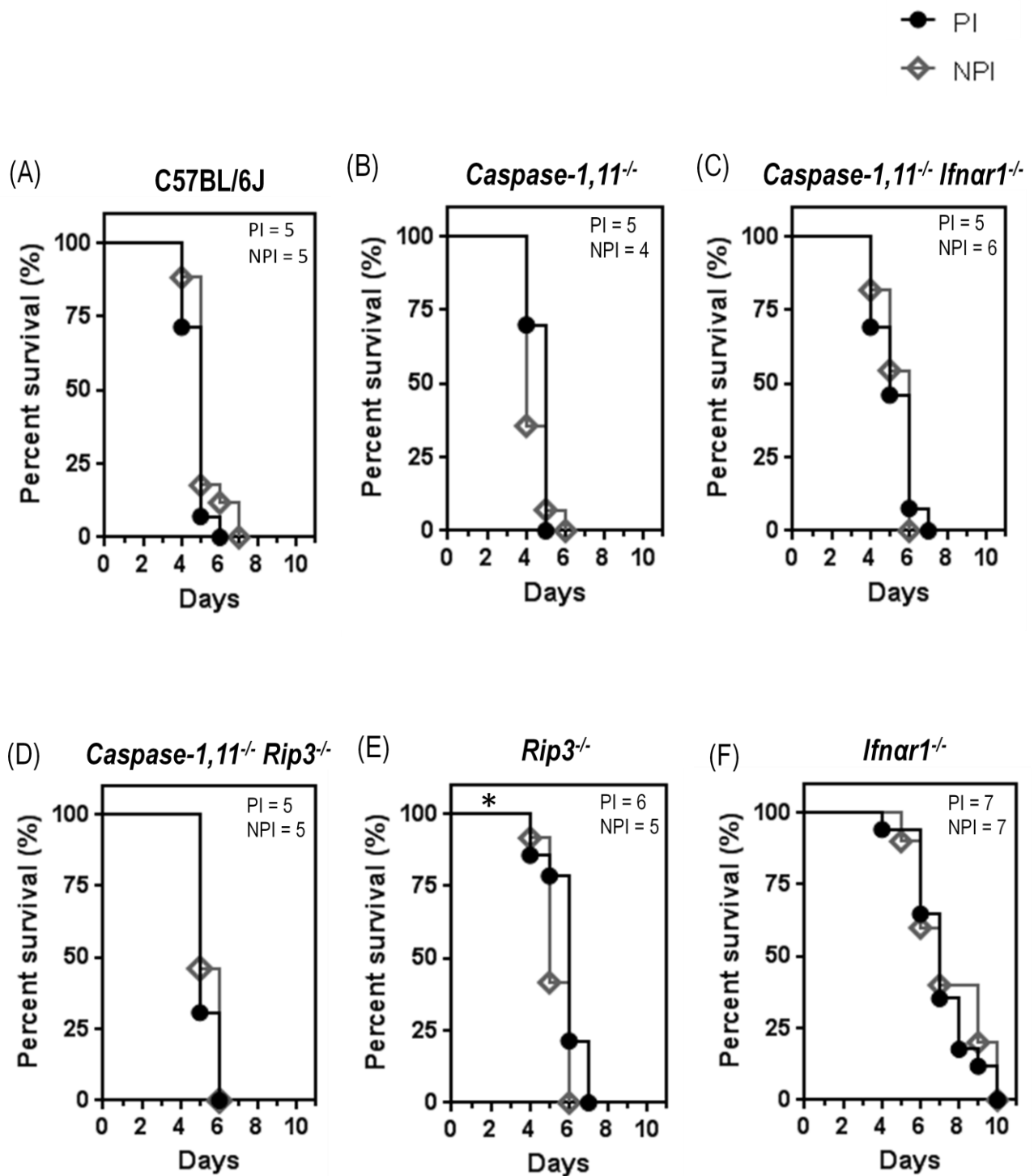


Figure 34

Figure 34. Survival kinetics of pregnant and non-pregnant cell death deficient strains versus C57BL/6J controls. A) C57BL/6J pregnant controls versus non-pregnant controls. B) *Caspase-1,11*^{-/-} pregnant versus non-pregnant mice. C) *Caspase-1,11*^{-/-}*Ifnar1*^{-/-} pregnant versus non-pregnant mice. D) *Caspase-1,11*^{-/-}*Rip3*^{-/-} pregnant versus non-pregnant mice. E) *Rip3*^{-/-} pregnant versus non-pregnant mice. Pregnant *Rip3*^{-/-} mice live longer (*p≤0.05) than non-pregnant infected *Rip3*^{-/-} mice; based on the log rank (Mantel-Cox) test. F) *Ifnar1*^{-/-} pregnant versus non-pregnant mice. Median days of survival are indicated in top right corner of each graph. Pregnant mice (gestational day 9 and 10) along with age-matched non-pregnant mice were streptomycin pre-treated 24 hours prior to oral infection with 10⁸ CFUs of S.Tm. Survival was repeated a total of 5 times to achieve 10 to 17 mice per category.

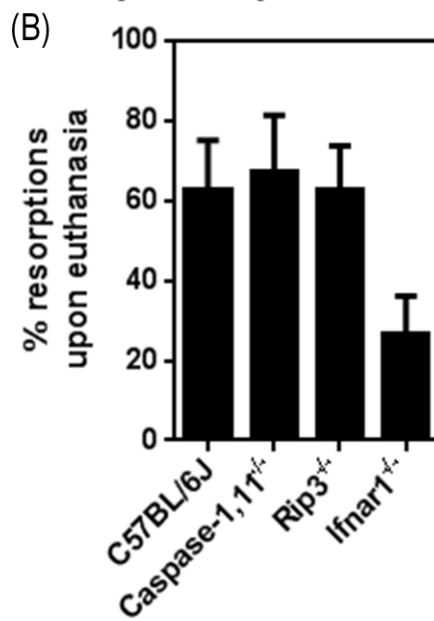
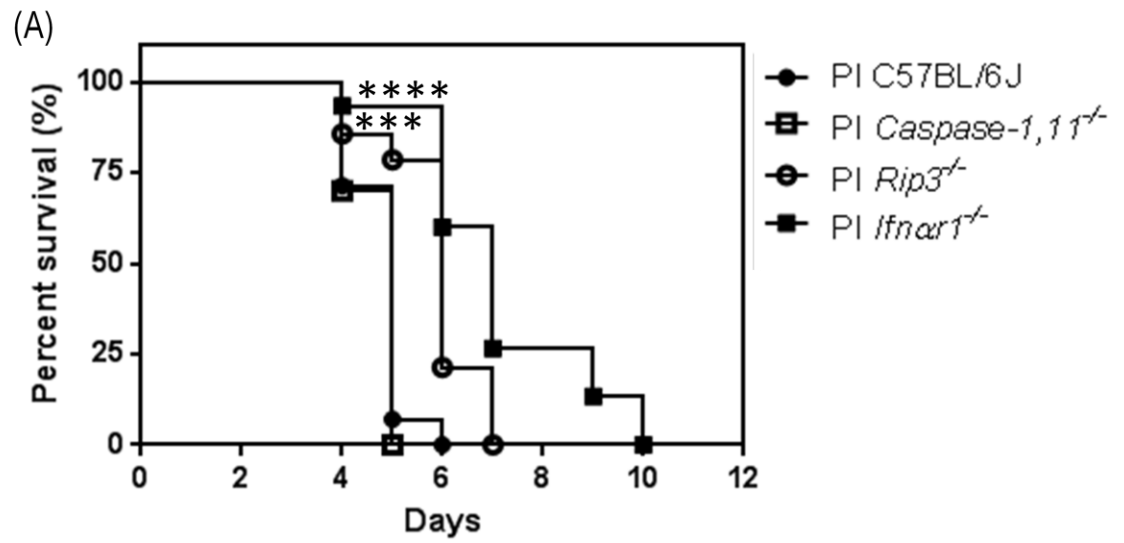


Figure 35

Figure 35. Survival kinetics of pregnant cell death deficient strains versus C57BL/6J controls and resorption rates upon euthanasia. A) Clinical course of oral infection in pregnant cell death deficient strains versus C57BL/6J pregnant controls. Pregnant *Rip3*^{-/-} mice live significantly longer (*** $p \leq 0.001$) than C57BL/6J controls; based on the log rank (Mantel-Cox) test. Pregnant *Ifnar1*^{-/-} mice live significantly longer (* $p \leq 0.05$) than *Rip3*^{-/-} mice and significantly longer (**** $p \leq 0.0001$) than C57BL/6J controls; based on the log rank (Mantel-Cox) test. B) Resorption rates of pregnant infected mice upon euthanasia. N numbers are as follows: 14 C57BL/6J mice, 10 *Caspase-1,11*^{-/-} mice, 14 *Rip3*^{-/-} mice, 15 *Ifnar1*^{-/-} mice.

Ifnar1^{-/-} mice to determine potential differences in bacterial burden and the rate of resorption.

Finally, throughout the survival studies conducted, resorption rates upon euthanasia of ill mice were recorded. Based on resorptions at the time of euthanasia, results indicate that *Ifnar1*^{-/-} showed a reduction, although not significant, in the rate of resorption compared to *Caspase-1,11*^{-/-} and *Rip3*^{-/-} mice and C57BL/6J controls (Fig. 35 B).

4.9 Oral infection outcome in C57BL/6J cell death deficient strains

From the survival results collected, we decided to investigate bacterial burden after oral infection in *Caspase-1,11*^{-/-}, *Rip3*^{-/-} and *Ifnar1*^{-/-} cell death deficient strains and compare bacterial titres and resorption rates to C57BL/6J controls.

Oral infection in *Caspase-1,11*^{-/-} mice (deficient in pyroptosis) resulted in a differential outcome in bacterial titres compared to C57BL/6J mice. PI *Caspase-1,11*^{-/-} mice had higher bacterial titres in their mesenteric lymph nodes ($p \leq 0.01$) and pooled placentas ($p \leq 0.01$) compared to PI C57BL/6J mice (Fig. 36 A & B). Moreover, NPI *Caspase-1,11*^{-/-} mice displayed increased bacterial burden in their mesenteric lymph nodes ($p \leq 0.001$) and spleens ($p \leq 0.001$) compared to NPI C57BL/6J mice highlighting that *Caspase-1,11* deficiency results in increased S.Tm susceptibility and replication regardless of pregnant/non-pregnant state. Increased bacterial burden in the placentas of PI *Caspase-1,11*^{-/-} mice was correlated with significantly higher resorption rates compared to C57BL/6J controls (Fig. 36 C).

Oral challenge of *Rip3*^{-/-} (deficient in necroptosis) and C57BL/6J controls revealed no differences in bacterial burden amongst mesenteric lymph nodes, spleens and pooled

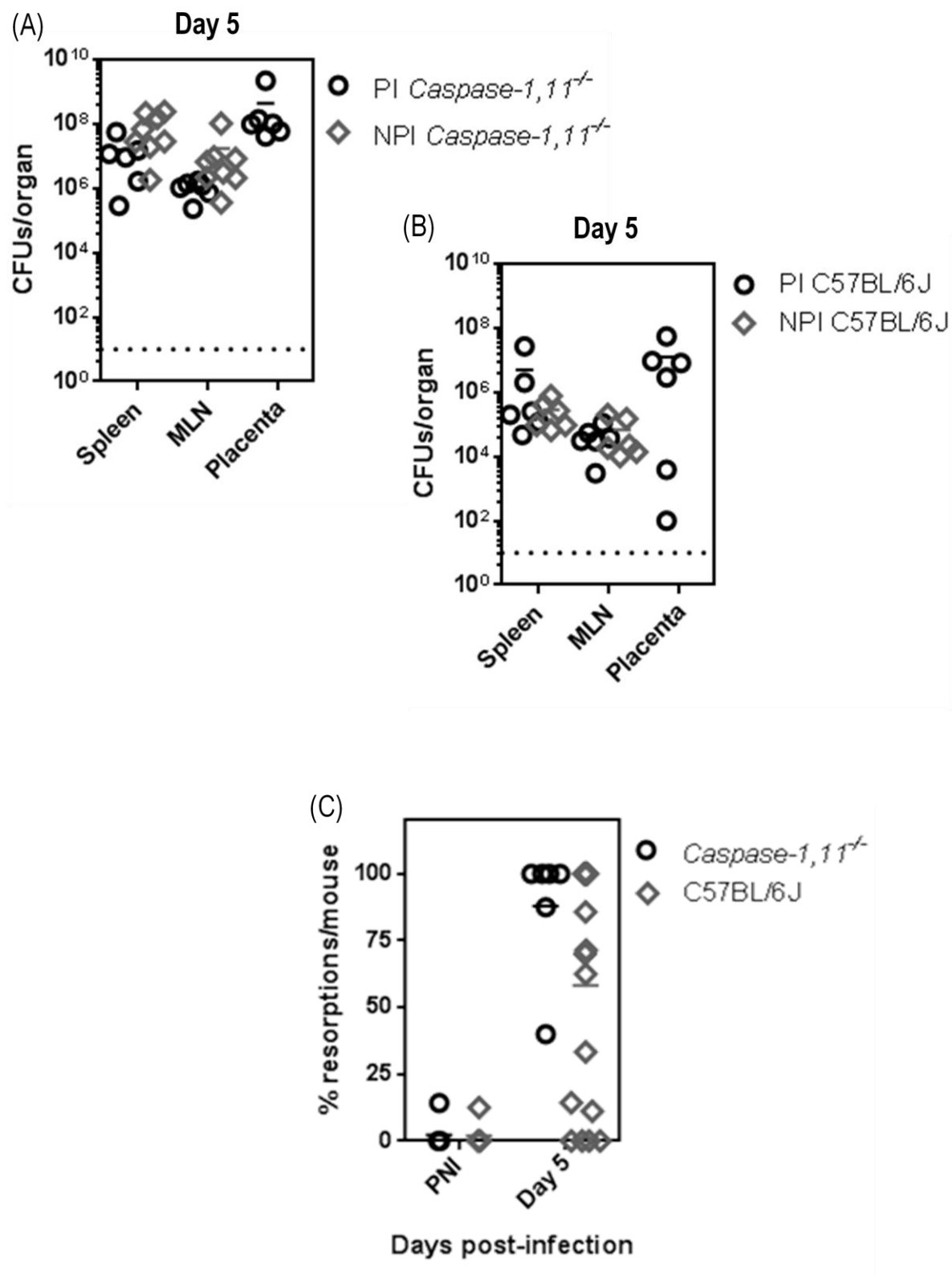


Figure 36

Figure 36. Infection outcome in *Caspase-1,11*^{-/-} mice compared to C57BL/6J controls.

A) Bacterial burden in pregnant infected and non-pregnant infected age-matched *Caspase-1,11*^{-/-} mice. B) Bacterial burden in pregnant infected and non-pregnant infected age-matched C57BL/6J control mice. C) Percent resorptions in *Caspase-1,11*^{-/-} mice compared to C57BL/6J mice. Pregnant mice (*Caspase-1,11* and C57BL/6J gestational day 9 and 10) along with non-pregnant age matched controls were pre-treated with 0.2 mg/μL streptomycin 24 hours prior to oral infection with 10⁸ S.Tm. Mice were euthanized 5 days after infection with collection of spleen, mesenteric lymph nodes and pooled placenta tissue. Resorption rates were also recorded. Each data point is indicative of bacterial burden or resorption rate of a single mouse. Mean (horizontal line) and detection limit (dotted line) are shown. Experiment was repeated four times; 6-8 mice per category. PI *Caspase-1,11*^{-/-} mice have significantly higher bacterial burden in their mesenteric lymph nodes ($p \leq 0.01$) and in their placentas ($p \leq 0.01$) compared to PI C57BL/6J mice; based on a two tailed Mann Whitney *U* test.

placentas between PI *Rip3*^{-/-} mice and PI C57BL/6J mice (Fig. 37 A & B). Furthermore, no differences in bacterial burden amongst NPI mice were seen (Fig. 37 A & B). Similarly, resorption rates were similar between *RIP3* deficient mice and C57BL/6J controls. This suggests that RIP3 deficiency in mice does not increase susceptibility to S.Tm infection during pregnancy.

It was recently shown that IFNAR1 signalling was vital for necroptosis to ensue within *Salmonella* Typhimurium infected macrophages. We therefore decided to examine S.Tm outcome in terms of bacterial burden and resorption rate in pregnant *Ifnar1*^{-/-} mice compared to C57BL/6J controls. Studies involving *Ifnar1*^{-/-} mice five days after oral challenge revealed interesting results; PI *Ifnar1*^{-/-} had significantly higher bacterial titres within their mesenteric lymph nodes and spleens in comparison to their placentas which had no detectable bacteria within pooled placenta homogenates (Fig. 38 A). As a result, bacterial burden was significantly lower in pooled placentas of *Ifnar1*^{-/-} mice compared to C57BL/6J controls (Fig. 38 A & B). This was correlated with a resorption rate of close to 0% in comparison to C57BL/6J controls which had a mean resorption rate of >50% (Fig. 38 C). Moreover, such resorptions rates were in stark contrast to other cell death deficient strains (Table 2).

Taken together, the intravenous and oral infection models reveal a differential outcome in pregnant cell death deficient strains during infection. *Caspase-1/11*^{-/-}*Nramp1*^{+/+} mice were as susceptible to I.V. S.Tm infection compared to *Nramp1*^{+/+} controls, while oral infection of *Caspase-1,11*^{-/-} mice revealed increased susceptibility, with higher bacterial burdens compared to C57BL/6J controls. *Rip3*^{-/-}*Nramp1*^{+/+} mice showed delayed placental inflammation compared to *Nramp1*^{+/+} controls during intravenous infection even though

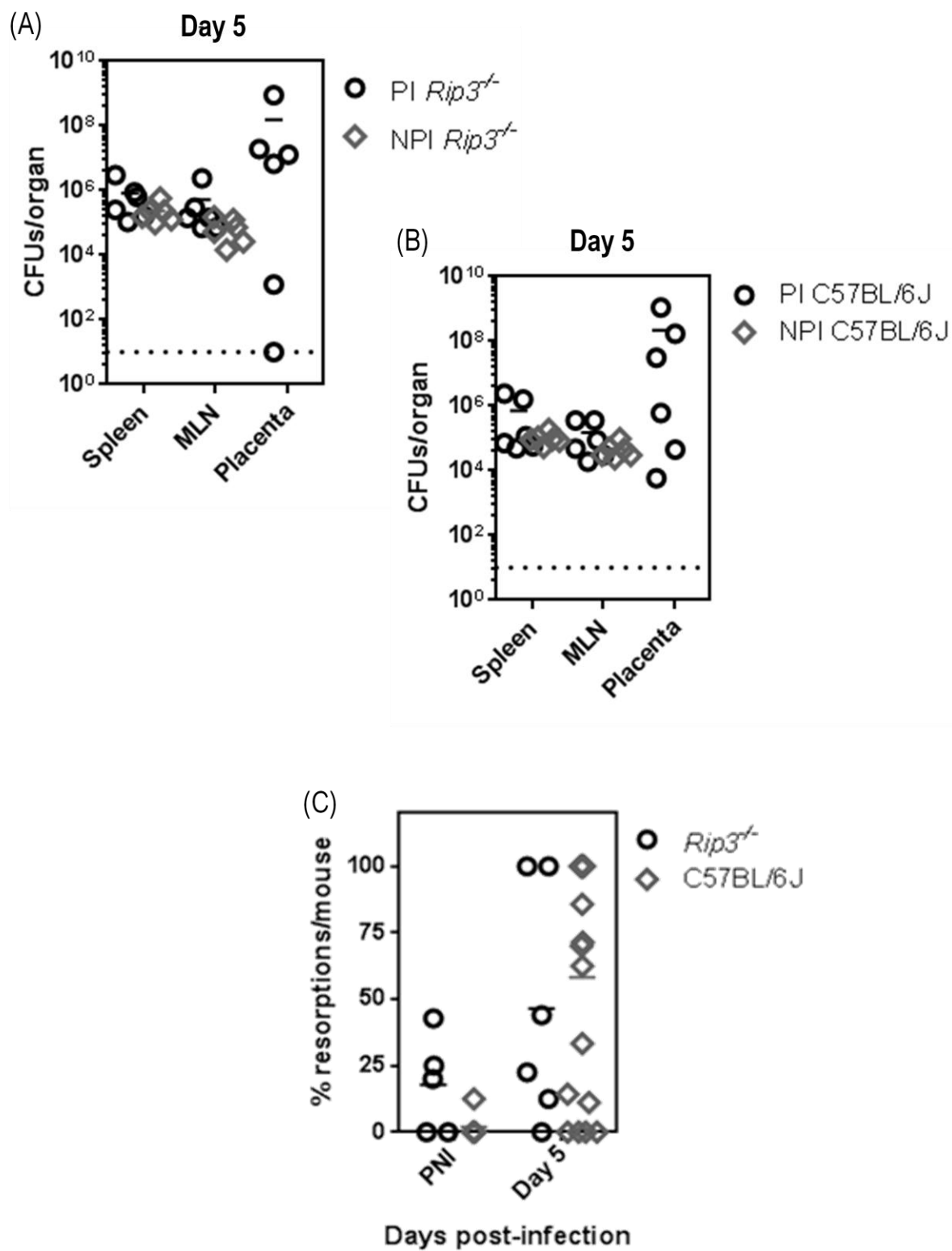


Figure 37

Figure 37. Infection outcome in *Rip3*^{-/-} mice compared to C57BL/6J controls. A) Bacterial burden in pregnant infected and non-pregnant infected age-matched *Rip3*^{-/-} mice. B) Bacterial burden in pregnant infected and non-pregnant infected C57BL/6J control mice. C) Percent resorptions in *Rip3*^{-/-} mice compared to C57BL/6J mice. Pregnant mice (*Rip3*^{-/-} and C57BL/6J gestational day 9 and 10) along with non-pregnant age-matched controls were pre-treated with 0.2 mg/μL streptomycin 24 hours prior to oral infection with 10⁸ S.Tm. Mice were euthanized 5 days after infection with collection of spleen, mesenteric lymph nodes and pooled placenta tissue. Resorption rates were also recorded. Each data point is indicative of bacterial burden or resorption rate of a single mouse. Experiment was conducted three times; 6 mice per category. Mean (horizontal line) and detection limit (dotted line) are shown.

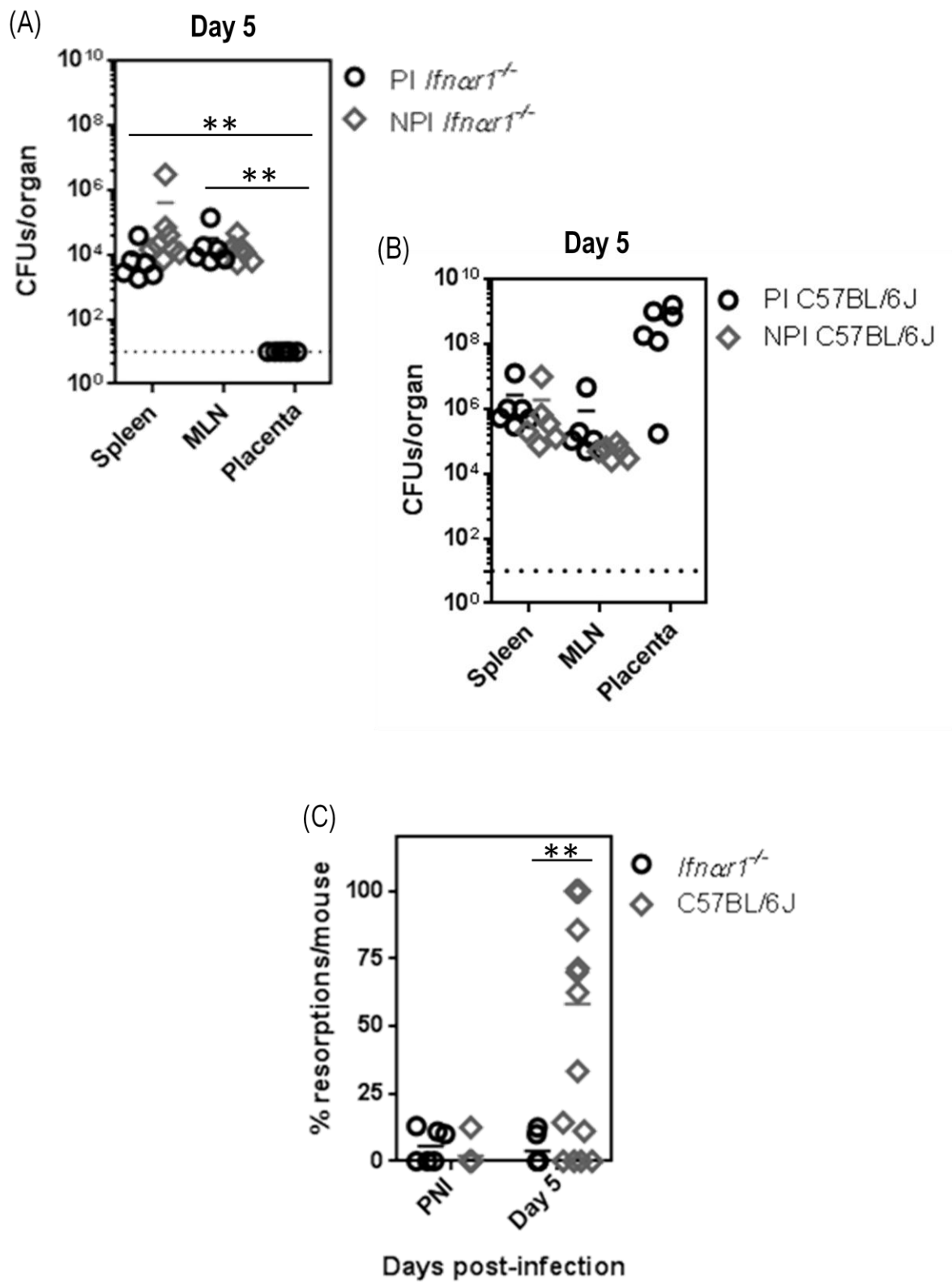


Figure 38

Figure 38. Infection outcome in *Ifnar1*^{-/-} mice compared to C57BL/6J controls. A) Bacterial burden in pregnant infected and non-pregnant infected age matched *Ifnar1*^{-/-} mice. B) Bacterial burden in pregnant infected and non-pregnant infected C57BL/6J control mice. C) Percent resorptions in *Ifnar1*^{-/-} mice compared to C57BL/6J mice. Pregnant mice (*Ifnar1*^{-/-} and C57BL/6J gestational day 9 and 10) along with non-pregnant age matched controls were pre-treated with 0.2 mg/μL streptomycin 24 hours prior to oral infection with 10⁸ S.Tm. Mice were euthanized 5 days after infection with collection of spleen, mesenteric lymph nodes and pooled placenta tissue. Resorption rates were also recorded. Bacterial burden in the placentas of PI *Ifnar1*^{-/-} mice is significantly lower (**p≤0.01) than the spleens and mesenteric lymph nodes of the same host and significantly lower (p≤0.01) in comparison to the placentas of PI C57BL/6J control mice. PI *Ifnar1*^{-/-} mice also have significantly less resorptions (**p≤0.01) in comparison to C57BL/6J mice 5 days post oral infection. Statistics based on a two-tailed Mann Whitney *U* test. Each data point is indicative of bacterial burden or resorption rate of a single mouse. Experiment was repeated four times; 6-8 mice per category. Mean (horizontal line) and detection limit (dotted line) are shown.

Table 3

Average resorption rates amongst cell death deficient strains compared to C57BL/6J controls 5 days after oral infection (Mean $\pm$ SEM)

Strain	Groups	
	PNI	Pregnant Infected
C57BL/6J	2.08 $\pm$ 2.08	58.24 $\pm$ 10.17
<i>Caspase-1,11</i> ^{-/-}	2.38 $\pm$ 2.38	87.92 $\pm$ 9.80
<i>Rip3</i> ^{-/-}	17.97 $\pm$ 6.63	46.50 $\pm$ 19.91
<i>Ifnar1</i> ^{-/-}	5.67 $\pm$ 2.57	***3.75 $\pm$ 2.39

***p $\leq$ 0.001: *Ifnar1*^{-/-} mice have a significantly lower resorption rate compared to C57BL/6J controls 5 days post-infection; based on a one-way ANOVA and post-hoc (Sidak's multiple comparisons) test.

bacterial burden in spleen, liver, and pooled placentas were comparable at each time point. This was not the case during oral infection as *Rip3*^{-/-} and C57BL/6J mice were equally susceptible. Lastly, PI *Ifnar1*^{-/-} on the C57BL/6J background, during oral infection, had no detectable bacteria within pooled placentas 5 days post-challenge. This was correlated with less placental inflammation compared to other cell death deficient strains and C57BL/6J controls.

5.0 Discussion

Outbreaks of foodborne pathogens are of great public health concern as mass production, packaging and distribution of food increases the risk of contamination. Although many case reports indicate that a variety of pregnancy complications can ensue as a result of *Salmonella* infection, there is a gap in our knowledge in the mechanisms involved in increased host susceptibility. Utilization of a pregnant mouse model during S.Tm infection enables the exploration of the interplay which exists between host and pathogen. Specifically, *in vivo* mechanisms such as host immune responses and bacterial immune evasion at the feto-maternal interface and in the maternal systemic compartment can be elucidated, complementing *in vitro* placental studies and human epidemiological studies. In the current *in vivo* study, we have begun to elucidate potential mechanisms of trophoblast cell death as a result of *Salmonella* Typhimurium placental infection during both intravenous and oral infection.

5.1 S.Tm infection during pregnancy in a mouse model

Previous studies have demonstrated that S.Tm infection in pregnant mice adversely affects the survival of both mother and fetus^{83,84}. Published studies utilized 129X1/SvJ mice which were resistant to S.Tm infection in the non-pregnant state but highly susceptible in the pregnant state, succumbing to infection within 7 days⁸³.

129X1/SvJ mice carry a functional *Nramp1* gene which confers resistance to S.Tm infection in a non-pregnant state. C57BL/6J mice have a point mutation in the *Nramp1* gene resulting in a non-functional divalent ion transporter and increased susceptibility to S.Tm infection.

The objective of our study was to determine if infection-induced death of trophoblast cells contributed to the increased susceptibility of pregnant mice to S.Tm infection. However, most gene-deficient mice are developed on the C57BL/6J background. In order to decipher the mechanism of trophoblast cell death through the use of various cell death deficient strains on the C57BL/6J background, the congenic C57BL/6J mouse carrying a functional *Nramp1* gene (*Nramp1*^{+/+}) was utilized for the current studies as a control, to enable a greater time frame to study the adverse effects attributed to S.Tm infection during pregnancy.

Nramp1^{+/+} mice in a non-pregnant state developed a chronic S.Tm infection (following systemic exposure) lasting 3-4 weeks compared to C57BL/6J mice with a median survival of 6 days (Fig. 8). Furthermore, in a pregnant state, *Nramp1*^{+/+} mice survived between 6-11 days post-infection (Fig. 8). As a result, *Nramp1*^{+/+} mice constituted a convenient model to study S.Tm infection during pregnancy, as there is a greater time frame in which the detrimental effects of infection can be studied.

The kinetics of infection in *Nramp1*^{+/+} mice indicate that S.Tm has a predilection for colonization and replication within the placentas of pregnant mice compared to other systemic tissues such as spleen and liver (Fig. 9 A-C). Intravenous infection with 10³ CFUs resulted in a mean burden of ~10⁶ bacteria within pooled placentas by 24 hours, whereas the systemic burden was quite low (~10³) (Fig. 9 A). By 72 hours post-infection, an astounding increase in bacterial burden was seen in pooled placental tissue (~10⁹), and systemic organs were also heavily infected in pregnant mice (~10⁵) (Fig. 9 C).

In vitro studies reveal that S.Tm proliferates substantially in JEG-3 human placenta-derived choriocarcinoma cells with a doubling time of 1 hour in comparison to HeLa human

cervical epithelial cells in which the doubling time is >4 hours⁸⁴. Previous studies have also demonstrated preferential placental proliferation of bacteria in 129X1/SvJ and the F1-cross strain (129X1xBL6)^{83, 84}. The current study in the *Nramp1* strain corroborates these results, that *Salmonella* species preferentially invade and/or survive within trophoblast cells. However, we observed that the systemic bacterial burden (spleen and liver) in pregnant mice was similar to non-pregnant mice. This contrasts with our previous results in other mouse strains wherein by 72 hours the splenic burden was also substantially higher in pregnant mice relative to non-pregnant infected controls. Thus, it appears that the *Nramp1*^{+/+} strain may show some increased systemic resistance even in the pregnant state to S.Tm infection.

In all previous studies, placentas from individual mothers were pooled to study infection at the feto-maternal interface. In this study, we determined the kinetics of progression of infection among individual placentas. Considering pooled placentas displayed substantial increases in bacterial burden from 24 to 72 hours post-infection, we hypothesized that the rapid increase may be attributable to equal invasion and replication within all placentas, or the establishment of a focus of infection within select placentas and subsequent spread. Investigation of S.Tm kinetics within individual placentas enabled an illustrative analysis of bacterial colonization and replication in relation to placental location within the uterine horns as well.

We have shown that the number of individual infected placentas increased progressively from 24-72 hours post-infection and correlated with increased fetal loss (Fig. 10 A & C). Given that after systemic infection of 1000 bacteria, it may be predicted that ~10% may reach the feto-maternal interface, it appears that a small focus of infection in a few concepti sites may be first established (Fig. 11-13). We did not notice any pattern

among the infected placentas in regards to their location in the uterine horn. Thus, it appears that few bacteria probably reach the placenta through the hematogenous route, and by random probability, cause an infection focus. However, the rapid proliferation of *S.Tm* then contributes to further infection at additional sites and rapid detrimental effects of the growing foetuses. Given the ability of *Salmonella* to double rapidly even *in vivo* (<2 hours)⁸⁴, few bacteria that reach the placenta may be able to establish productive infection. Thus, kinetics of infection in individual placentas revealed that bacterial titres and subsequent consequences of infection differ amongst placentas of a single host, highlighting the importance of individual placental analysis in delineating mechanisms of pathogen invasion and host immune responses at the feto-maternal interface.

Previously published *in vitro* results indicate that trophoblast cells actively take up *Salmonella* Typhimurium via receptor-mediated endocytosis¹⁹⁴. The increased production of IL-10 by trophoblasts prevents the maturation (phagosomal-lysosomal fusion) of the *Salmonella* containing vacuole, thereby providing a niche for which *Salmonella* can profoundly replicate and act as a potential reservoir for dissemination throughout the pregnant host¹⁹⁴.

Thus, *Salmonella*, an intracellular pathogen, may be similar to other pathogens such as *Listeria monocytogenes*, *Chlamydia* species, *Toxoplasma gondii*, etc. which are known to preferentially invade the placenta resulting in fetal demise.

5.2 Mechanism of invasion

The mechanism of *in vivo* invasion by which *Salmonella* gains entry to trophoblast cells remains to be elucidated. Maternal and fetal cells are in direct contact with each other

in specific areas where pathogen invasion may potentially occur: the syncytiotrophoblast-blood interface or the extravillous trophoblast-uterine interface in humans (trophoblast giant cell-uterine interface in mice)¹². The syncytiotrophoblasts, in both humans and mice, are bathed in maternal blood to enable nutrient and gas exchange, while the extravillous trophoblasts (trophoblast giant cells in mice) directly invade the maternal uterus¹². Syncytiotrophoblasts, which fuse to form a continuous multinucleated layer termed syncytium in both humans and mice, are exposed to a variety of pathogens circulating within the maternal blood compartment. *Salmonella* may gain direct entry to the feto-maternal interface by escaping the maternal blood. On the contrary, the extravillous trophoblasts (trophoblast giant cells) which are juxtaposed to the maternal decidua, may be a second site of potential infection via bacterial shuttling in infected macrophages or dendritic cells.

Interestingly, studies involving placental pathogens such as *Listeria monocytogenes* have shown that human syncytium is resistant to *L. monocytogenes* invasion⁹². A similar trend is seen with studies involving various viral and parasitic pathogens^{123, 245, 246}. A variety of unique mechanisms are hypothesized to aid in the prevention of syncytium pathogen invasion: lack of receptors required for internalization at the maternal blood interface and a lack of intracellular junctions to enable cell-cell spread¹². Moreover, the actin cytoskeleton within syncytiotrophoblasts prevents deformation compared to other trophoblast cell types, as disruption of the actin cytoskeleton contributed to decreased elasticity of syncytiotrophoblasts and an increase in *L. monocytogenes* colonization within human and mouse syncytium²⁴⁷.

Syncytial damage, as a result of infection from a variety of pathogens, has been shown to facilitate the spread of placental infection. Artificial enzymatic degradation of

syncytiotrophoblasts enables increased bacterial and parasitic colonization of the underlying cytotrophoblasts during *Listeria monocytogenes* and *Toxoplasma gondii* infection^{92, 123}. *Plasmodium falciparum* infection during pregnancy is associated with an increased accumulation of infected erythrocytes within the intervillous space of human placentas leading to syncytial degradation²⁴⁸. Such damage has been associated with increased HIV vertical transmission²⁴⁹. Thus, the syncytium forms an effective biophysical barrier against pathogen invasion and breach of this barrier may only occur once a certain level of cellular damage is incurred.

Surprisingly, the extravillous trophoblasts in humans (trophoblast giant cells in mice), are more susceptible to infection than syncytium. During *L. monocytogenes* or *T. gondii* infection in human placental explants, the extravillous trophoblasts are the preferred site of invasion^{92, 123}. Cytomegalovirus naturally infects the decidua and extravillous trophoblasts in human term placentas²⁵⁰. Murine studies have also revealed that trophoblast giant cells are the initial site of invasion and colonization during *Brucella abortus*⁸⁵, *Fusobacterium nucleatum*²⁵¹, *Coxiella burnetti*⁹⁷, *Listeria monocytogenes*⁹⁶ and *Toxoplasma gondii*²⁵² infection. Moreover, previous studies in our laboratory noted that wild-type *Salmonella* Typhimurium was capable of infiltrating deep within the labyrinth region of the murine placenta of 129X1/SvJ mice causing placental necrosis, whereas an attenuated mutant strain was restricted to the decidua. Thus, establishment of infection within the labyrinth may be essential for differential inflammation resulting in adverse fetal and maternal outcome⁸⁴.

Therefore, the more vulnerable site of invasion by pathogens may be the extravillous trophoblast (trophoblast giant cell)-uterine interface. Despite the increase in susceptibility of

the EVT-uterine interface, both the syncytiotrophoblast-blood and extravillous-uterine interfaces may be effective barriers until a threshold of cellular damage is incurred, permitting breach and subsequent invasion, colonization, and spread of pathogens (Fig. 39).

Although the localization of S.Tm species within the placenta, whether intracellular or extracellular, was not investigated in the current study presented, complimentary histological analyses are currently being conducted with our collaborator, Dr. Croy, at Queen's University. These studies, when completed, should elucidate the mechanism of *in vivo* placental infection, which may bear implication for bacterial escape and host pathogenesis.

5.3 Infection kinetics after systemic and oral S.Tm infection in pregnant hosts

Our use of both oral and intravenous models help to give insight into the potential ways in which S.Tm species may be reaching the feto-maternal interface. Considering that the syncytiotrophoblasts are bathed in maternal blood, intravenous infection permits direct bacterial translocation to the placenta (as well as other systemic tissues) and subsequent placental infection. On the other hand, oral infection studies give insight into the natural route of infection and the potential ways in which S.Tm are able to traffic from the gut to the placenta.

Standardization of the oral gavage protocol alone illustrated the many obstacles S.Tm species must overcome in order to colonize and replicate within placental tissues. Experimental oral *Salmonella* infection without streptomycin pre-treatment is often highly variable^{176, 253} and highlights the importance of gut microbiota and their role in the prevention

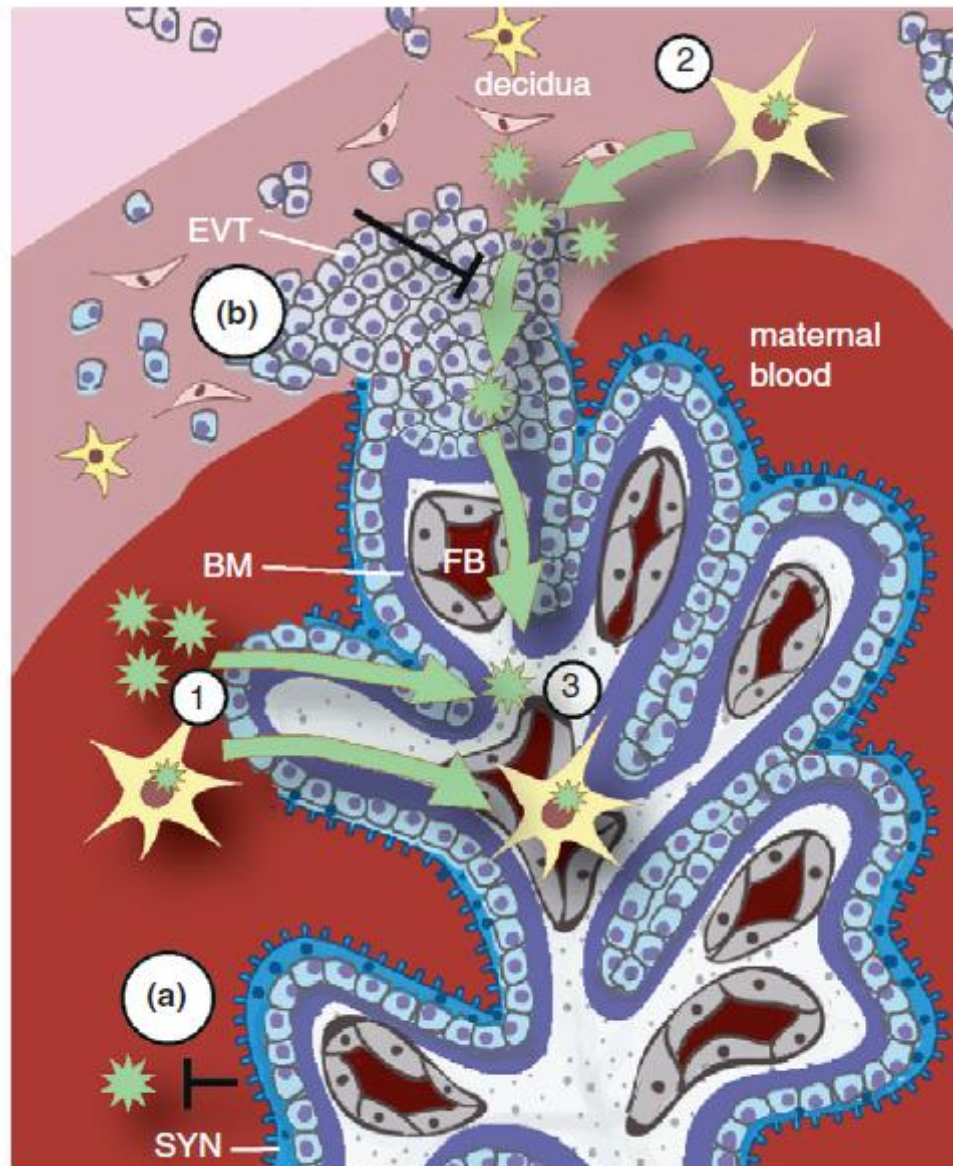


Figure 39

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Robbins, J.R. & Bakardjiev, A.I. Pathogens and the placental fortress. *Curr. Opin. Microbiol.* **15**, 36-43 (2012).

Figure 39. Schematic representation of the potential interfaces and mechanisms of invasion by pathogens tropic for the placenta. Illustrative representation of the human feto-maternal interface and the potential areas in which placental pathogen invasion may occur: the syncytiotrophoblast-blood interface (a) or the extravillous trophoblast (trophoblast giant cell in mice)-uterine interface in humans (b). Studies have shown that both the syncytiotrophoblasts and extravillous trophoblasts are effective barriers to pathogen invasion until a certain threshold of cellular damage is incurred, thereby permitting breach and subsequent invasion. 1) Syncytiotrophoblast damage enables pathogens (green stars) circulating within the maternal blood, or antigen presenting cells containing pathogens (yellow cells with green stars) to invade into placental tissues, potentially reaching the fetus by the fetal bloodstream. 2) Pathogen dissemination via leukocytes, such as macrophages or DCs, enables pathogens to reach the maternal decidua. Pathogen breach of the extravillous trophoblasts allows colonization and spread in the underlying cytotrophoblasts. 3) Vertical transmission may occur once the pathogen has reached the fetal blood. EVT: Extravillous trophoblast, SYN: syncytiotrophoblast, BM: basement membrane, FB: fetal blood

of intestinal infection²⁵⁴. A wide array of commensal bacteria colonize the human gut and assist in gut homeostasis, as well as create a barrier against invading pathogens, by competing with pathogens for nutrients and preventing pathogen adherence to the intestinal epithelium^{176, 255}. Mouse experiments demonstrate that S.Tm infection in the gut results in inflammation, thereby allowing S.Tm to successfully colonize the gut by outcompeting host microbiota²⁵⁶.

Treatment of mice with antibiotics, such as streptomycin, reduces host microbiota populations and leads to increased *Salmonella* colonization and inflammation in comparison to non-treated mice²⁵⁷. Pre-treatment of mice with streptomycin followed by *Salmonella* infection three weeks later still results in increased levels of inflammation and infection compared to non-treated mice, highlighting the importance of gut microbes and the detrimental and long-lasting effects antibiotics have on gut microflora populations. This has been further validated in an epidemiological case study in which individuals who recently used antibiotics had a significantly higher incidence of NTS diarrhea due to infection with both antimicrobial sensitive and resistant *Salmonella* strains compared to age and regional-matched controls²⁵⁸. Therefore, the use of antibiotics to treat other ailments results in prolonged alteration of the gut flora, allowing for increased colonization of the gut by pathogenic bacteria such as *Salmonella* species²⁵⁸. Such results stress the further increase in susceptibility of pregnant hosts to foodborne pathogens while on antibiotic medications.

Oral infection of C57BL/6J mice and *Nramp1*^{+/+} mice resulted in a differential outcome of infection, with *Nramp1*^{+/+} mice displaying increased resistance and a lack of placental infection, even after an increased infectious dose, compared to C57BL/6J mice (Fig. 31 D & Fig. 32 A & B). Since *Nramp1* is expressed on the phagosomes of both

macrophages and dendritic cells, and *Salmonella* species preferentially replicate within phagosomes, the preliminary oral infection studies involving *Nramp1*^{+/+} mice hint that S.Tm trafficking from the gut to the placental may occur through transport in infected macrophages and dendritic cells. Future studies involving the comparison of oral S.Tm infection between C57BL/6J mice and congenic *Nramp1*^{+/+} mice may provide insight into the main trafficking mechanisms utilized by S.Tm to reach the feto-maternal interface.

5.4 Differential immuno-modulation at the feto-maternal interface

The research presented indicates that S.Tm induced pathology at the feto-maternal interface results in two sequential steps, being profound bacterial growth within the placenta, followed by a significant decline in host resistance, in which a normally resistant mouse becomes highly susceptible during pregnancy. At 24, 48 and 72 hours post-infection in pregnant *Nramp1*^{+/+} hosts, splenic bacterial burden was preceded by substantial placental bacterial burden. It is plausible that S.Tm has a predilection to first colonize the placenta and then traffic to other peripheral organs such as the spleen. *Listeria monocytogenes* has also been shown to have a similar precedence for trafficking from placenta to spleen²⁵⁹.

At 48 hours post infection, neutrophil numbers within the spleens of pregnant infected hosts were comparable to pregnant non-infected hosts (Fig. 16). Furthermore, at 72 hours post infection, PI hosts had fewer, although not significant, splenic neutrophils versus NPI hosts, when compared to their respective non-infected controls (Fig. 20 B). The activation of neutrophils during bacterial infection assists in bacterial clearance through the induction of DNA containing neutrophil extracellular traps (NETs) which are capable of collecting and killing bacteria²⁶⁰. Therefore, the lack of adequate neutrophils within the

spleens of PI hosts may be one contributing factor of insufficient bacterial control and clearance in the maternal systemic compartment.

Despite the lack of neutrophil recruitment to the spleen during infection in pregnant hosts, there was a substantial increase in neutrophil percentages within individual placentas (Fig. 23 A). This coincides with published immunohistology data in the 129X1/SvJ strain involving infection with virulent *Salmonella*, but not mutant avirulent S.Tm, in which there was a massive influx of GR-1 positive neutrophils throughout the labyrinth trophoblasts of the murine placenta leading to tissue integrity loss and a highly necrotic labyrinth⁸⁴. Virulent S.Tm were dispersed throughout the necrotic labyrinth while avirulent bacteria were restricted to the decidua⁸⁴. Thus, establishment of an infection within the labyrinth trophoblasts may trigger overt inflammation.

The recruitment and activation of other immune cell subsets such as NK cells was also not evident in the spleen in comparison to individual placentas (Fig. 22 F & Fig. 23 F). Since perturbations in NK cells within the periphery during pregnancy can result in pregnancy loss and pre-eclampsia, prevention in the activation of certain immune subsets may be a pregnancy related mechanism in order to avoid an adverse outcome^{261, 262}. Furthermore, the lack of peripheral NK cell recruitment to the spleen during infection in pregnancy may be attributed to the redistribution of NK cells towards the placenta²⁶³.

Previously published results have revealed a significant increase in IFN- γ mRNA expression within pooled placentas of pregnant infected hosts⁸⁴. Considering that neutrophils are the first line of defence during microbial infection, and are the main source of IFN- γ during early infection, with IFN- γ production being vital for NK cell activation, the inflammation seen at the feto-maternal interface may be the compounding result of both

neutrophil and NK activation. Moreover, neutrophils and their induction of NETs have been implicated in the pathogenesis of pre-eclampsia, further validating such rationale for the lack of fetal rescue and the inflammation seen²⁶⁴.

That being said, it is important to keep in mind that there exists two types of NK cell populations during pregnancy, those that are cytotoxic and found systemically, and uterine NK cells which are not cytotoxic, playing an essential role during implantation.

Phenotypically, uterine NK cells are CD56^{bright}/CD16⁻ while peripheral NK cells are CD56^{dim}/CD16⁺. In healthy pregnant hosts, uterine NK cells decline substantially within the placenta by gestational day 9³³. Although uNK cells are not cytotoxic during normal pregnancy, and are not required in the resolution of placental *L. monocytogenes* infection, they have been implicated in triggering inflammation and fetal resorption during late gestation^{265, 266}.

The results presented thus far are too premature to identify exactly which NK cell population is increasing significantly in infected placentas. It is plausible that the uNK cells present within the placenta upon infection may be responding to S.Tm infection and contributing to placental pathology. In addition, the potential lack of trafficking of uNK cells from the placenta to the spleen may be causation for the lack of a rapid and robust systemic innate immune response. Further studies are required in profiling the NK cells present at the feto-maternal interface during S.Tm infection to definitely reveal their role.

During infection, there was a marked increase in CD11b⁺ CD11c⁻ cells at the feto-maternal interface (Fig. 23 B). These cells were also F4/80 positive. It is tempting to speculate that these cells are indeed monocytes or tissue macrophages, but it is important to note that F4/80 co-expression with CD11b has been found on DCs present within various

tissues such as spleen and lymph nodes²⁶⁷. In the spleen, both inflammatory monocytes and macrophages are phenotypically classified as CD11b^{high}CD11c⁻F4/80⁺ with distinction of the two populations being based on other surface markers such as Ly6C expression²⁶⁸.

Likewise, decidual DC characterization based upon CD11b, CD11c and F4/80 expression is also difficult. Collins and colleagues have characterized decidual DCs into two distinct populations, those that are F4/80 negative and either CD11b^{low}CD11c⁺ or CD11b^{high}CD11c⁺ and those that are F4/80 positive and CD11c^{low}⁵². Our results suggest that CD11b⁺CD11c⁻F4/80⁺ cells may be macrophages while the CD11c⁺F4/80⁺ cells are DCs (Fig. 23 B & C).

In response to placental S.Tm infection we surmise that the increase in neutrophils results in the secretion of inflammatory cytokines and/or chemokines, leading to the recruitment of macrophages. Based on our results, the responder macrophages have the potential to exhibit an inflammatory phenotype or an anti-inflammatory phenotype expressing IL-10. Inflammatory macrophages are associated with the secretion of pro-inflammatory mediators such as TNF to participate in the activation of antimicrobial mechanisms²⁶⁹. Oppositely, a CD11b⁺CD11c⁻F4/80⁺ macrophage population has been shown to secrete little to no inflammatory cytokines and instead produces copious amounts of IL-10²⁶⁹. Previously published studies in our lab have highlighted that there is a significant increase in the amount of inflammatory cytokines such as IL-6 and TNF- α as well as production of anti-inflammatory IL-10 within infected placental tissues⁸³. Such inflammatory cytokine production may be a result of infiltrating inflammatory CD11b⁺CD11c⁻F4/80⁺ macrophages or in contrast, the infiltrating CD11b⁺CD11c⁻F4/80⁺ cells may be secreting IL-10. IL-10 producing CD11b⁺CD11c⁻F4/80⁺ macrophages have been shown to promote the differentiation of FOXP3 regulatory T cells and the suppression

of Th17 cell responses²⁶⁹. As a significant increase in T cells occurred during infection in placental tissues, such a population may be Foxp3⁺ Tregs (Fig. 23 E). From a pregnancy perspective, the immune cells present at the feto-maternal interface act to protect and ensure a healthy pregnancy is carried through to term. Regulatory mechanisms may exist during pregnancy to prevent a deleterious outcome in response to infection. It would therefore seem plausible that down-modulation of overt immune responses in order to prevent overt inflammation would be necessary, the hallmark of regulatory T cell function.

Increased susceptibility to the intracellular pathogen *Listeria monocytogenes* during both human and rodent pregnancy has been attributed to pregnancy-induced Treg expansion^{91, 270-272}. This has been further exemplified in non-pregnant mice in which the accumulation of Foxp3⁺ Tregs to comparable allogeneic pregnancy levels resulted in paralleled infection susceptibility²⁷². Increased proportions of activated maternal Tregs have also been noted during *Plasmodium* infection. Interestingly, such Tregs were also shown to have an increased suppressive potency, most likely in an effort to minimize infection-induced inflammation at the expense of parasite replication^{273, 274}. Unfortunately, although maternal Treg depletion restores resistance to pathogens such as *L. monocytogenes*, the subsequent lack of Treg induced maternal tolerance results in fetal resorption²⁷².

Modifications to Treg suppressive potency can also ensue due to environmental cues such as the stimulation of toll-like receptors due to microbial ligands. Purified LPS or flagellin causes increased Treg suppressive potency *in vitro*^{275, 276}, paralleling *in vivo* suppressive potency to pathogens^{277, 278}.

While the expansion of immune-suppressive maternal Tregs is essential for fetal tolerance during pregnancy, it can also compromise effective host defences against prenatal

pathogens. Therefore, the pathogenesis at the fetomaternal interface may be dictated by the opposing balance of effective immune activation and immune suppression, to prevent fetal loss and immune mediated pregnancy complications. Thus, holes in host defense are most likely limited to intracellular pathogens which have a predilection for the placenta, since resistance against most pathogens is not weakened with pregnancy.

From an evolutionary perspective, it would be advantageous to promote a strong inflammatory response at the fetomaternal interface during infection to preserve the mother's well-being, rather than wasting resources on rescuing a potentially damaged and infected fetus. An increase in Th1/Th17 cell subsets would enable a strong cell mediated response to better fight infection. Th17 cells play an important role in host defense against bacterial, fungal and viral infections and the induction of inflammation^{279, 280}. They have also been implicated in a pathogenic role of various autoimmune disorders such as rheumatoid arthritis and multiple sclerosis²⁸⁰. Transforming growth factor- β is required for the differentiation of both Tregs and Th17 cells. While the pro-inflammatory cytokine IL-6 mediates the suppression of Treg function, IL-6 plays an important role in the conversion of Tregs to Th17 cells in mice^{59, 281}. Thus, while TGF- β is the central cytokine to induce differentiation of CD4⁺ cells to either Tregs or Th17 cells, IL-6 is the key cytokine which regulates the Treg/Th17 balance²⁸¹⁻²⁸³. As IL-6 is significantly higher in infected placentas⁸⁴, the increase in IL-6 secretion along with TGF- β may be promoting Th17 conversion and its associated inflammatory response. Furthermore, IL-17⁺ cell numbers have been correlated with that of neutrophils, suggesting IL-17 is involved with neutrophil infiltration⁵⁹. Oral infection of *Salmonella* Enteritidis during late stages of gestation resulted in the increased placental mRNA expression of the inflammatory cytokines TNF- α , IL-17,

and IFN- γ which paralleled cytokine levels from placental homogenates¹⁹³. Murine pregnancy studies during *Toxoplasma gondii* infection revealed an imbalance between Treg and Th17 cell populations¹²⁶. The ratio of Treg cells to Th17 cells was significantly lower in both the spleen and placenta of infected mice compared to pregnant non-infected controls¹²⁶. Moreover, placental mRNA expression levels of FOXP3 and TGF- β was significantly lower in infected mice compared to non-infected controls, while increased mRNA expression of IL-17A, ROR γ t and IL-6 was witnessed in infected placental tissue compared to non-infected tissue¹²⁶. Consequently, the imbalance of Treg/Th17 cells is suspected to contribute to fetal resorption during murine *T. gondii* infection¹²⁶. Thus, the increase in the TCR β ⁺ population within individual placenta tissue 72 hours after S.Tm infection may be attributable to an increase in Th17 cells (Fig. 23 E).

Elevated levels of IL-17 have been observed to accelerate graft rejection by suppressing Treg expansion in both clinical and experimental settings²⁸⁴⁻²⁸⁷. Thus, the increase in Th17 cells and elevated IL-17 as a result of *Salmonella* infection may be detrimental to pregnancy maintenance¹⁹³. Increased prevalence of Th17 cells within decidua and peripheral blood has been witnessed in patients with unexplained recurrent spontaneous abortion^{288, 289}. Furthermore, the Th17 transcription factor, ROR γ t, along with IL-23, which promotes Th17 cell expansion, is increased in human decidual tissue in recurrent spontaneous abortion cases²⁸⁹. This was further correlated with an inverse relationship between Treg and Th17 cell numbers in both peripheral blood and decidual tissue^{61, 289}. A recent study reported increased Tregs and decreased Th17 cells in peripheral blood during healthy pregnancy. However, this trend was reversed in patients with pre-eclampsia, with decreased Tregs and increased Th17 cells in peripheral blood²⁸⁷. Increased Th17 cells have

also been noted in preterm delivery cases with chorioamnionitis²⁹⁰. Moreover, Tregs isolated from women who present with repeated miscarriages display reduced suppressive capacity compared to fertile women²⁹¹. Thus, a reduction in Treg suppressive potency and an increase in Th17 cells may be attributable to placental tissue inflammation during infection resulting in adverse pregnancy outcomes. While a Th17 response is implicated in effective bacterial clearance, the literature suggests that a fine balance must be struck in which an effective, but not over suppressive Treg response is required for successful pregnancy, while at the same time an effective, but not overt, Th17 response is required for pathogen clearance. Future flow cytometric analysis involving intracellular staining is required to delineate the increased T cell population present within in the placenta during S.Tm infection.

In conclusion, a complex array of events occurs during S.Tm infection at the fetomaternal interface. Further mechanistic studies are required to determine the function of the various immune cells present at the fetomaternal interface to provide insight into host susceptibility to infection during pregnancy.

5.5 Placental pathology and inflammation

Over recent years, several studies have highlighted the role of innate immune mediators during infection in pregnancy and the complications which ensue²⁹²⁻²⁹⁴. Placental expression of pattern recognition receptors, specifically TLRs, suggests that the placenta is equipped to identify microbial pathogens and respond to such invasion^{14, 201, 295}. Human first trimester villous cytotrophoblasts and extravillous cytotrophoblasts highly express both TLR-2 and TLR-4²⁹⁶. Interestingly, first trimester syncytiotrophoblasts do not express TLR-

2 and 4 suggesting that the inner layers act as a highly specialized barrier, and only pathogens able to breach the outer syncytiotrophoblasts are able to evoke inflammatory damage²⁹⁵⁻²⁹⁷.

In the case of *Salmonella* infection, ligand recognition via TLR-2, 4 or 5 results in a kinase cascade, triggering the activation of the NFκB pathway²⁰². The outcome of such pathway is dependent upon which TLR(s) is/are ligated¹⁴. TLR-4 ligation promotes the production of inflammatory cytokines and chemokines by trophoblast cells which will have a modulatory effect on the maternal immune system, and potentially trigger apoptosis, while ligation of TLR-2 results in apoptosis of the infected cell¹⁴.

Although trophoblast secretion of inflammatory cytokines and chemokines would recruit various immune cells to the feto-maternal interface and induce an inflammatory immune response, such outcome seems to be dependent upon the localization of S.Tm species within the placenta. Murine studies involving an attenuated S.Tm strain (S.TmΔaroA) was mainly localized to the decidua while wild-type S.Tm was found deep within the labyrinth. Thus, the dichotomous localization of S.Tm species to distinct regions of the placenta may be the central mechanism for differences in inflammation at the feto-maternal interface⁸⁴.

S.Tm infection in various cell types has been examined throughout the years. *Salmonella* infected intestinal epithelia cells die via apoptosis²⁹⁸ while *Salmonella* induces caspase-1 dependent pyroptosis in infected macrophages²⁹⁹⁻³⁰¹. Furthermore, it was recently shown that type-1 interferon is capable of inducing necroptosis in S.Tm infected macrophages with the inhibition of RIP1 rescuing the infected cells from death²²¹. Our studies involving cell death deficient strains emphasize that although fetal death and

placental resorption ensue as a result of placental S.Tm infection, such cell death may not necessarily be attributed to apoptosis as the literature suggests. While LPS can bind TLR-4 signaling the production of inflammatory cytokines and chemokines, potentially triggering apoptosis and subsequent fetal loss, this outcome does not explain the severe maternal illness witnessed during infection. Thus, we hypothesized that S.Tm infection in placental tissues results in either necroptosis or pyroptosis of infected trophoblast cells.

5.5.1 Placental pathology during systemic infection

Intravenous survival kinetics of cell death deficient strains revealed graded susceptibility to *Salmonella* infection in a non-pregnant state (*Caspase-1,11*^{-/-}*Nramp1*^{+/+} > *Rip3*^{-/-}*Nramp1*^{+/+} > *Nramp1*^{+/+}) (Fig. 24 A-C). Although not as drastic, differences were also seen in pregnant cell death deficient strains.

We have shown that although intravenous infections in necroptotic (*Rip3*^{-/-} and *Ifnar1*^{-/-}) and pyroptotic (*Caspase-1,11*^{-/-}) cell death deficient strains on the *Nramp1*^{+/+} background displayed similar bacterial burdens in their placentas compared to *Nramp1*^{+/+} controls, there was a differential outcome in placental viability and a reduction in qualitative inflammation based on the observation of placental/fetal state. *Rip3*^{-/-}*Nramp1*^{+/+} mice displayed significantly less inflammation attributable to the increased percentage of viable placentas and fetuses from 48-96 hours in comparison to *Nramp1*^{+/+} controls (Fig. 30). This trend was not seen through the inhibition of caspase-1 dependent pyroptosis in *Caspase-1,11* deficient mice. Although the activation of specific cell death pathways may be important for appropriate host immune responses and pathogen control, overt inflammation as a result of

hyperactivation of specific cell death pathways may be the cause of severe placental resorption and fetal death.

Therefore, our intravenous model suggests that S.Tm grows rapidly within murine trophoblast cells and triggers necroptotic cell death, presumably enabling the release of large quantities of bacteria from the infected cells. This, in turn, recruits various host inflammatory cells such as neutrophils into the placenta, leading to overt tissue inflammation. Pregnant *Rip3^{-/-}Nramp1^{+/+}* mice, in which necroptosis is inhibited, exhibited delayed placental inflammation, although bacterial titres were similar in placental tissue compared to controls. Therefore, the prevention of RIP3 mediated necroptosis delays overt inflammation and potential placental pathology. Thus, necroptotic cell death may be the driver of potentially misdirected immune responses and the hyperactivation of inflammation, leading to dangerous pathology and collateral damage to surrounding healthy tissues.

5.5.2 Placental pathology during oral infection

Oral infection survival kinetics of cell death deficient strains revealed graded susceptibility, although not drastic, to *Salmonella* in a non-pregnant state (*Caspase-1,11^{-/-}* > *Caspase-1,11^{-/-}Rip3^{-/-}* = *Rip3^{-/-}* = C57BL/6J > *Caspase-1,11^{-/-}Ifnar1^{-/-}* > *Ifnar1^{-/-}*) (Fig. 34 A-F). Differences were also seen in pregnant infected mice (C57BL/6J = *Caspase-1,11^{-/-}* = *Caspase-1/11^{-/-}Ifnar1^{-/-}* = *Caspase-1/11^{-/-}Rip3^{-/-}* > *Rip3^{-/-}* > *Ifnar1^{-/-}*) with pregnant *Ifnar1^{-/-}* mice surviving the longest of all cell death deficient strains (Fig. 34 A-F). Furthermore, while *Rip3^{-/-}* mice did not display any differences in bacterial burden or resorption rates compared to controls, *Caspase-1,11^{-/-}* and *Ifnar1^{-/-}* mice showed differential outcomes during oral infection.

Caspase-1,11^{-/-} mice showed significantly higher bacterial burdens within their mesenteric lymph nodes and pooled placentas in comparison to controls (Fig. 36 A & B). Furthermore, although not significant, *Caspase-1,11*^{-/-} mice showed an increase in percent resorptions (Fig. 36 C). It therefore seems that the lack of caspase-1 dependent pyroptosis during oral infection enhances bacterial proliferation and subsequent pathology. The inhibition of pyroptotic cell death and lack of subsequent membrane rupture and release of intracellular contents may have a two-fold effect: I) preservation of the intracellular niche for *S.Tm* replication, II) lack of the pro-inflammatory cytokines IL-1 β and IL-18 in initiating an immune response.

Thus, initiation of pyroptosis during oral infection may provide protection by eliminating the niche exploited by *Salmonella* necessary for replication and enabling detection by extracellular immune surveillance. Hypothetically, pyroptotic death of infected trophoblast cells would expel the intracellular bacteria, enabling the detection and clearance of *Salmonella* by phagocytes such as neutrophils³⁰². The release of various DAMPs, such as IL-1 α or HMGB1, may act as stimulants of the adaptive immune system. Moreover, maturation and secretion of the pro-inflammatory cytokines IL-1 β and IL-18 may assist in appropriate pathogen clearance. IL-1 β has been shown to regulate local responses to infection and injury, such as the migration of leukocytes to sites of infection³⁰³. IL-18 has been shown to contribute to the polarization of Th1 responses, specifically IFN- γ production by NK cells and T cells as a result of IL-18 in the presence of IL-12^{303, 304}. Moreover, IL-18 has been shown to synergize with IL-23 inducing Th17 responses and IL-17 production. Therefore, the induction of pyroptotic cell death at the feto-maternal interface may assist in placental immune defence to *S.Tm*.

Cell death pathways can be viewed as a double-edged sword in which their tight regulation is required for successful pathogen clearance, while dysregulation can result in overt inflammation. Although such results indicate that pyroptosis may aid in pathogen clearance at the fetomaternal interface, other studies have highlighted the detrimental effects as a result of NLR and caspase-1 activation. Stimulation of NLRs with bacterial products in human first trimester trophoblasts induces inflammation³⁰⁵. Activation of NLRs in term placental explants resulted in an inflammatory cytokine profile³⁰⁵. Women with preterm labour and confirmed intrauterine infection presented with higher caspase-1 levels within amniotic fluid compared to at-term women or preterm women without infection³⁰⁶. Moreover, injection of high doses of bacterial products into mid pregnant C57BL/6J mice induces premature birth due to recognition by NLRs. Although low dose of bacterial products did not induce prematurity, a reduction in fetal weight was noted³⁰⁵.

While IL-1 β is an important cytokine involved in human parturition, the administration of IL-1 β either intra-amniotic or systemically induces premature labour in mice and monkeys^{307, 308}. Moreover, IL-1 β has been implicated in pre-term birth during intrauterine microbial infection and inflammation^{22, 200}. Although our results are pre-mature to determine if pyroptosis is beneficial in pathogen control and clearance at the fetomaternal interface, the literature suggests that a balance must be struck; initiation of inflammatory cell death is needed to prevent pathogen evasion while prevention of overt inflammation is necessary to mitigate pregnancy complications.

In contrast to *Caspase-1,11*^{-/-} mice, *Ifnar1*^{-/-} mice revealed no detectable bacteria within their pooled placentas, correlating with significantly less placental resorptions compared to controls. IFNAR1 signalling has been implicated in necroptosis of

macrophages due to sustained RIP3 activation^{221, 309}. Further in-depth experimentation will be needed to decipher the differential activation of immune pathology at the feto-maternal interface and oral infection in *Ifnar1*^{-/-} mice. We speculate that the lack of detectable bacteria within the placenta may be attributable to bacterial shuttling via macrophages from the gut to the placenta, rather than blood circulation. Macrophages may play a vital role in *Salmonella* clearance, either in the gut or at the feto-maternal interface. Considering S.Tm has been shown to induce type-I interferon expression in macrophages, type-I interferon mediated necroptosis of infected macrophages may diminish their role in effective innate immunity and pathogen control^{221, 310}. Thus, the inhibition of type-I mediated necroptosis during oral infection may be a means by which S.Tm proliferation and immune evasion may be curbed.

In conclusion, our intravenous and oral infection models using cell death deficient strains reveal that certain cell death pathways may be utilized either by the host to curb and fight pathogen replication or by the pathogen as a means to evade the host immune response. Although the prevention of necroptosis during intravenous infection did not reduce the amount of bacteria present within placental tissues, its inhibition resulted in a significant qualitative reduction in inflammation seen at the feto-maternal interface. Likewise, although the same trend was not seen during oral infection with *Rip3*^{-/-} mice, *Ifnar1*^{-/-} mice displayed reduced bacterial titres and qualitative inflammation. The differences between oral and systemic infection requires further study but may relate to the manner in which S.Tm reaches the placental interface (hematogenous extracellular bacteria after systemic infection, and infected APCs from the gut carrying bacteria to the placenta after oral infection). Thus, S.Tm may modulate necroptotic cell death and inflammation at the feto-maternal interface.

Such results represent a crucial first step in unravelling the mechanism of S.Tm induced trophoblast cell death as a possible trigger of overt inflammation and a cascade of detrimental downstream effects such as pregnancy complications, fetal loss and severe maternal illness and death.

6.0 Conclusion

As pregnancy is a meticulously orchestrated process accompanied by distinct immunological changes, these changes can lead to altered immunity to a variety of pathogens. Only a few evasive pathogens, such as S.Tm, which also have a predilection for invasion and colonization of the feto-maternal interface, are detrimental to the fetus and mother. Moreover, the striking similarities of pathogens (life cycle, preferred site of trophoblast invasion, etc.) capable of causing prenatal infections in humans and other mammals further highlights potential areas of host vulnerability most likely created by the reproductive process.

The results obtained from the current *in vivo* study have contributed to a better understanding of *Salmonella* Typhimurium infection during pregnancy. Briefly, the main findings include:

- 1) *Nramp1*^{+/+} mice, which are resistant to S.Tm infection in a non-pregnant state, are highly susceptible during pregnancy.
- 2) *Salmonella* Typhimurium has a predilection for the placenta in comparison to other systemic tissues. S.Tm rapidly proliferates within placental tissues, with subsequent placental pathology and fetal loss.
- 3) Murine S.Tm infection results in early (24 hrs) random focal bacterial burden in select placentas through the hematogenous route, with progression of infection to surrounding placentas.

- 4) Differences in innate immune responses in the systemic compartment exist between non-pregnant infected hosts and pregnant-infected hosts, with a lack of splenic neutrophil recruitment in pregnant infected hosts.
- 5) During infection, an increase in neutrophils, natural killer cells, CD11b⁺CD11c⁻F4/80⁺ macrophages and T cells was witnessed within individual placentas. The increase in such immune mediators may be contributing to overt immune responses and placental pathology during infection.
- 6) Reduced resorptions in *Rip3*^{-/-}*Nramp1*^{+/+} and *Ifnar1*^{-/-} mice during systemic and oral infection respectively, suggests modulation of necroptotic inflammation and cell death in placental pathogenesis.

Thus, a complex interplay between immunity and inflammation during pregnancy, S.Tm virulence, and bacterial burden impacts maternal and fetal survival. The data presented in this study, has shown that placental infection by *Salmonella* Typhimurium leads to profound bacterial replication triggering necroptotic cell death. This correlates to the recruitment of various host inflammatory cells, such as neutrophils and macrophages, into the placenta which then contributes to tissue inflammation and fetal loss (Fig. 40). This study complements current *in vitro* and epidemiological studies to bridge the gap in our knowledge of placental infection.

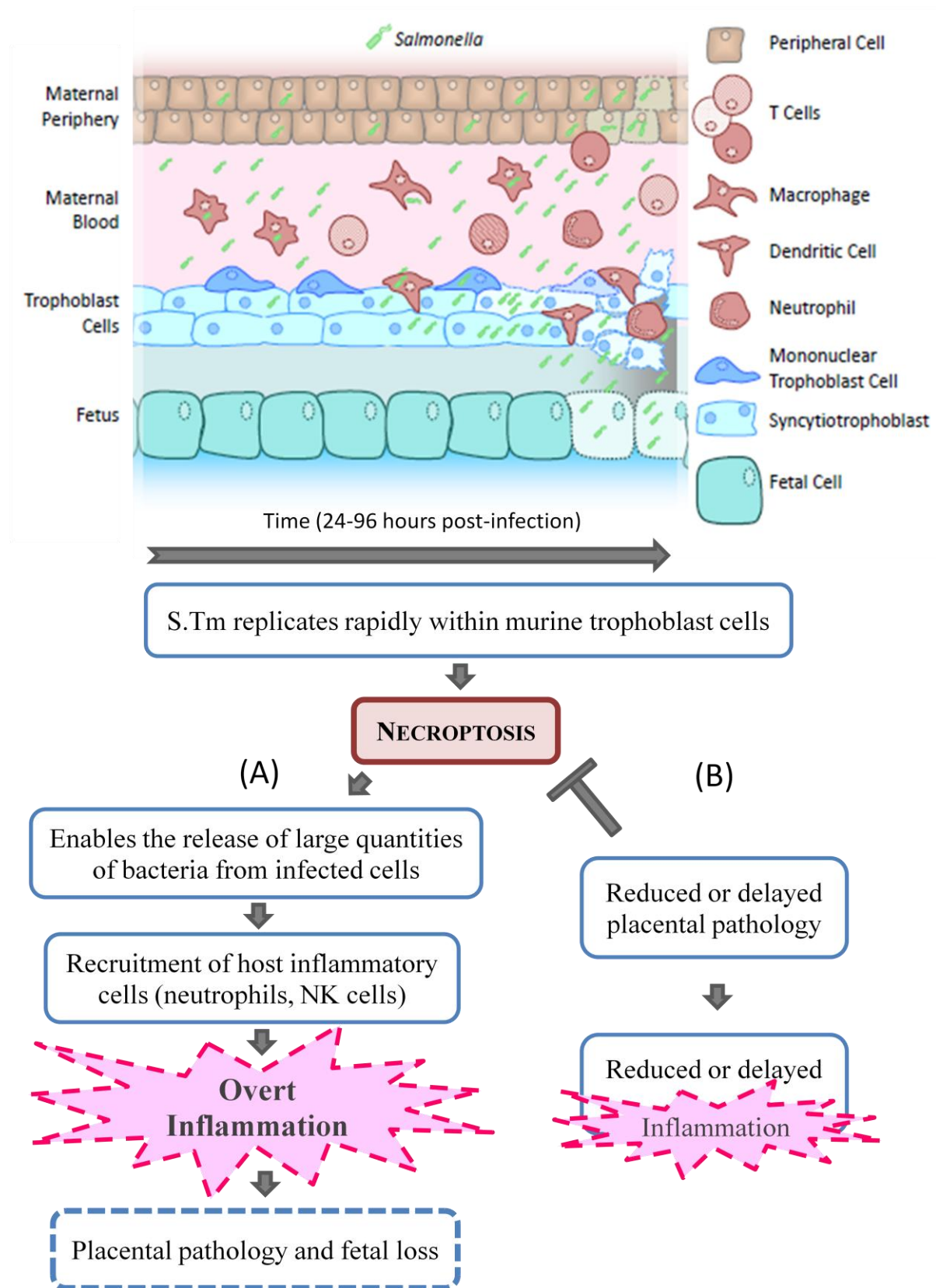


Figure 40

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Figure 40. Summary of the main thesis findings. An illustrative representation of the progression and consequences attributed to S.Tm infection at the feto-maternal interface from 24 to 96 hours post-infection. Infection and profound S.Tm proliferation occurs within murine trophoblast cells leading to necroptotic cell death of the trophoblast cells. A) Death of the infected trophoblast cells enables the release of large quantities of bacteria. This in turn, leads to the recruitment of various host inflammatory cells such as neutrophils and NK cells to the feto-maternal interface which contributes to overt inflammation resulting in placental pathology and fetal loss. B) The inhibition of necroptosis, through the use of necroptotic cell death gene-deficient mouse strains (*Rip3*^{-/-} or *Ifnar1*^{-/-}), results in reduced/delayed placental pathology and fetal loss which may be attributable to reduced/delayed inflammation.

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Curriculum Vitae

KRISTINA LORA CATHERINE WACHHOLZ

Education

2013 January – Present: Masters candidate, Department of Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa, ON

- Research based thesis under the supervision of Dr. Lakshmi Krishnan at the National Research Council (NRC) Canada to be completed Fall 2015
- Investigating mechanisms of increased host susceptibility and immune responses to *Salmonella* Typhimurium infection during pregnancy
- Experience in microbiological, immunological and cell culture techniques (cell isolation assays, cell culture, colony forming unit assays, immunophenotyping by flow cytometry, cytometric bead array assay) molecular biological techniques (DNA and RNA isolation, cDNA synthesis, polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR), gel electrophoresis)
- Technical experience includes Biosafety Level 1 and Level 2 laboratory experience, laboratory animal experience (mouse handling, breeding colony maintenance, mating, dissection and infection techniques), data entry and analysis using FlowJo and GraphPad. Data presentation using Microsoft Word and Powerpoint.

2008 September – 2012 April: Bachelor of Science, Honours specialization in Biology, McMaster University, Hamilton, ON

- Course and lab based undergraduate degree program
- Dean's Honour List

Academic Awards

2014 September: Top ten poster presenter at the International Federation of Placenta Associations (IFPA) 2014 conference, Paris, France.

2013 September – 2014 August: CIHR Reproduction, Early Development and the Impact on Health (REDIH) scholarship of \$11,900

2011 September – 2012 April: McMaster Honours Biology Dean's List Award
Award granted for 4th year of university due to the sessional average being above a 10.0 on a 12.0 scale.

2008 September – 2009 April: McMaster Honour Award LV 3 of \$2,000
Award granted for grade 12 average of 90%-94% for 6 final grades (prerequisites for program plus next best grade 12 university level courses)

Manuscripts

Patel, R., Shutinoski, B., **Wachholz, K.**, Krishnan, L., Sad, S. (2015). Synergism of Caspase-1/11 and RipK3 signaling platforms promotes cell death and IL-1 processing for comprehensive control of intracellular bacteria. *Manuscript submitted*

Agbayani, G., **Wachholz, K.**, Chattopadhyay, A., Gurnani, K., Krishnan, K. (2015). Pregnancy impairs Th17 cell responses against *Salmonella* Typhimurium infection. *Manuscript in preparation.*

Krishnan, L., Nguyen, T., **Wachholz, K.**, Agbayani, G. (2015) *Salmonella* infections in pregnancy: pathogenesis and host immunity. *Manuscript in preparation.*

Published Abstracts

KLC Wachholz, K Gurnani, S Mohammad, SP Murphy, BA Croy, L Krishnan. (2015). Murine placental infection by *Salmonella enterica* serovar Typhimurium: pathogenic mechanisms and the role of inflammatory cell death. General Abstracts. American Journal of Reproductive Immunology, 73: 34–89. Page 58.

GP Agbayani, **KLC Wachholz**, A Chattopadhyay, K Gurnani, L Krishnan. (2015). Regulation of T regulatory cell and Th17 responses in pregnant mice infected with *Salmonella* Typhimurium infection. General Abstracts. American Journal of Reproductive Immunology, 73: 34–89. Page 34.

S Mohammad, **KLC Wachholz**, L Krishnan, SP Murphy, BA Croy. (2015). *Salmonella enterica* serovar Typhimurium infection and pregnancy disruption. General Abstracts. American Journal of Reproductive Immunology, 73: 34–89. Page 80.

L Krishnan, T Nguyen, GP Agbayani, **KLC Wachholz**. (2015). The battle of wits: the placental barrier and virulent *Salmonella enterica* Typhimurium infection. Speakers' Abstracts Opening Session. American Journal of Reproductive Immunology, 73: 15–33. Page 30.

Wachholz, K., Agbayani, G., Nguyen, T., Gurnani, K., and Krishnan, L. (2014). Placental infection by *Salmonella enterica* Typhimurium in a murine model: mechanisms of pathogenesis and role of inflammatory cell death. Placenta, 35 (9), Page A64.

Conferences

June 2nd – 5th 2015: American Society for Reproductive Immunology (ASRI) 35th Annual Meeting Kingston, ON

September 9th – 12th 2014: International Federation of Placenta Associations (IFPA) Paris, France

Presentations

June 2nd – 5th 2015: Poster presentation at the American Society for Reproductive Immunology (ASRI) Conference, Kingston, ON

- KLC Wachholz et al. “Murine placental infection by *Salmonella enterica* serovar Typhimurium: pathogenic mechanisms and the role of inflammatory cell death”

September 9th – 12th 2014: Poster presentation at the International Federation of Placenta Associations (IFPA) 2014 conference, Paris, France.

- Wachholz, K. et al. “Placental infection by *Salmonella enterica* Typhimurium in a murine model: mechanisms of pathogenesis and role of inflammatory cell death”

June 2014: Reproduction, Early Development and the Impact on Health (REDIH) poster presentation, Montreal, QB

- Wachholz, K. et al. “Murine placental infection by *Salmonella* Typhimurium and the mechanisms of pathogenesis and inflammatory cell death at the fetal-maternal interface”

March 2014: Biochemistry, Microbiology and Immunology Annual Seminar Day, oral presentation, University of Ottawa, Ottawa, ON

- Wachholz, K. et al. “Placental infection by *Salmonella* Typhimurium in a murine model: profiling the innate immune mediators involved in cell death at the fetal-maternal interface”

December 2013: Reproduction, Early Development and the Impact on Health (REDIH) oral presentation, Ottawa, ON

- Wachholz, K. et al. “Placental infection by *Salmonella* Typhimurium in a murine model”

May 2013: Biochemistry, Microbiology and Immunology Annual Poster Day, poster presentation, University of Ottawa, Ottawa, ON

- Wachholz, K. et al. “Placental infection by *Salmonella* Typhimurium in a murine model: The role of innate immune mediators in cell death at the fetal-maternal interface”

Work Experience

2006 November to 2012 December: PT Swim Instructor/Lifeguard/Supervisor – Cawthra Pool, Mississauga, ON

- Guarded fun/length swims; Taught swim lessons; Oversaw guards/instructors when aquatic supervisor absent; Opened and closed pool facility; Responsible for ensuring proper water quality (chlorine and pH levels); Oversaw cashiers and cash handling procedures

2006 September – 2008 June, 2012 September – 2012 December: PT Swim Instructor/Lifeguard/Supervisor – South Common Pool, Mississauga, ON

- Guarded fun/length swims; Taught swim lessons; Oversaw guards/instructors when aquatic supervisor absent; Opened and closed pool facility

2012, 2011, 2010, 2009, 2008 June to September: FT Guard/Instructor/Coach/Supervisor- Westacres Pool, Mississauga, ON

- 44 hours per week during summers of 2008-2012
- Guarded swims; Taught swim lessons; Coached swim team; Oversaw guards when aquatic supervisor absent; Opened and closed pool facility; Responsible for ensuring proper water quality (chlorine and pH levels); Oversaw cashiers and cash handling procedures

2007 June to 2007 September: FT Cashier/Guard/Instructor/Coach-Westacres Pool, Mississauga, ON

- 44 hours per week during summer of 2007
- Handled cash, registrations, payments; Guarded swims; Taught swim lessons; Coached swim team

Volunteer experiences

2010 May to 2011 August: Volunteer at the Summerville Health Team Office, Mississauga, ON

2010 June to 2011 August: Volunteer at the Oakville Well Life Center, Oakville, ON

2010 February to 2010 February: Volunteer at the Mississauga Seniors' Centre, Mississauga, ON

2008 March to 2008 June: Prom Committee Member for Fr. Goetz '08 Prom, Mississauga, ON

2007 December to 2008 January: Volunteer at the Daily Bread Food Bank, Toronto, ON

2005 July to 2005 August: Volunteer at Westacres Outdoor Pool, Mississauga, ON

2003 September to 2004 June: Volunteer at Open Door in Square One, Mississauga, ON

2000 October to 2012 September: Neighbourhood clean-up of Woodington Green Park, Mississauga, ON