

**MODE OF ENTRY AND SURVIVAL OF *SALMONELLA*
ENTERICA SEROVAR TYPHIMURIUM IN
TROPHOBLAST CELLS**

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ABSTRACT

Salmonella enterica species are intracellular bacteria causative agents of gastroenteritis and typhoid fever in humans. Pregnancy poses an increased risk of severe Salmonellosis in many mammalian species contributing to miscarriage and/or maternal illness. Previous studies indicated that *Salmonella* infection in pregnant mice caused rapid fetal and maternal death due to massive bacterial proliferation in the placenta. However, the susceptibility of human primary trophoblast cells (cTBCs) to *Salmonella* infection was not known. We hypothesized that human placental trophoblast cells are productively infected and provide a unique intracellular niche that permits uncontrolled *Salmonella* replication due to an ineffective maternal innate immune response to the virulent bacteria resulting in placental death. Firstly, we observed that *S.Tm* strains defective in the *Salmonella* pathogenicity island (SPI)-1 type III secretion system (TTSS) (*S.Tm-ΔinvA*) were unable to enter epithelial cells, but efficiently infected placental choriocarcinoma cell lines through scavenger receptor-mediated endocytosis. Next, we observed that *S.Tm* failed to grow vigorously in macrophages, but replicated rapidly within epithelial and placental trophoblast cells. Further examination of intracellular localization of *S.Tm* indicated that bacteria were arrested in early Rab5 expressing phagosomal vesicles within trophoblast cells, whereas phagosomal maturation progressed steadily in macrophages (with expression of lysosomal-associated membrane protein-1 (LAMP-1) and cathepsin D). Moreover, human primary cTBCs harboring *S.Tm* underwent rapid death of the cells. Infected cTBCs expressed phosphorylated-receptor-interacting serine/threonine-protein kinase (RIPK)-1 protein and phosphorylated-mixed lineage kinase domain-like (MLKL), suggesting induction of the necroptosis pathway of cell death. Furthermore, specific inhibition of necroptosis rescued *S.Tm*-induced death of cTBCs. Finally, *S.Tm* infected trophoblast cells produced interleukin (IL)-10,

and signal transducer and activator of transcription (STAT)-3 signalling. This correlated to delayed phagosomal maturation which consequently facilitated intracellular pathogen proliferation. Overall, human trophoblast cells may act as reservoirs for *S.Tm* survival and may aid dissemination in the pregnant host.

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LIST OF ABBREVIATIONS

Ab	Antibody
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Analysis of variance
APAF-1	Apoptotic protease-activating factor 1
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-protein containing
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
β-ME	β-mercaptoethanol
B	CFU at the start time
b	CFU at the end time
BAK	BCL-2-antagonist/killer
BAX	BCL-2-associated X protein
BCL-2	B-cell lymphoma 2
BD	Becton Dickinson
BHI	Brain and heart infusion
BSA	Bovine serum albumin
Buffer RLT	Qiagen RNeasy lysis buffer
Buffer RPE	Qiagen RNeasy wash buffer for membrane-bound RNA
Buffer RW1	Qiagen RNeasy wash buffer
CARD	Caspase activator and recruitment domain
CBS	Canadian Blood Services
CCAC	Canadian Council on Animal Care
CD	Cluster of differentiation
cDNA	Complementary DNA
c-FLIP	Cellular FADD-like IL-1β-converting enzyme-inhibitory protein
CFU	Colony forming units
CIHR	Canadian Institutes of Health Research
CMV	Cytomegalovirus
CO₂	Carbon dioxide
c-SRC	Cellular sarcoma kinase
CSF	Colony-stimulating factor
cTBC	Cytotrophoblast cell
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EEA1	Early endosome antigen 1
EGTA	Ethylene glycol-bis (β-aminoethyl ethyl)-N,N,N',N'- tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting

FADD	Fas-associated protein with death domain
FasL	Fas ligand
FasR	Fas receptor
FBS	Fetal bovine serum
Fc	Fragment, crystallisable
FDG	5-Dodecanoylaminofluorescein Di- β -D-Galactopyranoside
FITC	Fluorescein isothiocyanate
FV	Fluoview
G	Generation time
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	Hour
HBSS	Hank's Balance Salt
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEV	Hepatitis E virus
HHV	Human herpesvirus
HIV	Human Immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
IFN	Interferon
IFNAR	Type I interferon receptor
IFN-γR	IFN- γ receptor
IgG	Immunoglobulin G
IL	Interleukin
IL-10R	IL-10 receptor
InI	Internalin
<i>InvA</i>	<i>Salmonella</i> invasion gene A
IPAF	Ice protease-activating factor, also known as NLRC4
IRF3	Interferon regulator factor 3
Jak	Janus family tyrosine kinase
KCl	Potassium chloride
kDa	KiloDalton
LB	Lysogeny Broth
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
M	Molar
M cell	Microfold cell
M-CSF	Macrophage colony-stimulating factor (M-CSF)
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
mg	Milligram
MgCl₂	Magnesium chloride
min	Minute

mL	Millilitre
mm	millimeter
MHC	Major histocompatibility complex
MLKL	Mixed lineage kinase domain-like
MOI	Multiplicity of infection
Mϕ	Macrophage
mRNA	Messenger RNA
MSR1	Macrophage scavenger receptor 1
MyD88	Myeloid differentiation primary response gene 88
N/A	Not applicable
NaCl	Sodium chloride
NaHCO₃	Sodium bicarbonate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIAID	National Institute of Allergy and Infectious Disease
NI	Non-infected
NIH	National Institutes of Health
NK	Natural killer
NLR	NOD-like receptor
NLRC4	NLR family CARD domain-containing protein 4
NLRP3	NLR, LRR- and pyrin containing domain containing 3
nm	Nanometers
nM	Nanomolar
NOD	Nucleotide-binding oligomerization domain
NRC	National Research Council of Canada
<i>Nramp-1</i>	Natural resistance associated macrophage protein-1
OD	Optical density
OGS	Ontario Graduate Scholarship
OHRI	Ottawa Hospital Research Institute
PAMPs	Pathogen-associated molecular patterns
PARP	Poly-ADP-ribose polymerase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
Ph.D.	Doctor of Philosophy
Phospho	Phosphorylated
PI3K	Phosphoinositide 3-kinase
PIPES	Piperazine- <i>N,N'</i> bis [2-ethanesulfinic acid]
PMA	Phorbol-12-myristate-13-acetate
PRR	Pathogen recognition receptor
PVDF	Polyvinyl difluoride
REDIH	Reproduction, Early Development and Impact on Health
RILP	Rab7-interacting-lysosomal-protein
RIPA	Radioimmunoprecipitation assay
RIPK	Receptor-interacting serine/threonine-protein kinase

ROS	Reactive oxygen species
rpm	Rotation per minute
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
R10	RPMI + 10% FBS medium
s	Second
SCV	<i>Salmonella</i> containing vacuole
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SH2	Src homology 2
<i>Slc11a1</i>	Solute carrier family 11 member a1 (also known as <i>Nramp1</i>)
SPI	<i>Salmonella</i> pathogenicity island
SR	Scavenger receptor
STAT	Signal transducers and activators of transcription
<i>S.Tm</i>	<i>Salmonella enterica</i> serovar Typhimurium
<i>S.Tm-ΔaroA</i>	Auxotrophic mutant <i>Salmonella enterica</i> serovar Typhimurium
<i>t</i>	Time elapsed
TBS	Tris-buffered saline
TCPS CORE	Tri-Council Policy Statement: Ethical Conduct for Research Involving Human Courses on Research Ethics
TCR	T cell receptor
TGF	Transforming growth factor
TIR	Toll/IL-1 receptor
T_H	T helper
T_{H1}	T helper 1
T_{H2}	T helper 2
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	TNF receptor
TRADD	TNFR-1-associated death domain
Tregs	T regulatory
TRIF	TIR-domain containing adaptor-inducing interferon-β
Tris	Tris(hydroxymethyl)aminomethane
Tris HCL	Tris(hydroxymethyl)aminomethane-hydrochloric
TTSS	Type three secretion system
Tyk2	Tyrosine kinase 2
μg	Microgram
μm	Micrometer
μM	Micromolar
μl	Microliter
VZV	Varicella zoster virus
WHMIS	Workplace Hazardous Materials Information System
WHO	World Health Organization
WT	Wild-type
129.B6F1	129X1/SvJ x C57BL/6J F1 hybrid mice

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CHAPTER 1: **INTRODUCTION**

1.0 INTRODUCTION

Mammalian pregnancy poses a unique physiological situation where the mother has to accept a fetus, which is half foreign, as expression of paternal major histocompatibility complex (MHC) antigens by the fetal tissue could potentially lead to rejection by the immune system. Fetal tolerance is facilitated by a number of immune alterations during pregnancy including unique physiological adaptations of the placenta, and changes in immune cell function at the feto-maternal interface. This may pose a challenge for the maternal host to preserve the ability to counter infections that may occur during pregnancy. Thus, an intricate balance of immunoregulatory pathways and adaptation of maternal immune and placental cells appears to be the hallmark of successful mammalian pregnancy. In the following sections, the placental structure and function, immunoregulation and interaction of pathogens with the feto-maternal interface will be described.

1.1 PREGNANCY

1.1.1 PLACENTA

The placenta (Greek word meaning flat cake) is named of its anatomical appearance. The placenta is made of maternal and fetal tissues and forms a connection between the fetal membrane and the inner uterine wall. The mature human placenta is a discoid organ 20-25 cm in diameter, 3 cm thick and weighing 400-600g (Strachan 1923; Benirschke 1962). Throughout fetal development, the placenta functions both as a unique agent of symbiosis and as the fetal renal, respiratory, hepatic, gastrointestinal, endocrine, and immune systems. The chorionic villi of the fetus are bathed in maternal blood spaces, which allow gaseous exchange, uptake of nutrients, regulating fluid volume and elimination of waste metabolites (Gude et al. 2004;

Dilworth and Sibley 2013; Maltepe and Fisher 2015). The placenta releases steroid and peptide hormones into the maternal and fetal circulations (Korgun et al. 2012), and also protects the fetus from both pathogens and from maternal immune rejection (Fisher et al. 2000; Robbins et al. 2010; Zeldovich et al. 2011; Delorme-Axford et al. 2013).

1.1.2 PLACENTAL CLASSIFICATION

The placenta can be classified into hemochorial, endotheliochorial, or epitheliochorial based on the structural organization and the maternal and fetal circulation (PrabhuDas et al. 2015). Hemochorial describes the placenta where the chorion or the fetal derived trophoblast cells comes in direct contact with the maternal blood (human, higher order primates, guinea pigs, mice and rats). 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 placenta. The second group, endotheliochorial describes the placenta where maternal endometrial blood vessels are bare to the endothelium and these come in contact with the chorion (dogs and cats). Lastly, epitheliochorial describes the placenta where the maternal epithelium of the uterus comes in contact with the chorion (pigs and cows) (Furukawa, Kuroda, and Sugiyama 2014).

1.1.3 HUMAN PLACENTA STRUCTURE AND DEVELOPMENT

Human gestation is about 280 days long. Implantation occurs between 5 and 9 days after ovulation. The placenta begins to develop upon implantation of the trophoblast cells of the blastocyst into the maternal endometrium, or decidua of the pregnant uterus. The progression of placentation involves proliferation, invasion and differentiation of extraembryonic trophoblast cells. Following implantation, the trophoblast cells can differentiate into two pathways: extravillous at one junction and villous trophoblast cells at another junction (Aplin 1991; Roby

and Soares 1993; Red-Horse et al. 2004). The extravillous trophoblast cells proliferate and differentiate to invade the maternal decidual stroma and the spiral arteries of the myometrium to establish the uteroplacental circulation. The villous trophoblast cells proliferate and fuse to form the multinucleated syncytiotrophoblast cells with openings into the maternal space that form the outer surface of the fetal placental villi. The syncytiotrophoblast cells become compressed and are overlaid by a continuous layer of multinucleated sheet, covering each villous and forming intervillous space where nutrient and gas exchange between the mother and fetus occurs. The two main regions within the placenta in which fetal and maternal tissues are in direct contact include the syncytiotrophoblast-maternal blood interface and the extravillous trophoblast-decidua interface (Pijnenborg 1988).

In the first trimester of human pregnancy, the placenta has a villous structure. The placenta or floating villi contain fetal blood vessels and occasional macrophages (Hofbauer cells) in a core of mesenchymal connective tissue, surrounded by a syncytiotrophoblast layer. In the second trimester, the placenta matures and increases in size. The villi become smaller and more vascular. The villi are bathed in maternal blood. The syncytiotrophoblast is a multinucleated continuous cell layer that covers the surface of the placenta and contributes to the barrier function of the placenta. It forms as a result of differentiation and fusion of the underlying cytotrophoblast cells (cTBCs), a process that continues throughout placental development (Fig. 1). In the third trimester, the placenta has small and highly vascularized chorionic villi to support the blood gas and nutrient exchange of the maternal-fetal circulation required by the growing fetus approaching term gestation.

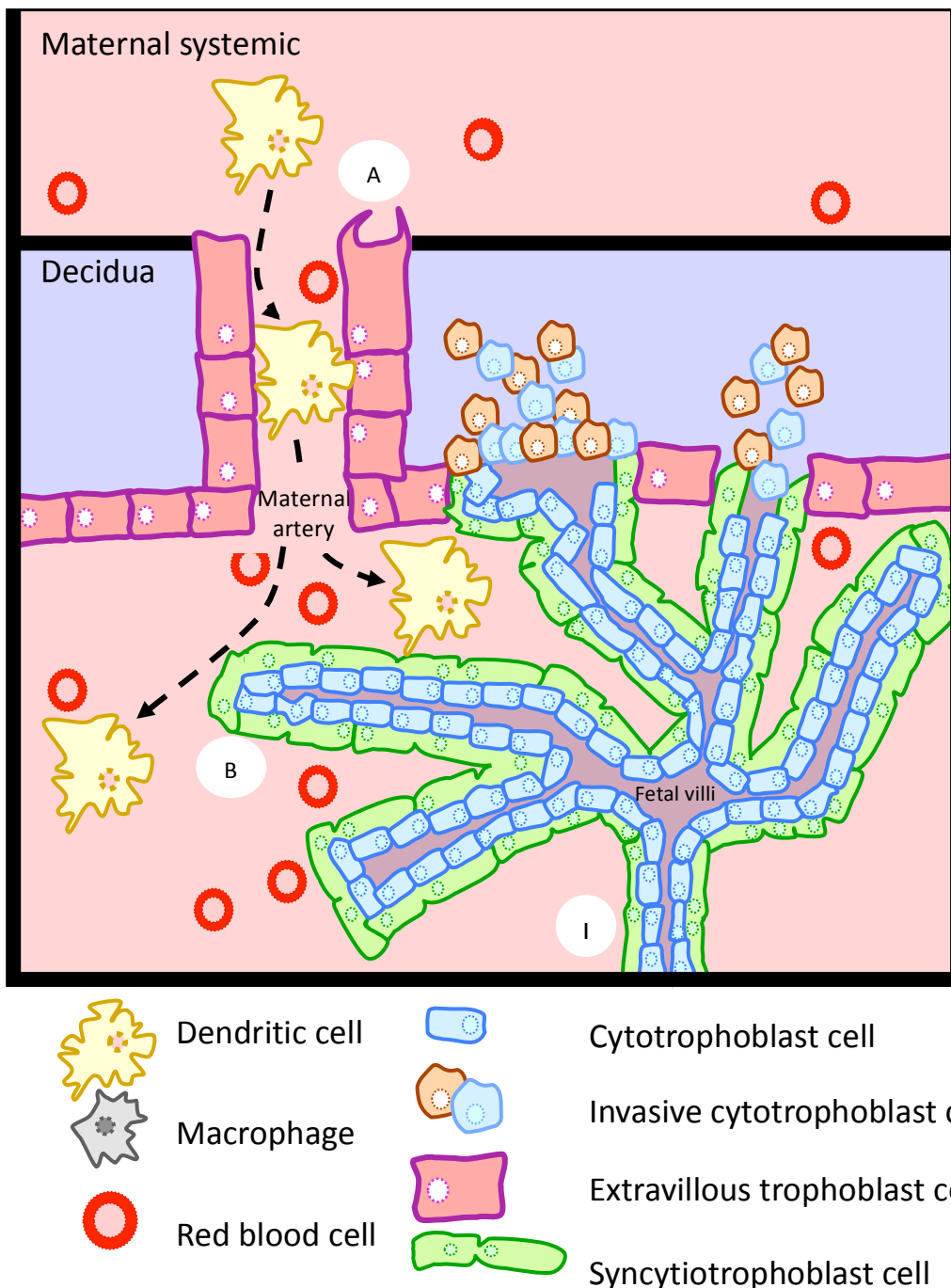


Figure 1. Schematic representation of the maternal-fetal interface. I Following implantation, the trophoblast cells can differentiate into two pathways: extravillous and villous trophoblast cells. The extravillous trophoblast cells proliferate and differentiate into an invasive phenotype that invades the maternal decidual stroma and the spiral arteries of the myometrium to establish the uteroplacental circulation. The villous trophoblast cells proliferate and fuse to form the multinucleated syncytiotrophoblast cells that develop openings into the maternal space that form the outer surface of the fetal villi. The syncytiotrophoblast cells become compressed and are overlaid by a continuous layer of multinucleated sheet, covering each villus and forming intervillous spaces where nutrient and gas exchange between the mother and fetus occurs. The two main regions within the placenta in which fetal and maternal tissues are in direct contact is at **A** the extravillous trophoblast-decidua interface and at **B** the syncytiotrophoblast cell-maternal blood interface.

The maternal surface of the placenta at term is divided into cotyledons. The fetal surface of the placenta is translucent and with visible villous tissue. Thus, the human placenta is a dynamic physiological structure comprised of distinct cellular types that undergoes adaptation to serve specific functions throughout the duration of pregnancy (Kaufmann, Sen, and Schweikhart 1979; Wells and Bulmer 1988).

1.1.4 PLACENTAL CIRCULATION

Placental circulation can be divided into two parts: the maternal-placental and the feto-placental. For the maternal placental-circulation, the uterine endometrium undergoes a process termed decidualisation in preparation for implantation of the blastocyst. The spiral arteries, small arteries which temporarily supply blood to the decidua are remodeled to become less convoluted and their diameter is increased to allow increased maternal blood flow to the placenta. The maternal blood fills the intervillous space through these spiral arteries and bathes the fetal villi in blood, allowing an exchange of gases to take place. The feto-placental circulation consists of deoxygenated fetal blood that passes through umbilical arteries to the placenta. At the junction of umbilical cord and placenta, the umbilical arteries branch radially to form chorionic arteries. Chorionic arteries, in turn, branch into cotyledon arteries. In the villi, these vessels eventually branch to form an extensive arterio-capillary-venous system, bringing the fetal blood extremely close to the maternal blood; but no intermingling of fetal and maternal blood occurs. In the placenta, there are 3 separate aortic/venous circulatory systems: umbilical, systemic or embryonic (remodeled to form the mature cardiovascular system) and vitelline. Nutrition of the fetus is derived from 100-150 maternal uterine spiral arteries located in the basal plate (Fig. 2) (Kearns 1934; Wallenburg 1981; Ramsey 1985; Pijnenborg 1988; Carter 1999).

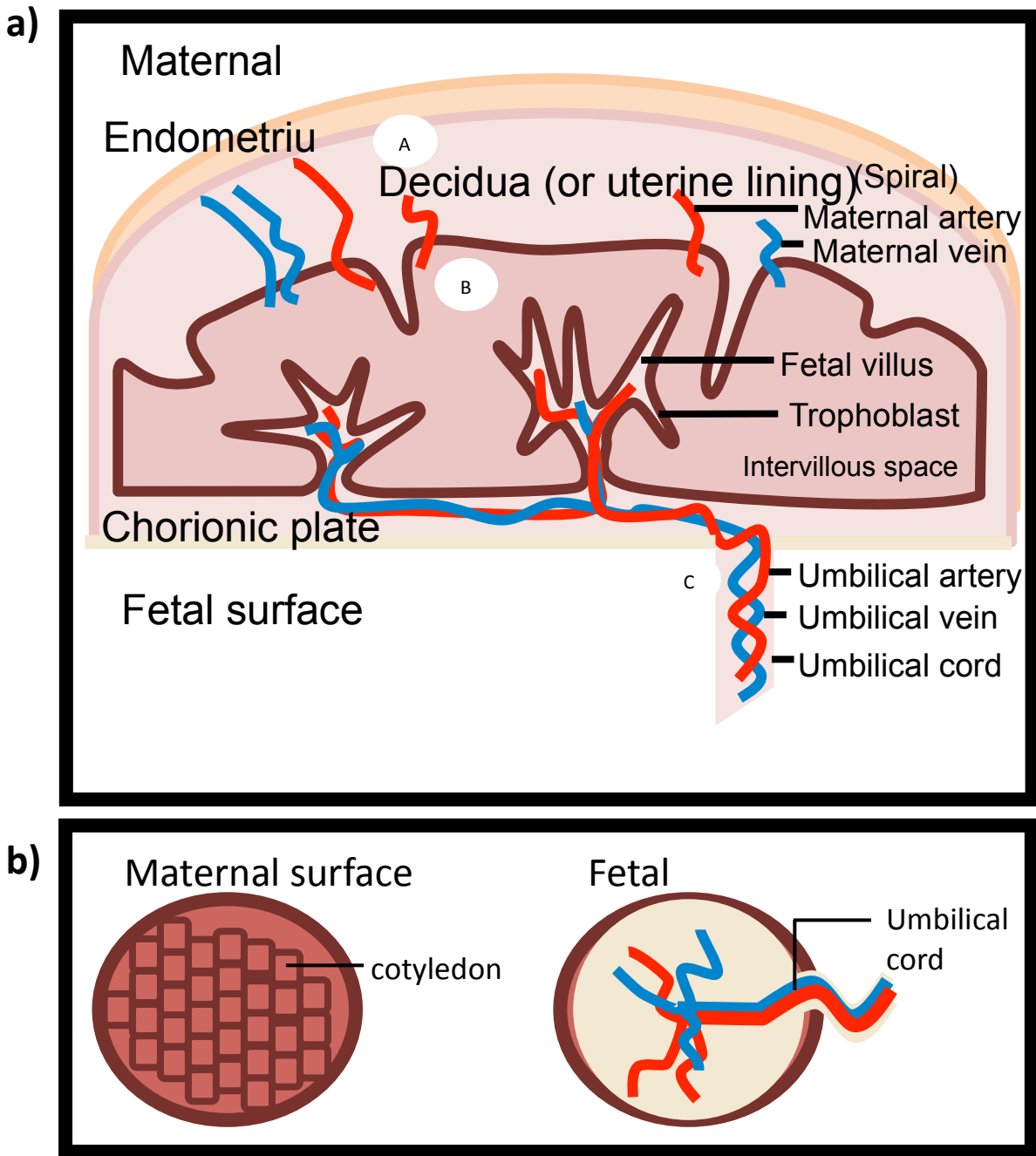


Figure 2. Schematic representation of the hemochorial placenta. a) Placental circulation can be divided into two parts: the maternal-placental and the fetoplacental. **A** The spiral arteries, or maternal arteries, supply blood to the endometrium of the uterus and allows maternal blood flow to the placenta. **B** The maternal blood fills the intervillous space through the spiral arteries and bathes the fetal villi in blood, allowing an exchange of gases to take place. **C** The fetoplacental circulation consists of deoxygenated fetal blood that passes through umbilical arteries to the placenta. b) The maternal surface of the placenta at term is divided into cotyledons. The fetal surface of the placenta is translucent. At the junction of umbilical cord and placenta, the umbilical arteries branch radially to form chorionic arteries. Chorionic arteries, in turn, branch into cotyledon arteries.

1.1.5 IMMUNOLOGICAL TOLERANCE IN PREGNANCY

Wegmann and colleagues first developed the concept that there is a shift from a T helper 1 (T_H1) immune response to a T helper (T_H2)/ anti-inflammatory bias during pregnancy that functionally induces maternal tolerance, based on studies in murine pregnancy (Wegmann et al. 1993). His observations were first based on the production of T_H2 cytokines such as IL-10 by trophoblast cells. Furthermore, the detrimental effects of T_H1 cytokines on murine pregnancy were demonstrated in experimental models. Moreover, distinct phenotypes of maternal decidua immune cells were noted.

The immune composition of the maternal decidua is distinct from the peripheral immune cells. The maternal decidua is comprised of roughly 30-40% leukocytes, most of which are uterine natural killer (NK) cells (~70%) and macrophages (~20%). T cells are also present (10%) whereas dendritic cells (DCs), B cells, and NK T cells are rare (Erlebacher 2013). Uterine NK cells have a distinct phenotype: they are cluster of differentiation (CD)56^{high} and CD16^{negative} in comparison to peripheral blood NK cells which are CD56^{low} and CD16^{positive}. Additionally, they also have functional differences: peripheral NK cells express CD16 which is involved in triggering the lysis of target cells, while uterine NK cells lack the expression of CD16 resulting in a reduced cytotoxic phenotype (Redline 2000; Croy et al. 2003; Eriksson et al. 2004). Moreover, decidual macrophages assist with tissue remodelling at the maternal-fetal interface (Mor and Abrahams 2003). These cells produce elevated levels of anti-inflammatory interleukin (IL)-10 (Heikkinen et al. 2003). Overall, the cytokine profile at the maternal-fetal interface consists of anti-inflammatory cytokines such as, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF), IL-3, transforming growth factor-beta (TGF-β) and colony stimulating factor (CSF)-1 produced by placental and/or maternal immune cells, which may

down regulate maternal immunity (Bowen et al. 2002). IL-10 is a key cytokine for the maintenance of pregnancy by tightly regulating pro-inflammatory cytokines at the maternal-fetal interface and has pleiotropic activities (Roth et al. 1996). Moreover, secretion of IL-10 by a diverse set of maternal and fetal cells has proven to aid in the orchestration of normal processes of pregnancy. Villous cTBCs produce IL-10 (Naruse et al. 2010). Whereas, extravillous trophoblast cells are intrinsically poor in IL-10 production (Thaxton and Sharma 2010). Uterine NK cells and monocytes in the decidua produce IL-10; although these cells normally are identified as cytotoxic killers, they are altered in the context of pregnancy where they aid in angiogenesis and placental regulation (Viganò et al. 2001). Furthermore, regulatory T cells (Tregs) in the decidua are able to produce IL-10 (Nancy and Erlebacher 2014).

Hormones during pregnancy also aid tolerance (Tuckey 2005). Progesterone promotes T_H2 cell expansion (Miyaura and Iwata 2002), decreases NK cell cytotoxicity (Dosiou and Giudice 2005), inhibits nitric oxide (Coughlan, Gibson, and Murphy 2005), inhibits inflammatory tumour necrosis factor-alpha ($TNF-\alpha$) production in activated macrophages, and enhances production of anti-inflammatory IL-4 (Ragusa et al. 2004; Lissauer et al. 2015). Estrogen decreases NK cell activity (Nilsson and Carlsten 1994) and decreases production of both inflammatory $TNF-\alpha$ (Ito et al. 2001) and interferon-gamma ($IFN-\gamma$) (Nakaya, Tachibana, and Yamada 2006). Inflammatory cytokines such as $TNF-\alpha$ and $IFN-\gamma$ can exert detrimental effects in the placenta and tend to be present at low concentrations (Hauguel-de Mouzon and Guerre-Millo 2006).

The trophoblast cells also have unique properties to prevent proto-typical allograft responses of the maternal host. Extravillous trophoblast cells invade the endometrium and change its cell adhesion receptors to mimic those of the vascular cells (Soundararajan and Rao

2004). Second, trophoblast cells fail to express classical human leukocyte antigen (HLA) class I (HLA-A and -B) and II molecules. Instead, in early pregnancy, cTBCs express non-classical truncated class Ib HLA molecules named HLA-G and HLA-E in addition to the classical class Ia HLA-C (Blaschitz, Hutter, and Dohr 2001). HLA-G is unique and is highly expressed in cTBCs in the placenta (Hunt et al. 2005). The lack of expression of HLA class I and II on trophoblast cells prevent recognition by maternal T lymphocytes. However, due to the lack of HLA class I molecules, trophoblast cells should be susceptible to killing by the uterine NK cells, but this is not the case due to HLA-G expression. HLA-G binds to specific inhibitory receptors found on NK cells and macrophages (Hunt et al. 2006). Lastly, pregnant women make immunoglobulin G (IgG) antibodies to paternal antigens such as HLA class I and II molecules that do not cross the placenta. Maternal IgG is selectively transferred into fetal circulation via fragment, crystallizable (Fc)- γ receptors expressed by trophoblast cells and maternal antibodies directed to paternal antigens on the fetus are trapped locally in the placental stroma (Moffett and Loke 2004).

The new paradigm $T_H1/T_H2/T_H17$ and regulatory T cells

The simplistic concept of T_H2 predominance has been used to describe the phenomenon of fetal-maternal tolerance. However, recurrent spontaneous abortions and preeclampsia have been observed and reported in both T_H1 and T_H2 predominant immunity. Therefore, the T_H1/T_H2 paradigm does not adequately explain the mechanisms of tolerance at the fetal maternal interface. Further identification of T_H -type populations, such as T_H17 and regulatory T cells (Tregs), adds to the paradox of tolerance to the fetus (Fu, Tian, and Wei 2014). T_H17 cells and Tregs differentiate from a common T helper progenitor cell.

TGF- β causes the proliferation of both Treg and T_H17 cells. During inflammation, enhanced production of IL-6 inhibits the induction of forkhead box P3 (FOXP3), halting the generation of Tregs and activates the expression of ROR γ , driving the proliferation of T_H17 cells. T_H17 cells produce an array of pro-inflammatory cytokines and host defense molecules and play an important role in preventing pathological infection in pregnancy. The frequency of T_H17 in the decidua is higher compared to that in peripheral blood. The uterine cavity is not completely sterile and therefore T_H17 cells play a role to induce protective immune response against extracellular microbes. Excessive T_H17 cell numbers and high levels of IL-17, IL-16 and IL-1 β have been identified in the cytotrophoblast and syncytiotrophoblast cells in the decidua in recurrent pregnancy loss and preeclampsia (Piccinni 2011; Piccinni et al. 2015). This indicates that uncontrolled T_H17 cells may be an important mediator of inflammation and tissue damage in the disease of pregnancy.

In addition to effector cells, T_H cells are regulated by Treg cells. In the last few years, it has been observed that Treg cells are essential in promoting fetal survival and avoiding the recognition of paternal semi-allogenic tissues by maternal immune system. Treg capacity to produce cytokines is suppressed by immunoregulatory cytokines such as TGF- β and IL-10 or by cell-to-cell interaction. Several functional studies have shown that unexplained infertility, miscarriage and preeclampsia are often associated with deficit in Treg cell number and function while normal pregnancy selectively stimulates the accumulation of maternal FoxP3⁺CD4⁺ Treg cells with fetal specificity (Sharma 2014; Ruocco et al. 2014). *CTLA4* molecules are expressed on Tregs and play a role in the suppressive function of these cells. Decreased expression of *CTLA4* is reported in fetal loss in humans (Svensson-Arvelund et al. 2015; Walker 2013). The PD1/PDL1 pathway is one of the major negative costimulatory pathways, along with *CTLA4*,

that regulates T cell activation and has been shown to play a role in the *in vivo* plasticity of T_H cells, resulting in conversion of T_H1 cells to Treg type and inhibiting T_H17 cell responses. PDL1 is expressed on the trophoblast cells of the placenta and PD1 is expressed on the maternal effector and Tregs (Guleria et al. 2005; Francisco, Sage, and Sharpe 2010).

1.2 PATHOGEN INFECTION DURING PREGNANCY

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 countries (WHO | The Worldwide Incidence of Preterm Birth: A Systematic Review of Maternal Mortality and Morbidity 2016). Bacterial, viral, and parasitic infections acquired during pregnancy have the potential to pose a significant threat to the health of the mother and fetus. Of the pathogens, only select intracellular infections are able to cross the placental barrier (Vigliani and Bakardjiev 2014; Robbins and Bakardjiev 2012; Robbins et al. 2012; Doran et al. 2013; Ribet and Cossart 2015; Robbins et al. 2010), suggesting that pregnancy does not fully compromise immunity to all infections.

Infections during pregnancy have the potential to cause a variety of complications. Fifteen million preterm infants are born annually worldwide, with infection and inflammation as the leading cause of preterm birth (Bastek, Gómez, and Elovitz 2011; Burdet et al. 2014; Kemp 2014). Preterm birth, or birth prior to 37 weeks of gestation, is a major cause of morbidity and mortality, accounting for 65% of neonatal deaths and 50% of childhood neurologic disability world-wide. Preterm birth accounts for 11% of pregnancies in the United States and remains a leading cause of long-term neurological handicap (Hadders-Algra, Huisjes, and Touwen 1988; Moore et al. 2012). Although the mechanisms leading to pre-term birth are multifactorial, a

causative association has been established with intra-uterine infection and inflammation. It is estimated that infections and inflammation are responsible for up to 80% of the cases of preterm birth (Agrawal and Hirsch 2012). Placental infections can also cause other complications such as spontaneous abortion, still birth, chorioamnionitis, fetal infection and severe maternal illness (Galinsky et al. 2013; McClure et al. 2010; Giakoumelou et al. 2016). Furthermore, infection with certain pathogens during pregnancy may cause maternal death (Schantz-Dunn and Nour 2009).

Pathogens can infect the placenta and fetus by three different routes: first, pathogens in the lower genital tract may ascend through the cervix; second, pathogens in the maternal blood or uterus can colonize the placenta by breaching the maternal-fetal barrier and third, pathogens can descend into the uterus from the peritoneal cavity. (Zeldovich and Bakardjiev 2012; McDonagh et al. 2004; Coyne and Lazear 2016; Adams Waldorf and McAdams 2013).

1.2.1 VIRUSES

Various acute and chronic viral infections during pregnancy can result in a variety of complications. Examples of specific viruses and their impact on pregnancy are described in this section.

1.2.1.1 CYTOMEGALOVIRUS

Human cytomegalovirus (CMV), also known as human herpesvirus-5 (HHV-5), causes prenatal viral infection and infection-related congenital disabilities (Demmler 1991). The placenta acts as a viral reservoir for CMV and the virus can cross the placenta and infect the fetus (Yinon et al. 2010). Infected cTBCs of the placenta show defective invasion capacity

(Fisher et al. 2000; Tabata et al. 2012). Cultures of syncytiotrophoblast cells can also be permissively infected with CMV *in vitro*. Infection of term syncytiotrophoblast cells required a high virus inoculum compared to first trimester syncytiotrophoblast cells, which occurred at higher frequency and progressed more rapidly (Schleiss, Aronow, and Handwerger 2007). Consequently, a primary infection in the first trimester of pregnancy may result in more severe fetal consequences than one occurring in the third (Gindes et al. 2008). The risk of CMV infection-induced pregnancy complications has resulted in the interest to develop vaccines for maternal immunization.

1.2.1.2 ZIKA VIRUS

Zika virus is an emerging flavivirus that belongs to the same family as the dengue, West Nile and yellow fever viruses (Petersen et al. 2016). In the past year, more than 1,500,000 cases of Zika-infection are estimated to exist in Brazil and Zika has spread to other South and Central American countries (Jamali Moghadam et al. 2016). Zika is spreading rapidly and the risk for pregnant women was highlighted earlier this year. From October 2015 onward, a high incidence of microcephaly in newborns was observed. Most of the women who delivered these children presented symptoms of Zika infection (Noronha et al. 2016) and the entire Zika genome was recovered from the brain of a fetus from an infected mother (Jurado et al. 2016). Zika ribonucleic acid (RNA) has been reported in amniotic fluid, placenta and fetal neural tissue from women weeks to months after being infected during gestation (Martines et al. 2016; Meaney-Delman et al. 2016). Zika infects primary human placental cells and explants such as cTBCs, endothelial cells, fibroblasts and Hofbauer cells in chorionic villi and amniotic epithelial cells and trophoblast progenitors in amniochorionic membranes, that are known to express Axl, Tyro,

and/or TIM1 viral entry cofactors (Tabata et al. 2016). Furthermore, Zika is able to replicate in a log-fold manner in human syncytiotrophoblast cell lines and not in primary human syncytiotrophoblast cells (Bayer et al. 2016; Miner et al. 2016). There were fundamental differences between the approach of the two groups, such as the use of current endemic virus versus high passage historic strains and the duration of both infectivity and culture. Nevertheless, Zika infection during pregnancy has become a serious threat and the mechanism of fetal neuronal development defects are under intense investigation.

1.2.1.3 HUMAN PAPILLOMAVIRUS

Human papillomavirus (HPV) is a small double stranded deoxyribonucleic acid (DNA) virus and is considered to be the main cause of cervical cancer (Burd 2003). HPV has been reported to infect placenta (Rombaldi et al. 2008). HPV was detected in trophoblast cells from early pregnancy losses and spontaneous abortions (Hermonat et al. 1998; Ambühl et al. 2016; You et al. 2003). In the placenta, studies have shown that HPV DNA are localized in syncytiotrophoblast cells (Sarkola et al. 2008; Weyn et al. 2011) and HPV DNA was detected at birth of neonates (S. M. Lee et al. 2013) (Chisanga et al. 2015).

1.2.1.4. VARICELLA-ZOSTER VIRUS

Varicella zoster virus (VZV) is highly infectious and is transmitted by direct contact and through respiratory droplets (Pergam and Limaye 2009). About 90% of infections are contracted during childhood, and immunity to VZV is lifelong (A. M. Arvin 1996; Ann M. Arvin 2008). Morbidity and mortality are much higher among older persons who are newly exposed to VZV (Yoshikawa and Schmader 2001). Pregnant women who are not immune to VSV may develop

pneumonia and encephalitis and can transmit varicella to the fetus via the placenta. The incidence of primary VSV in pregnancy has been estimated to be between 1 to 5 cases per 10,000 pregnancies (Lamont, Sobel, Carrington, et al. 2011; Pupco, Bozzo, and Koren 2011; Charlier et al. 2014). Vertical transmission is believed to occur through maternal viremia and placental infection (Wilson et al. 2008) and first trimester maternal varicella infection may lead to fetal infection with congenital varicella syndrome (Balducci et al. 1992)..

1.2.1.5 HEPATITIS E VIRUS

Hepatitis E virus (HEV) is a water-borne pathogen that has fecal-oral transmission (Mirazo et al. 2014). HEV infection during third trimester pregnancy is associated with severe infection and can lead to fulminant hepatic failure and maternal death, as well as adverse perinatal outcomes, such as preterm birth, stillbirth and neonatal death (Navaneethan, Mohajer, and Shata 2008; Kamar et al. 2014; Krain et al. 2014). There is increasing evidence that HEV is an important contributor to maternal morbidity and mortality in South Asia (Chaudhry, Verma, and Koren 2015). The placenta is shown to be the replication site of HEV in humans (Krain et al. 2014) similar to CMV.

1.2.1.6 RUBELLA

Rubella infection is generally mild, self-limited illness characterized by rash, lymphadenopathy, and low-grade fever. Even though routine vaccination has made prenatal infection rare in the developed world (Hardt et al. 2016), infection with rubella virus causes severe damage when the mother is infected early in pregnancy. Pregnant women who contract rubella are at risk for miscarriage or stillbirth, and the developing fetuses that survive are at risk

for severe birth defects with devastating lifelong consequences (Watson et al. 1998). Rubella does infect the placenta (Garcia et al. 1985) even though there is fetal demise.

1.2.1.7 INFLUENZA VIRUS

Influenza viruses are human respiratory pathogens that cause both seasonal, endemic infections and periodic unpredictable pandemics (Taubenberger and Morens 2008). Influenza infection during pregnancy results in increased disease severity and a higher incidence of maternal mortality in comparison to non-pregnant individuals (Meijer et al. 2015). Second trimester fetal demise occurred after exposure to seasonal influenza A virus (H1N1) early in pregnancy confirming transplacental passage. Pre-term birth and still birth rates have been reported regarding to 2009 influenza A/H1N1 infection (Lieberman et al. 2011; Carlson, Thung, and Norwitz 2009). Highly pathogenic strains of influenza virus, such as avian influenza A H5N1, can be transmitted across the placenta to the fetal lungs and liver (Xu et al. 2011). Although influenza crosses the placenta, replication does not occur.

1.2.1.8 HERPES SIMPLEX VIRUS

Genital herpes caused by transmission of herpes simplex virus (HSV) in pregnancy can cause maternal morbidity (Hyde and Giacoia 1993). Primary infections with HSV-1 or HSV-2 near delivery carry the highest risk of neonatal herpes infection, as newborn infants can become infected with herpes virus by passing through the birth canal (Enright and Prober 2002). There has been an occurrence of HSV-2 infection of the placenta that was limited to maternal-derived cells of the subchorionic tissue and decidualized cells in the decidua capsularis (Finger-Jardim et al. 2014). Replication has not been observed.

1.2.1.9 HUMAN IMMUNODEFICIENCY VIRUS

Human immunodeficiency virus (HIV) is a virus that causes AIDS (Acquired Immunodeficiency Syndrome). If a woman is infected with HIV, her risk of transmitting the virus to her baby is reduced if she is treated and has low viral load in the blood (Moodley and Wennberg 2005). Transmission through the placenta may occur through CD4⁺ endothelial tissues or CD4⁺ Hofbauer cells (Al-husaini 2008).

1.2.2 PARASITES

Parasitic pathogens can pose a threat during pregnancy and may either be exacerbated during pregnancy or result in congenital parasitic infection.

1.2.2.1 TOXOPLASMA GONDII

Toxoplasma gondii infection is acquired primarily through ingestion of cysts in infected, undercooked meat or oocysts that may contaminate soil, water, and food (McConkey et al. 2013). Worldwide, the risk of infection with *Toxoplasma gondii* is 0.1-1% of all pregnancies (Lopes et al. 2007). Transmission to the fetus occurs predominantly in women who acquire their primary infection during gestation varies from 20% in the first trimester to 70% in the third trimester (Abbasi et al. 2003). Fetuses infected early in development are more severely affected than the third (Adams Waldorf and McAdams 2013). *Toxoplasma gondii* was shown to infect villous trophoblast from placenta associated with congenitally infected infants (Abbasi et al. 2003). Extravillous trophoblast cells are susceptible to *Toxoplasma gondii* infection and are more permissive to *Toxoplasma gondii* replication in comparison to syncytiotrophoblast cells (Robbins

et al. 2012). Cultures of cTBCs and syncytiotrophoblast cells have been shown to be infected and the infection was followed by apoptosis (Zhao et al. 2013; Abbasi et al. 2003; Xu et al. 2012).

1.2.2.2 PLASMODIUM SPECIES

Plasmodium falciparum is the major species that causes malaria in humans (Andrews and Lanzer 2002). Malaria during pregnancy is a health concern and a contributor to maternal and infant morbidity and mortality (Ahmed et al. 2014). *Plasmodium vivax* is also a significant cause of maternal morbidity during pregnancy and of low birth weight (Carmona-Fonseca, Arango, and Maestre 2013). *Plasmodium falciparum* can adhere and sequester in the placenta (Andrews and Lanzer 2002), specifically binding to chondroitin sulfate A on the syncytiotrophoblast cells (Ahmed et al. 2014; Pereira et al. 2016). Placental malaria is characterized by the presence of parasites and leucocytes within the intervillous spaces, pigment within macrophages, fibrin deposits and trophoblast cells, proliferation of cTBCs and thickening of the trophoblast basement membrane (Uneke 2007).

1.2.2.3 SCHISTOSOMA SPECIES

Schistosomiasis currently affects ~207 million people in tropical countries, of this, 20 million have severe illness (Barsoum, Esmat, and El-Baz 2013; Gray et al. 2011; Grimes et al. 2015). Ten million women in Africa per year have schistosomiasis during pregnancy. Schistosomes infect approximately 40 million women of child-bearing age and are associated with morbidity in pregnant women and of their offspring (Friedman et al. 2007). *Schistosoma mansoni* has been found in the placenta of a 28-year-old German woman who had been exposed to schistosomiasis in Lake Malawi one year prior to gestation (Schleenvoigt et al. 2014). There have been 4 reported cases of placental involvement in Brazilian women infected with s

Schistosoma mansoni. One of the pregnancies resulted in a normal baby, the other 3 in stillbirths (Bittencourt et al. 1980). Furthermore, in another two cases, adult flukes were found in the intervillous space of the placenta in one case and in the decidual vessels in another (Nour 2010). In another case of schistosomiasis infection, eggs were found in the placenta, in the intervillous space, within villi or in the decidua (Friedman et al. 2007). Despite infection of the placenta, there is no evidence of infection of the fetus.

1.2.3 BACTERIA

1.2.3.1 COXIELLA BURNETII

Coxiella burnetii, a Gram-negative and obligate intracellular bacteria, is the causative agent of Q fever (van Schaik et al. 2013). *Coxiella burnetii* causes miscarriage, low birth weight, and premature birth, and fetal death in cattle, sheep, and goats (Roest et al. 2012). In humans, *Coxiella burnetii*-infection during pregnancy is often followed by abortion and stillbirth (Munster et al. 2012; Prasad, Chandiramani, and Wagle 1986). Among published cases of Q fever during human pregnancy, *Coxiella burnetii* was isolated from placentas (Bildfell et al. 2000). In cattle and sheep, *Coxiella burnetii* was often detected in placenta and aborted fetuses (Jones et al. 2010; Munster et al. 2012; Agerholm 2013). Once *Coxiella burnetii* invades the placenta, studies have shown that infection results in inhibition of apoptosis of the trophoblast cells, which allows for bacterial replication within the cells. Thus, trophoblast cells could be a niche for *Coxiella burnetii* proliferation (Ben Amara et al. 2010; Voth, Howe, and Heinzen 2007).

1.2.3.2 CHLAMYDIA TRACHOMATIS AND CHLAMYDOPHILA ABORTUS

Infection by *Chlamydia trachomatis* and *Chlamydophila abortus*, Gram-negative and obligate intracellular bacteria, can lead to adverse pregnancy outcomes in horse, rabbit, guinea pigs, mice, pigs and humans (Gómez and Parry 2009). In pregnant women, the common clinical manifestations of this infection include cervicitis, pelvic inflammatory disease and tubal factor infertility (Allaire, Nathan, and Martens 1995; Howie, Horner, and Horne 2011; Adachi, Nielsen-Saines, and Klausner 2016). Furthermore, chlamydia infection has been associated with increased risk of ectopic pregnancy, preterm delivery, spontaneous abortions, low birth weight, premature rupture of membranes, perinatal mortality, and postpartum endometritis (McGregor and French 1991; Rours et al. 2011; Bakken, Skjeldestad, and Nordbø 2007). *Chlamydia* DNA was detected in placental tissues and in fetal fluid (Baboonian et al. 2003). Although *Chlamydia* infects the placenta, growth of the bacteria within the placenta has not been observed.

1.2.3.3 BRUCELLA ABORTUS

Brucella, Gram-negative and facultative intracellular bacteria, causes brucellosis, a zoonosis transmitted by ingesting contaminated food, direct contact with an infected animal, or inhalation of aerosols (de Figueiredo et al. 2015; Christopher, Umopathy, and Ravikumar 2010). *Brucella abortus* is found in cattle populations such as buffalo and causes abortion (Carvalho Neta et al. 2008). *Brucella abortus* can colonize mouse placentas of inoculated pregnant mice (Tobias, Cordes, and Schurig 1993; Bosseray 1980; T. M. A. Silva et al. 2011). In infected animals of various species, there is preferential growth of *Brucella* within trophoblast cells, resulting in placental damage, fetal infection, and fetal loss (Salcedo et al. 2013; O'Callaghan 2013; Fernández et al. 2016).

1.2.3.4 PERIODONTAL BACTERIA

Porphyromonas gingivalis is a major periodontal bacterium (How, Song, and Chan 2016). Human studies in patients with periodontitis and animal studies have suggested an association of this pathogen with preterm birth (Shanthi et al. 2012; Katz et al. 2009). In mice, *Porphyromonas gingivalis* infection can disseminate to the placenta and is associated with a shift of the placental T_H2-type humoral immunity to T_H1-type cellular immunity and also fetal death. Furthermore, translocation of *Porphyromonas gingivalis* into the placenta induces local immune responses that impair placental function which correlated with fetuses that exhibit fetal growth restriction (D. Lin, Smith, Champagne, et al. 2003). *Porphyromonas gingivalis* induces IL-8 and IFN- γ secretion in HTR-8/SVneo cells through the induction of mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ β) pathways. *Porphyromonas gingivalis* has also been shown to induce apoptosis of HTR8/SVneo cells (Ren et al. 2016). *Porphyromonas gingivalis* induced preterm birth in mice (Ao et al. 2015).

Fusobacterium nucleatum is a Gram-negative anaerobic oral species and an opportunistic human pathogen associated with various forms of periodontal disease and is detected in 83% of preterm birth (Han et al. 2004). Oral infection of pregnant mice resulted in localized infections of the fetoplacental unit. The infections initiated at the decidua followed by spreading to the fetal membranes and fetus (H. Liu, Redline, and Han 2007).

1.2.3.5 LISTERIA MONOCYTOGENES

Listeria monocytogenes is a Gram-positive bacterium that can cause invasive disease in immunocompromised individuals including pregnant women. Listeriosis during pregnancy can

result in severe complications such as preterm labour, spontaneous abortion, stillbirth, or infection of the neonate resulting in high morbidity and mortality (Lamont, Sobel, Mazaki-Tovi, et al. 2011; Janakiraman 2008; Mateus et al. 2013). Murine infection with *Listeria monocytogenes* resulted in vertical transmission and resorption of murine fetuses (Abram et al. 2003; Monnier et al. 2006). Intravenous inoculation of *Listeria monocytogenes* in BALB/c mice at mid-gestation produced consistent placenta infection with rapid fetal loss. As early as 6 h post infection, bacteria were detected in the placenta, followed by ~2-log units increase per day. By day 2, the bacteria were visualized in both the proximal and distal central arterial canal and had spread to the decidua, spongiotrophoblast and trophoblast giant cells. By day 3, bacteria invaded all layers of the placenta including the labyrinth (Monnier et al. 2006). In human placenta, extracellular *Listeria monocytogenes* present in the maternal blood that bathes placental villi binds to InIA or InIB, leading to penetration of the syncytiotrophoblast cells and subsequently invasion of the fetal vascular compartments (Pamer 2004). *Listeria monocytogenes* was found in the intervillous spaces, on surfaces of syncytiotrophoblast cells and cTBCs, and in the cytoplasm of syncytiotrophoblast cells (Robbins et al. 2010). Human invasive extravillous trophoblast cells and syncytiotrophoblast cells have been shown to restrict intracellular spread of *Listeria monocytogenes* (Zeldovich et al. 2011). *Listeria monocytogenes*-infection during pregnancy results in induction of adaptive immunity in the host which can clear infection (Pamer 2004).

1.2.3.6 CAMPYLOBACTER

Campylobacter species are Gram-negative bacteria that can infect humans and animals. *Campylobacter jejuni* is the most common species that can cause food-borne gastroenteritis (J. Silva et al. 2011). Infections are most often self-limiting and treatment is not necessary (J. L.

Smith 2002). *Campylobacter fetus* is a cause of spontaneous abortions in cattle and sheep, as well as an opportunistic pathogen in humans (Simor and Ferro 1990). *Campylobacter jejuni* infections tend to be milder than those of *Campylobacter fetus* (Simor and Ferro 1990). *Campylobacter jejuni*-induced intra-uterine infection during pregnancy can lead to abortion, stillbirth, or early neonatal death (McDonald and Gruslin 2001).

1.2.3.7 SALMONELLA SPECIES

Salmonella species are Gram-negative, rod-shaped, flagellated facultative intracellular bacteria. *Salmonella* serovars can be divided into two main groups, typhoidal and non-typhoidal *Salmonella*. Typhoidal serovars include *Salmonella* Typhi and *Salmonella* Paratyphi. Non-typhoidal salmonellosis refers to illness caused by all serotypes of *Salmonella* except for Typhi, Paratyphi A, Paratyphi B, and Paratyphi C (Coburn, Grassl, and Finlay 2007). In developed countries, non-typhoidal serovars present mostly as gastrointestinal disease. However in sub-Saharan Africa, where there are other co-morbidities prevalent, non-typhoidal *Salmonella* can become invasive and systemic, particularly in immunocompromised individuals (Morpeh, Ramadhani, and Crump 2009; Mahon and Fields 2016). Invasive non-typhoidal *Salmonella* infection is mostly caused by *Salmonella* Typhimurium or *Salmonella enteritidis*. The incubation period of non-typhoidal salmonellosis is 6-72 hours (h), but illness usually occurs within 12-36 h after exposure (Fournier et al. 2015). Illness is commonly manifested by acute diarrhea, abdominal pain, fever, and sometimes vomiting. The illness usually lasts 4-7 days, and most people recover without treatment (Glynn and Palmer 1992). Strains of *Salmonella* with resistance to antimicrobial drugs are widespread in both developed and developing countries and have become a worldwide health problem. A distinct strain of *Salmonella enterica* serovar

Typhimurium (*S.Tm*), known as definitive type 104, is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline and has become a major cause of illness in humans and animals (Rajashekara et al. 2000; Weese et al. 2001).

Between 200 million and 1.3 billion cases of intestinal disease including 3 million deaths occur in humans due to non-typhoidal *Salmonella* each year worldwide, however, the real incidence rate is estimated to be 30 fold higher based on missed surveillance of unreported mild cases. In the developing world, non-typhoidal *Salmonella* infections are the leading cause of bloodstream infection in both children and adults with a fatality rate of 20-25%. Non-typhoidal *Salmonella* is also the leading cause of meningitis with a case fatality rate of 52% in children and 80% in adults.

1.3 SALMONELLA

1.3.1 SALMONELLA VIRULENCE MECHANISM

The molecular mechanisms of *Salmonella* pathogenicity are complex. *Salmonella* species virulence genes are organized as specific pathogenicity islands. The major virulence genes include those of Type III secretion systems (TTSSs) found in many Gram-negative pathogens, which consist of an apparatus that resembles a needle like structure that allows translocation of effector proteins from the bacterial cytoplasm into the host cell. *S.Tm* virulence is dependent on two TTSS. The genes encoding the TTSS structural components as well as the effector proteins are located on *Salmonella* pathogenicity islands (SPI)s -1 and -2. SPI-1 effector proteins cause actin cytoskeleton rearrangement of non-phagocytic host cells such as epithelial cells. The effector proteins result in cell membrane ruffling and eventually internalization of the bacteria inside the host cell (LaRock, Chaudhary, and Miller 2015). Inside the cells, *Salmonella* resides in

Salmonella containing vacuoles (SCV), where SPI-2 effector proteins modify the intracellular environment to allow growth of the bacteria (Coburn, Grassl, and Finlay 2007).

1.3.2 SALMONELLA INVASION INTO NON-PHAGOCYtic CELLS

Salmonella can gain entry into non-phagocytic cells by two mechanisms, a TTSS-dependent mechanism or -independent mechanism also known as receptor mediated zipper-like internalization. Upon contact with non-phagocytic cells, *Salmonella* injects a set of effector proteins into the host cell cytoplasm via the SPI-1-TTSS to induce bacterial uptake. The SPI-1-TTSS consists of a set of proteins and adenosine triphosphate (ATP)ase in the inner membrane of the bacteria that forms a “syringe” and extends into the cytoplasm of the host cell. *Salmonella* invasion A (InvA) protein forms the core of the TTSS “syringe” and is crucial to the functioning of the TTSS (Coburn, Sekirov, and Finlay 2007; Bueno et al. 2010; Jepson and Clark 2001; Galán, Ginocchio, and Costeas 1992). *Salmonella* can also invade non-phagocytic cells by the Rck outer membrane proteins. Rck is encoded on the *S.Tm* virulence plasmid and is a member of a family of related 17- to 19-(kiloDalton) kDa outer membrane proteins of Enterobacteriaceae, *Yersinia enterocolitica*. Rck interacts with an epidermal growth factor receptor, which induces cellular signals, including phosphorylation of tyrosine proteins, and activation of cellular sarcoma kinase (c-Src) and phosphoinositide 3-kinase (PI3K). Microvillus-like extensions then forms a zipper-like structure, engulfing the adherent bacteria. *Salmonella* is the first bacterium found to be able to induce both receptor mediated and non-receptor mediated invasion into host cells (Mijouin et al. 2012; Rosselin et al. 2010; Cirillo et al. 1996).

1.3.3 SALMONELLA PATHOGENESIS

After *Salmonella* ingestion by the host, the bacteria can invade the intestinal epithelial barrier of the gastrointestinal tract by three methods. Firstly, *Salmonella* can invade non-phagocytic enterocytes. Shortly after *Salmonella* adhere to the apical epithelial surface, *Salmonella* utilizes its SPI-1 T3SS which injects effector proteins into the host cells and results in profound cytoskeletal rearrangements in the host cells, disrupting the normal epithelial brush border and inducing formation of membrane ruffles that reach out and enclose the bacteria in a vacuole. Secondly, *Salmonella* may passively cross the intestinal epithelial barrier following phagocytosis by migrating DCs that are specialized to penetrate the tight junctions between gut epithelial cells. Thirdly, *Salmonella* may adhere to and enter microfold cells (M cells) of the intestinal epithelial that overlay the Peyer's patches and gain access to the lymphoid follicles and lamina propria, thereby infecting macrophages (Fig. 3) (Coburn, Sekirov, and Finlay 2007; Bueno et al. 2010; Jepson and Clark 2001).

1.4 SALMONELLA INFECTION DURING PREGNANCY AND TRANSMISSION ACROSS THE PLACENTA

Salmonella infection during pregnancy can result in various complications to both the mother and fetus. Many *Salmonella* species can cross the placental barrier to the fetus and increase the risk for spontaneous abortion, stillbirth, preterm birth, or perinatal complications and a few reported cases of life-threatening septicemia in the mother. *Salmonella* is a well-known cause of abortion in livestock, resulting in significant economic damages (Hinton 1977; Ravneet and Purabi 2011; Schloesser, Schaefer, and Groll 2004; Scialli and Rarick 1992; van der Klooster and Roelofs 1997).

Below are various case reports which highlight the need to study *Salmonella* infection during pregnancy in order to understand the host-pathogen interaction with the hopes of developing new and effective therapies.

1.4.1 SALMONELLA TYPHI-INFECTION DURING PREGNANCY IN HUMANS

Typhoid fever is a major health problem in developing countries (Buckle, Walker, and Black 2012; Zaki and Karande 2011; Mogasale et al. 2016; Dougan and Baker 2014). Cases of vertical transmission have been reported of *Salmonella typhi*. Gluck B *et al.*, reported positive blood cultures for *S. typhi* from a woman at 26 weeks gestation who was pregnant for the first time (Gluck, Ramin, and Ramin 1994). Ozer *et al.* reported a case of a 27-year-old pregnant woman with aortic valve infective endocarditis caused by *S. typhi* (Ozer et al. 2009). Hedriana *et al.* reported a case of intrapartum *Salmonella typhi* infection of an HIV-infected pregnant woman. The maternal blood, cervical and uterine cultures, and placental surface and intra-membraneous space cultures grew *Salmonella typhi*. However, the mother responded to antibiotic therapy and the infant did not show signs or symptoms of *Salmonella* sepsis (Hedriana, Mitchell, and Williams 1995). Vigliani *et al.* reported a case in which placental abruption occurred at 16 weeks following first trimester diagnosis and treatment of typhoid fever of a 23-year-old HIV-negative Cambodian female (Vigliani and Bakardjiev 2013). In this case, *S. Typhi* was found in fetal tissues at autopsy, here the placenta did not act as a barrier to *Salmonella*-infection.

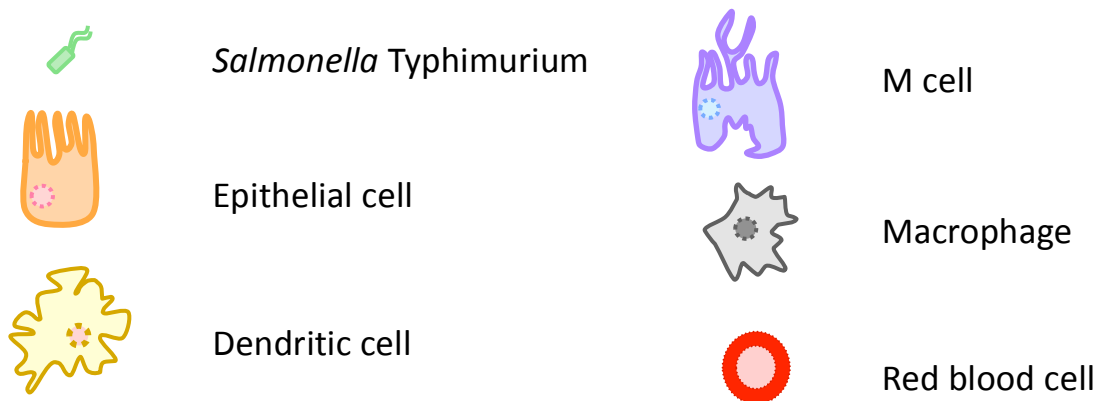
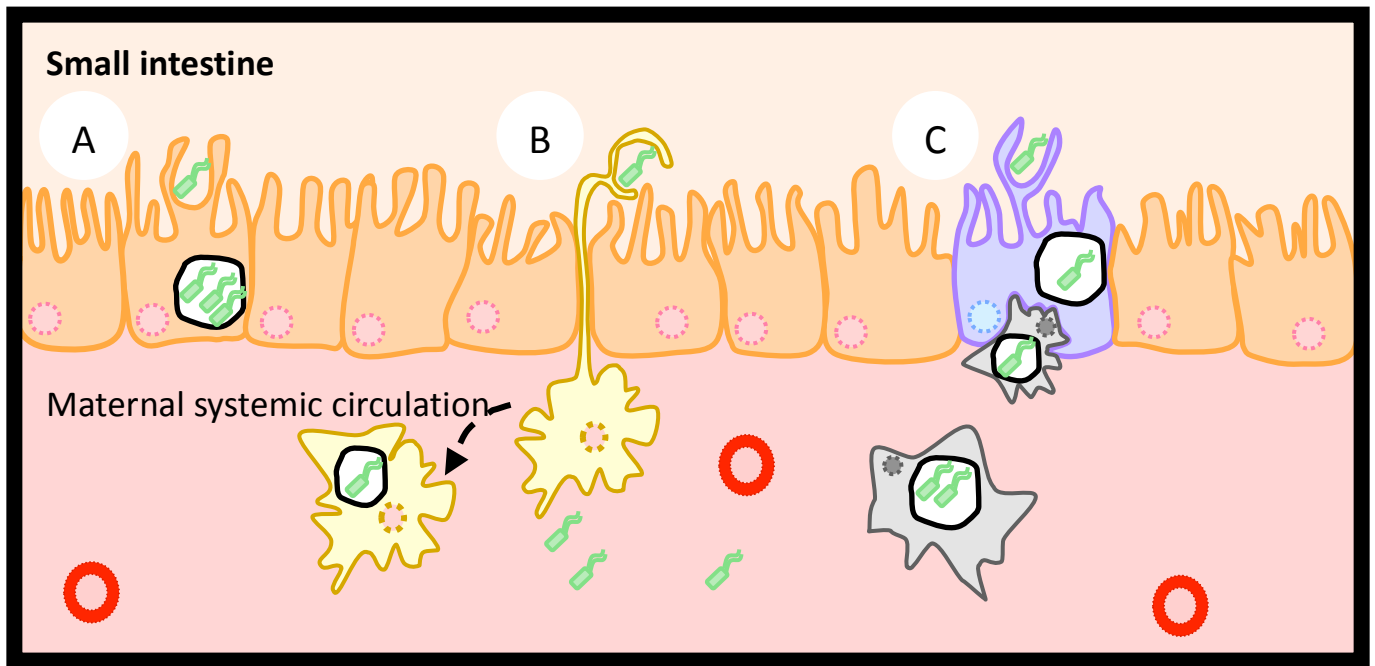


Figure 3. Schematic representation of *Salmonella* pathogenesis in the gut. *Salmonella* can invade the small intestine in one of three pathways: through the small intestinal epithelial cells (orange), dendritic cells (yellow) or M cell (purple). **A** *Salmonella* employs its SP1-TTSS to induce membrane ruffling of the cell which allows internalization of the bacterium into the non-phagocytic epithelial cells. **B** Migrating DCs can penetrate the tight junctions between the epithelial cells and phagocytose *Salmonella*. **C** *Salmonella* may adhere to and enter M cells that overlay the Peyer's patches, thereby infecting macrophages.

1.4.2 NON-TYPHOIDAL SALMONELLA-INFECTION DURING PREGNANCY IN HUMANS

A number of non-typhoidal related pregnancy complications have been cited in the literature. Case reports have revealed a variety of fetal outcomes: fetal or infant death, septic abortion, or premature birth. Some of these are described in this section.

A case history of a neonatal infection caused by non-typhoidal *Salmonella* was presented by van der Klooster and Roelofs, where the mother suffered from gastroenteritis 1 week before delivery. The newborn was treated with ampicillin, gentamicin, and later amoxicillin but 5 months later was still a carrier of *Salmonella* (van der Klooster and Roelofs 1997). Scialli *et al.* presented a case of second-trimester fetal loss associated with non-typhoidal *Salmonella* (Scialli and Rarick 1992). Dalaker *et al.* reported a case of septic abortion due to non-typhoidal *Salmonella* (Dalaker *et al.* 1988).

Non typhoidal *Salmonella*-related preterm births are also reported in literature: Roll *et al.* reported a case of a 29 year old woman with severe non-typhoidal gastroenteritis that crossed the placental barrier and resulted in infection of the fetus and neonatal death of the cesarian-delivered premature infant (Roll *et al.* 1996). In another case, Schloesser *et al.* reported a case of transplacentally acquired neonatal sepsis with non-typhoidal *Salmonella*. The infant's mother, a 36-year-old woman, presented with fever and malaise in the 25th week of gestation. Caesarean section was performed. The premature infant had clinical signs of sepsis with multiorgan failure and died 4 days later despite intensive medical care (Schloesser, Schaefer, and Groll 2004). In another case report, Gyang *et al.* reported transplacental transmission of non-typhoidal *Salmonella* resulting in spontaneous miscarriage at 18 weeks gestation that occurred 1 week after resolution of an acute diarrheal illness (Gyang and Saunders 2008).

Case reports have shown that non-typhoidal *Salmonella* can infect the fetus *in utero*. Ault *et al.*, reported a 25-year-old woman at 28 weeks of gestation where the mother and neonate were infected with non-typhoidal *Salmonella* (Ault et al. 2013). Bobylev *et al.* reported a case of intrapericardial infection resulting from nontyphoidal *Salmonella* in a 2-month-old baby (Bobylev et al. 2016).

1.4.3 NON-TYPHOIDAL SALMONELLA –INFECTION IN IMMUNOCOMPROMISED HOST

Non-typhoidal *Salmonella* causes diarrhoeal disease, invasive bacteremia illness, focal suppurative infection, and asymptomatic carriage in the stool (Acheson and Hohmann 2001). In an immunocompetent host, non-typhoidal *Salmonella* serovars cause self-limiting diarrhoeal disease. However, immunocompromised individuals are more likely to present with primary bacteremia and an absence of diarrhoeal disease (Feasey et al. 2012). Adults with diabetes, long term steroid medication, haematological malignancy, advanced or disseminated solid cancers, autoimmune disease, liver disease, renal transplantation, and those taking immunosuppressive drugs are susceptible to non-typhoidal *Salmonella* bacteremia (Acheson and Hohmann 2001; Dhanoa and Fatt 2009; Galanakis et al. 2007; Chen et al. 2013). Moreover, non-typhoidal *Salmonella* have a dramatically more severe and invasive presentation in HIV-infected individuals (Gordon 2008).

Non-typhoidal *Salmonella* is the leading etiology of community acquired bacteremia in patients with HIV infection in developed or developing countries. The incidence of non-typhoidal *Salmonella* bacteremia was found to be 20- to 100-fold higher among HIV-infected

patients than among patients not infected with HIV. (Feasey et al. 2012; Levine and Farag 2011; Preziosi et al. 2012).

1.4.4 PRETERM DELIVERY OF NON-TYPHOIDAL *SALMONELLA*

A male infant was born at 34 weeks gestation to a primigravida mother infected with *Salmonella* serovar *Montevideo*. The mother had a history of 1 day of diarrhea and mild fever 8 days prior to delivery. Her blood culture was negative during the illness and her stool did not grow any pathological organism. The blood culture of the baby and placental swab from the mother grew the bacteria (Rai, Utekar, and Ray 2014).

1.5 HOST IMMUNE RESPONSE TO *SALMONELLA*-INFECTION

The innate immune system plays an essential role in the early responses to *Salmonella* by inducing a variety of inflammatory and antimicrobial responses. Nevertheless, the acquired immune system is also important for clearing the infection as well as providing effective protection to subsequent challenge with related *Salmonella* strains. The following sections will introduce the innate and the adaptive immune responses to *Salmonella*.

1.5.1 INNATE IMMUNE RESPONSE TO *SALMONELLA*-INFECTION

After crossing the intestinal barrier at the site of the Peyer Patches, *Salmonella* is taken up by macrophages (Jensen, Harty, and Jones 1998; B. D. Jones 1997).

1.5.1.1 MACROPHAGE PHAGOCYTOSIS

Eukaryotic cells internalize a variety of particles during their lifetime. The uptake of particles $>0.5 \mu\text{m}$ in size is termed phagocytosis, whereas particles $<0.5 \mu\text{m}$ are taken up by receptor-mediated endocytosis or pinocytosis. Macrophages destroy bacteria by engulfing them in intracellular compartments which they then acidify to kill or neutralize the bacteria (Weiss and Schaible 2015). Distinct types of phagocytosis tend to be ligand specific: bacteria ($\sim 0.5\text{-}3 \mu\text{m}$) or yeast ($\sim 3\text{-}4 \mu\text{m}$) are internalized by macrophages through scavenger receptors (Greaves and Gordon 2009; Winther et al. 2000; Platt and Gordon 2001). Scavenger receptor A (SR-A) are expressed by macrophages, dendritic cells and certain endothelial cells and play a role in uptake and clearance of modified host molecules and apoptotic cells (Abdul Zani et al. 2015). Scavenger receptor A molecules are divided into two groups, SR-A I and SR-A II, which differ in regard to the presence or absence of the SR cysteine-rich domain (Canton, Neculai, and Grinstein 2013). This domain has a role in binding to specific ligands. SR-A I and SR-A II can bind to Gram-positive bacterial lipoteichoic acid and Gram-negative bacterial lipopolysaccharide (LPS), which then internalize *Salmonella* (Fenton and Golenbock 1998; van Oosten et al. 2001).

1.5.1.2 PATHOGEN ASSOCIATED MOLECULAR PATTERN MOLECULES RECOGNITION BY PATHOGEN RECOGNITION RECEPTOR AND CYTOKINE SECRETION

Innate immune receptors detect infection by recognizing conserved microbial features common to broad classes of pathogenic and non-pathogenic microbes known as the pathogen-associated molecular patterns (PAMPs) via pathogen recognition receptor (PRRs). In mammals, Gram negative bacteria are recognized by systems such as the toll-like receptor (TLR) and

nucleotide-domain oligomerization domain (NOD)-like receptor (NLR) families (H. Kumar, Kawai, and Akira 2011; Akira and Takeda 2004; Beutler 2009).

The TLRs target a range of microbial ligands, including lipoprotein (TLR2), lipopolysaccharide (TLR4) and flagellin (TLR5) (Akira and Takeda 2004). Recognition of extracellular *Salmonella* by the cell components of *Salmonella*, is largely mediated by these TLRs. TLR4 is also capable of binding endogenous danger-associated molecular patterns (DAMPs) such as heat shock proteins and reactive oxygen species. Upon ligand binding, TLRs engage the signaling adaptor myeloid differentiation primary response gene 88 (MyD88) and Toll/IL-1 receptor (TIR)-domain-containing adapter-inducing interferon- β (TRIF), which initiate signaling cascades leading to the activation of the transcription factors and interferon regulator factor 3 (IRF3) that induce the production of proinflammatory cytokines such as TNF- α , IL-1, IL-6, IL-8, and IL-12 as well as type I IFN response and a variety of chemokines that recruit cells of the immune system to the site (Vaure and Liu 2014; Lu, Yeh, and Ohashi 2008).

Intracellular *Salmonella* and can be recognized by intracellular PRR such as the NLR family. NLR stimulation results in the induction of an inflammatory pathway, pyroptosis, described in detail in another section below (Behnsen et al. 2015; Elinav et al. 2011).

At the epithelial barrier region of M cells, *Salmonella* also encounters the next layer of innate immune defenses, macrophages and dendritic cells of the gut-associated lymphoid tissue: These cells remove invading microbes by phagocytosis and alert other immune cells of the infection directly or by secreting pro-inflammatory cytokines. Monocytic cells express an array of germline-encoded PRR, which enable them to detect PAMPs such as LPS, lipoprotein, and flagellin (Mabbott et al. 2013).

1.5.1.3 CYTOKINE RESPONSES AND JAK-STAT SIGNALING PATHWAY

Cytokines mediate their function by utilizing the Janus family tyrosine kinase (Jak)-signal transducers and activators of transcription (STAT) signaling pathway; Jaks are receptor-associated protein tyrosine kinases, and STATs are latent cytoplasmic transcription factors that are activated by tyrosine phosphorylation (Schindler, Levy, and Decker 2007). STATs play a critical role in mediating cellular transcriptional responses to cytokines, cell activation, survival and proliferation. For example, IFN- γ binds to the IFN- γ receptor (IFN- γ R). The IFN- γ R1 subunit is associated with Jak1 and IFN- γ R2 subunit is associated with Jak2. Activation of the receptors, following ligand binding, results in dimerization and rearrangement of receptor units leading to the activation of associated Jaks by auto-phosphorylation. STAT-1 binds to the cytoplasmic portion of the ligand-activated IFN- γ receptor and is phosphorylated by Jak proteins. Phosphorylation causes Src Homology 2 (SH2) domain-mediated dimerization, nuclear translocation and binding of STAT-1 to its cognate promoter DNA, the γ -IFN-activated site, and the transcription of IFN-stimulated genes is then initiated. Complete activation of macrophages during immune responses results from stimulation with IFN- γ and a second stimulus, usually a microbial product. Bacterial infection of macrophages, or treatment with bacterial LPS, results in rapid STAT-1 phosphorylation (Fig. 4) (Ivashkiv and Donlin 2014; Schneider, Chevillotte, and Rice 2014).

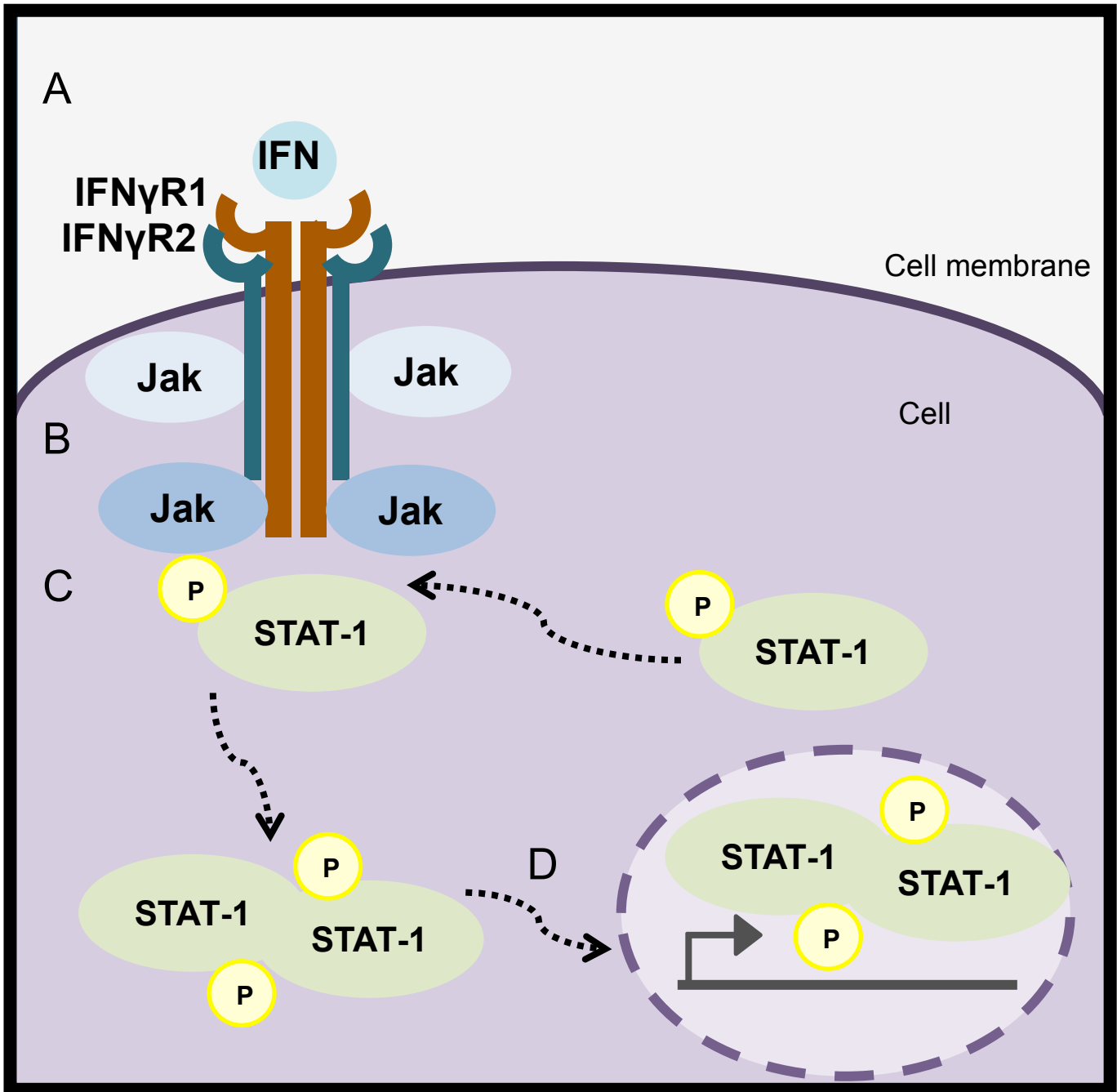


Figure 4. Schematic representation of the IFN- γ signaling pathway. **A** IFN- γ binds to the IFN- γ receptor (IFN- γ R). **B** The IFN- γ R1 subunit is associated with Jak1 and IFN- γ R2 is associated with Jak2. **C** Activation of the receptors leads to Jak auto-phosphorylation and phosphorylation of STAT-1. **D** Phosphorylated STAT-1 translocates to the nucleus and binds to the γ -IFN-activated site. The transcription of IFN-stimulated genes is then initiated.

IL-10 signals via two receptors that belong to the IFN receptor family. The most well described signaling pathway specific for IL-10 binding is that of the Jak/STAT pathway. IL-10RI is mainly necessary for the binding of the IL-10 protein, is constitutively associated with Jak1 and is mainly involved in downstream signaling. IL-10RII acts as an accessory subunit that recruits the second kinase, tyrosine kinase 2 (Tyk2), to the receptor complex. Upon binding of IL-10 to the IL-10R subunits, the Jaks are activated by transphosphorylation (Riley et al. 1999). The activated Jaks phosphorylate IL-10RI, thereby creating docking sites for STAT transcription factors such as STAT-3. The intracellular part of the IL-10R1 contains only two tyrosine motifs. Each of the single motifs is sufficient for full STAT-3 activation. STAT-3 becomes activated at the receptor by tyrosine phosphorylation. STAT-3 then homodimerizes and subsequently translocates into the nucleus to induce STAT-3-responsive genes (Fig. 5). IL-10 is a cytokine with potent anti-inflammatory properties (Saraiva and O'Garra 2010). IL-10-induced STAT-3 activation results in decreased expression of inflammatory cytokines such as TNF- α , IL-6, and IL-1 β in macrophages (Rawlings, Rosler, and Harrison 2004; Murray 2007; Jatiani et al. 2010; Stark and Darnell 2012; Carey, Tan, and Ulett 2012; Hutchins, Diez, and Miranda-Saavedra 2013).

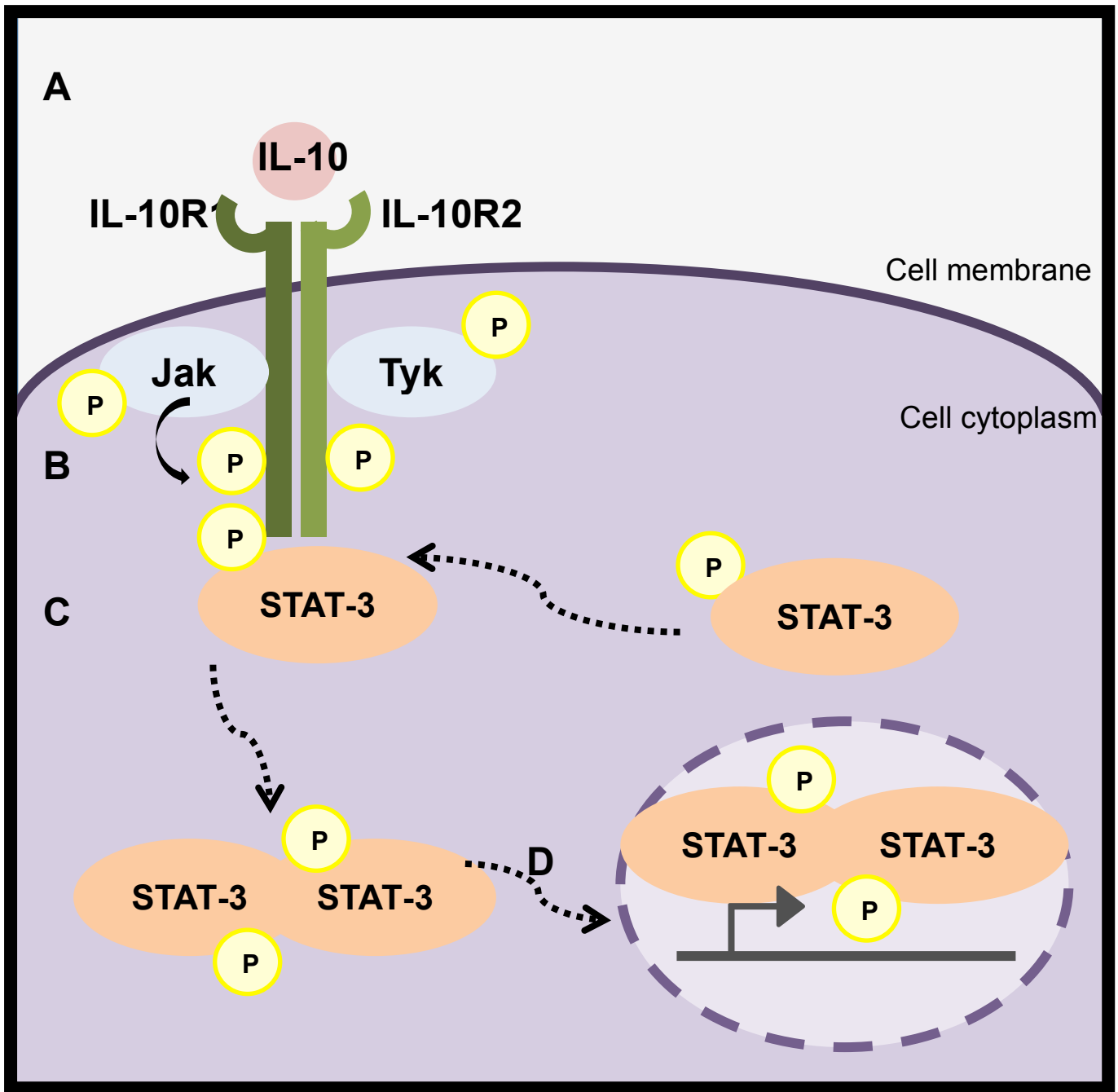


Figure 5. Schematic representation of the IL-10 signaling pathway. **A** IL-10 binds to IL-10 receptor (IL-10R). IL-10RI is constitutively associated with Jak1. **B** The Jaks are then activated by transphosphorylation. **C** The activated Jaks phosphorylate IL-10RI, thereby creating docking sites for STAT transcription factors such as STAT-3. STAT-3 becomes activated at the receptor by tyrosine phosphorylation. **D** STAT-3 then homodimerizes and subsequently translocates into the nucleus to induce STAT-3-responsive genes.

1.5.1.4 PHAGOSOME MATURATION PROCESS

Once *Salmonella* gains entry into macrophages, *Salmonella* can grow inside the cells in SCV by employing its SPI-2 T3SS (Figueira and Holden 2012; Waterman and Holden 2003).

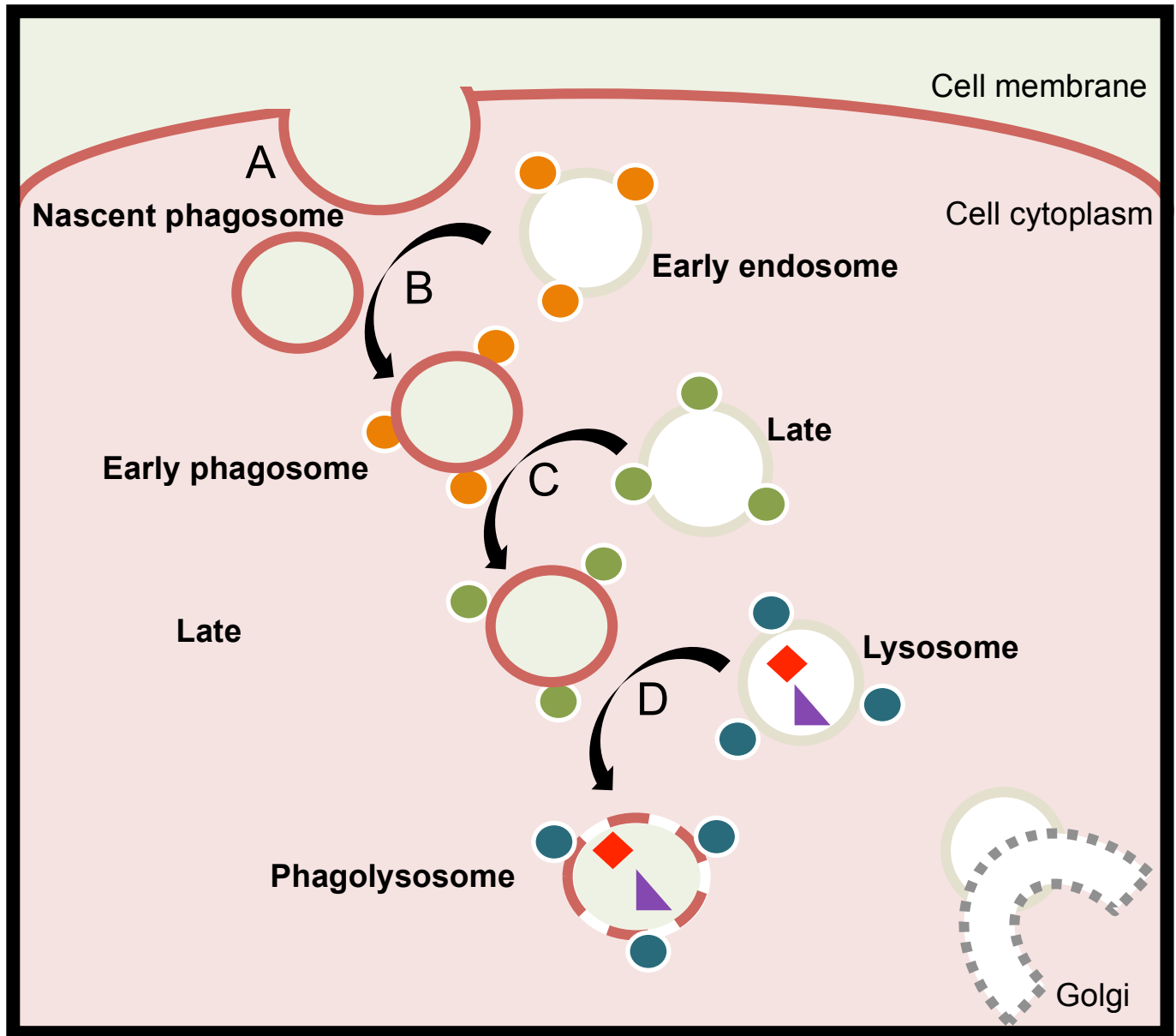
Immediately after internalization, the particle resides in a nascent phagosome, where the membrane is largely derived from the plasma membrane and the luminal milieu is similar to the extracellular medium. The nascent phagosome undergoes maturation in a series of sequential membrane fusion and fission interactions with other membrane-bound endocytic compartments.

The internalized particles are trafficked into a series of increasingly acidic membrane-bound structures, eventually fusing with the lysosome, a vesicle comprised of enzymes to form the phagolysosome, which then leads to degradation of the intracellular components (Kinchen and Ravichandran 2008; Vieira, Botelho, and Grinstein 2002). The stages of phagosome maturation

can be identified using markers such as Rab GTPases. Rab5 is associated with phagosomes immediately after phagocytosis and facilitates the recruitment of Rab5 effector proteins, early endosome antigen 1 (EEA1) and PI3K. Membrane bound Rab5 is rapidly dissociated from the phagosome after its maturation. Rab7 appears on the phagosome maturation after Rab5

dissociation and remains on the membrane during phagosome maturation. After acquisition of Rab7, phagolysosome biogenesis is accelerated by the recruitment of Rab7-interacting-lysosomal-protein (RILP) to the phagosome which mediates the fusion of the phagosome with

late endosomes and ultimately, lysosomes, and aids in pathogen degradation (Fig. 6). Degraded pathogenic components can be presented by antigen presenting cells (APC) to the T cells for induction of adaptive immunity (A. C. Smith et al. 2007; Seto, Tsujimura, and Koide 2011; Fairn and Grinstein 2012).



- | | | |
|--------|----------|---------------|
| ● Rab5 | ● LAMP-1 | ▲ Cathepsin D |
| ● Rab7 | ◆ Beta- | |

Figure 6. Schematic representation of the phagosome maturation pathway. Upon internalization of a particle, **A** the nascent phagosome undergoes maturation in a series of sequential membrane fusion and fission interactions with other membrane-bound endocytic compartments. **B** The nascent phagosome acquires the GTPase Rab5 from fusion with endosomes. **C** During the course of phagosomal maturation, early endosomal markers, such as Rab5, are lost, and the phagosome fuses with late endosomes and thereby acquires a second GTPase, Rab7. **D** Late phagosomes fuse with lysosomes to form phagolysosomes, which are characterized by the presence of hydrolytic proteases, such as cathepsin D.

1.5.1.5 CELL DEATH MECHANISMS

Intracellular infection of mammalian cells can result in triggering of cell death, a mechanism that eliminates the host cell and consequently the pathogen. Cell death can occur through a number of extrinsically and/or intrinsically programmed mechanisms, which may be modulated by the pathogens. Apoptotic, necroptotic, and pyroptotic cell death are critical defense mechanisms of the host against microbial infection; however, successful bacterial pathogens use many strategies to manipulate these host cell pathways to enhance their replication and survival. There are two broad categories of cell death pathways: non-inflammatory and inflammatory, the latter resulting in more collateral damage than the former. Discriminating non-inflammatory cell death and inflammatory cell death pathways may provide insights into the pathophysiology of infectious diseases caused by intracellular pathogens (Ashida et al. 2011).

1.5.1.5.1 NON-INFLAMMATORY CELL DEATH PATHWAY

1.5.1.5.1.1 APOPTOSIS

Apoptosis is a conserved programmed cell death. Apoptosis is the default programmed cell death pathway and occurs during embryonic development and homeostasis and apoptosis leads to intracellular disassembly without changes in membrane integrity. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Elmore 2007; Taylor, Cullen, and Martin 2008).

The extrinsic pathway is initiated at the plasma membrane by the death receptor-mediated interactions. This pathway involves death receptors that are members of the TNF receptor gene superfamily. Members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the “death domain”. The

death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways (Wajant, Pfizenmaier, and Scheurich 2003). The best-characterized ligands and corresponding death receptors include FasL/FasR, and TNF- α /TNFR-1. The binding of PAMPs to PRRs such as TLRs can also initiate apoptosis (Elmore 2007). The binding of Fas ligand (FasL) to Fas receptor (FasR) results in the binding of the adaptor protein Fas-associated protein with death domain (FADD), and the binding of TNF ligand to TNF receptor (TNFR) results in the binding of the adapter protein TNFR-1-associated death domain (TRADD) with recruitment of FADD and the receptor-interacting serine/threonine-protein kinase (RIPK)-1 and RIPK-3, preventing their phosphorylation and subsequent activation (Kasof et al. 2000). FADD associates with procaspase-8 via dimerization of the death effector domain. A death-inducing signaling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8. Once caspase-8 is activated, the execution phase of apoptosis is triggered. Death receptor-mediated apoptosis can be inhibited by a protein called cellular (FLICE) FADD-like IL-1 β -converting enzyme-inhibitory protein (c-FLIP) which will bind to FADD and caspase-8, rendering them ineffective (Portt et al. 2011).

The intrinsic pathway is initiated at the mitochondria. The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor mediated stimuli such as cellular stress, for example, DNA damage, radiation, growth factor depletion, or cytotoxic drugs. This pathway is regulated by members of the B-cell lymphoma 2 (BCL-2) family of regulator proteins. 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 cytoplasm. 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-9 (Fig. 7) (Fulda and Debatin 2006; Czabotar et al. 2014).

The apoptosis initiator caspases -8, and -9 via the extrinsic and intrinsic pathways respectively, enable both pathways to converge with the processing of the executioner caspases -3, which leads to various downstream effects such as DNA degradation into oligonucleosomal fragments, chromatin condensation, externalization of the membrane lipid phosphatidylserine and membrane blebbing. The membrane-bound apoptotic bodies can then be taken up by neighbouring phagocytic cells, allowing for degradation of cellular components without tissue damage. A characteristic feature of apoptosis is the lack of accompanying inflammatory response (McIlwain, Berger, and Mak 2013; Ziegler and Groscurth 2004; Saraste and Pulkki 2000).

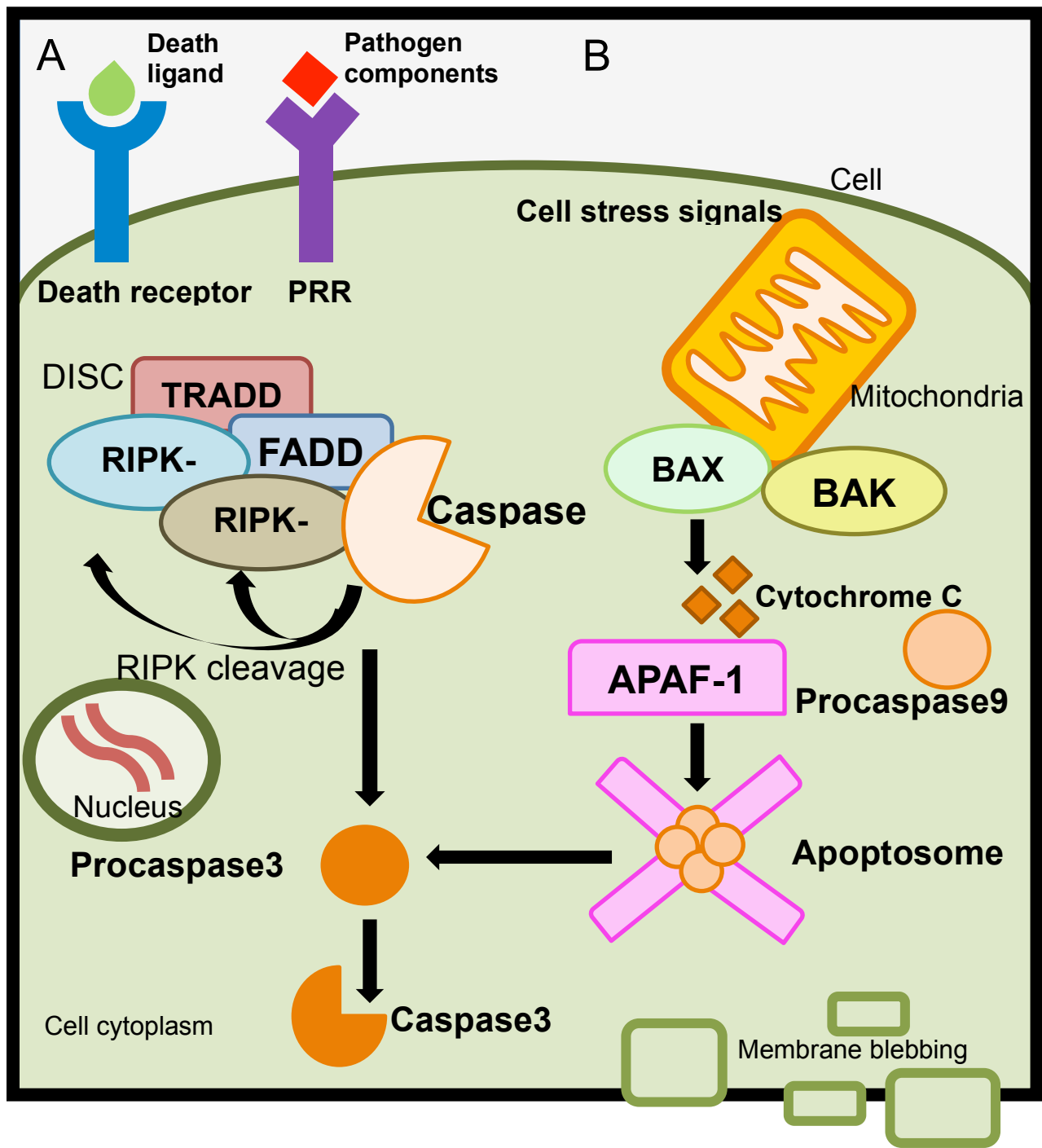


Figure 7. Schematic representation of the extrinsic and intrinsic apoptosis signaling pathways. **A** The extrinsic pathway of apoptosis is activated upon stimulation of death receptors or pattern recognition receptors (PRR) by their respective ligands, which results in the formation of the death inducing signaling complex (DISC) in which procaspase-8 is recruited and activated. Caspase-8 cleaves RIPK-1 and RIPK-3 preventing their phosphorylation and activation. **B** The intrinsic pathway of apoptosis is activated upon intracellular stress signals. 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 with activates caspase-9. Activation of the initiator caspase-8 and/or-9 enables the processing of the executioner caspase-3, ultimately resulting in the morphological and biochemical characteristic features of apoptosis.

1.5.1.5.2 INFLAMMATORY CELL DEATH PATHWAYS

1.5.1.5.2.1 PYROPTOSIS

Pyroptosis is referred to as caspase-1 mediated pro-inflammatory programmed cell death. NLR recognize PAMPs inside the cell, which then forms a multi-molecular complex termed the pyroptosome consisting of an apoptosis-associated speck-protein containing (ASC), a caspase activator and recruitment domain (CARD), and caspase-1 (Guo, Callaway, and Ting 2015). Bacterial LPS is detected by NLR, leucine-rich repeat (LRR)- and pyrin containing domain containing 3 (NLRP3), while ice protease-activating factor (IPAF) also known as NLRC4 detects bacterial flagellin components of Gram-negative bacteria (Matusiak et al. 2015). Upon stimulation, the NLR undergoes oligomerization and associates with ASC. The CARD on the ASC molecules enables oligomerization through CARD-CARD interactions with subsequent recruitment and proximity-induced auto-activation of caspase-1 (Denes, Lopez-Castejon, and Brough 2012). The pyroptosome cleaves caspase-1, leading to an active form which then cleaves the inactive precursors IL-1B and IL-18 into active inflammatory cytokines (Sollberger et al. 2014). 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 milieu. The active inflammatory cytokines are then secreted which leads to recruitment of neutrophils to the infection site to aid pathogen clearance. Unlike necroptosis but similar to apoptosis, nuclear condensation and DNA fragmentation occur (Fig. 8) (Fink and Cookson 2007; Tait, Ichim, and Green 2014; Jorgensen and Miao 2015)

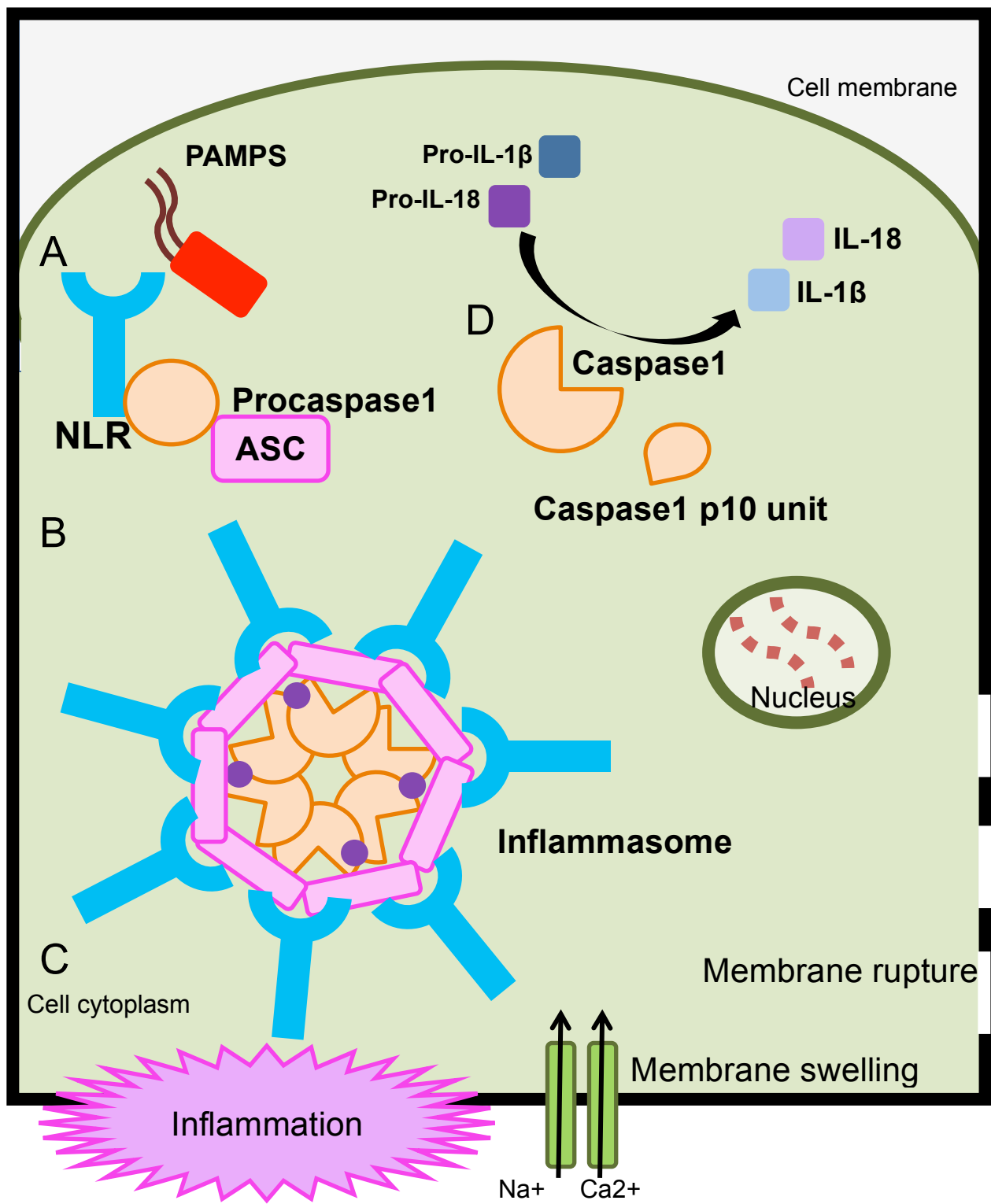


Figure 8. Schematic representation of the pyroptosis signaling pathway. **A** Pyroptosis can be triggered upon detection and stimulation of NLRs by PAMPs within the cell cytoplasm. **B** Assembly of the inflammasome enables the activation of caspase-1. **C** Active caspase-1 leads to membrane pore formation with an influx of ions and subsequent membrane rupture. **D** 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.

1.5.1.5.2.2 NECROPTOSIS

Necroptosis is a programmed form of necrosis. Necroptosis can be triggered by ligation of various death receptors: apoptosis antigen 1 (Fas), members of the TNF family, TNFR-1 and TNFR-2, TLRs and type I interferon receptor (IFNAR) signaling (Vandenabeele et al. 2010). Necroptosis is independently regulated by caspase-8 and is dependent on the kinase activity of RIPK-1 and RIPK-3 (Pasparakis and Vandenabeele 2015). The term necroptosis was coined when small-molecule inhibitors of necroptosis called necrostatins were identified. The necrostatins were found to inhibit RIPK-1 which was consistent with earlier studies highlighting the importance of RIPK-1 (Ofengeim and Yuan 2013). Production of TNF- α during viral infection leads to stimulation of its receptor, TNFR-1. The TRADD signals to RIPK-1 which recruits RIPK-3 forming the necrosome. Caspases repress necrotic or necroptotic cell death by cleaving and inactivating poly-ADP-ribose polymerase (PARP) (Shao et al. 2015, 3). In the absence of caspase-8, due to its inactivity, RIPK-1, RIPK-3 interact with mixed lineage kinase domain-like (MLKL), forming the necrosome. Phosphorylation of MLKL drives oligomerization of MLKL, allowing MLKL to insert into and permeabilize plasma membranes and organelles, and binds to phosphatidylinositol phosphates and forms a pore-forming complex. 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 swelling. Integration of MLKL leads to the inflammatory diseases through the release of DAMPs (Murphy et al. 2013). Caspases repress necrotic or necroptotic cell death by cleaving and inactivating PARP, activation of PARP is required for necroptosis. A variety of downstream effectors induce necroptosis through the induction of increased reactive oxygen species (ROS) production, and lysosomal destabilization (Christofferson and Yuan 2010). Necroptosis leads to collateral damage of the host due to the release of harmful chemicals

that damage other cells and due to the lack of signaling to recruit immune cells (Fig. 9) (Strzyz 2016; Silke, Rickard, and Gerlic 2015).

1.5.1.5.3 CELL DEATH IN *SALMONELLA*-INFECTION

After *Salmonella* ingestion by the host, epithelial cells in the gastrointestinal tract are the initial site of cellular contact. Studies have shown that only after prolonged exposure of *Salmonella*, 24 h post infection, apoptosis occurred in intestinal epithelial cells, which suggests that *Salmonella* is capable of suppressing apoptotic cell death at the initial stage of infection (L. A. Knodler and Finlay 2001; Leigh A. Knodler, Finlay, and Steele-Mortimer 2005). Macrophages are the second cell type in the lumen that become sites of *Salmonella* residence in vivo (Geddes, Iii, and Heffron 2007). However, *Salmonella*-infected macrophages do not undergo classical apoptosis. Intracellular *S.t*m flagellin is recognized by NLRC4 in macrophages and initiates inflammasome assembly and caspase-1 activation. This evokes pyroptosis (Puri et al. 2012; Fink and Cookson 2007; Lara-Tejero et al. 2006; Franchi 2011; Qu et al. 2016; Broz et al. 2010). *Salmonella* induced macrophage pyroptosis features pore formation of the plasma membrane which results in the release of cytoplasmic contents such as p10 or p20 subunits of caspase-1, mature IL-18, and mature IL-1 β . Macrophage pyroptosis leads to release of *Salmonella* into the extracellular milieu, which then is followed by neutrophil uptake. Neutrophils mediate killing of *S.T*m, leading to bacterial clearance (Guiney 2005; Boise and Collins 2001; Thurston et al. 2016).

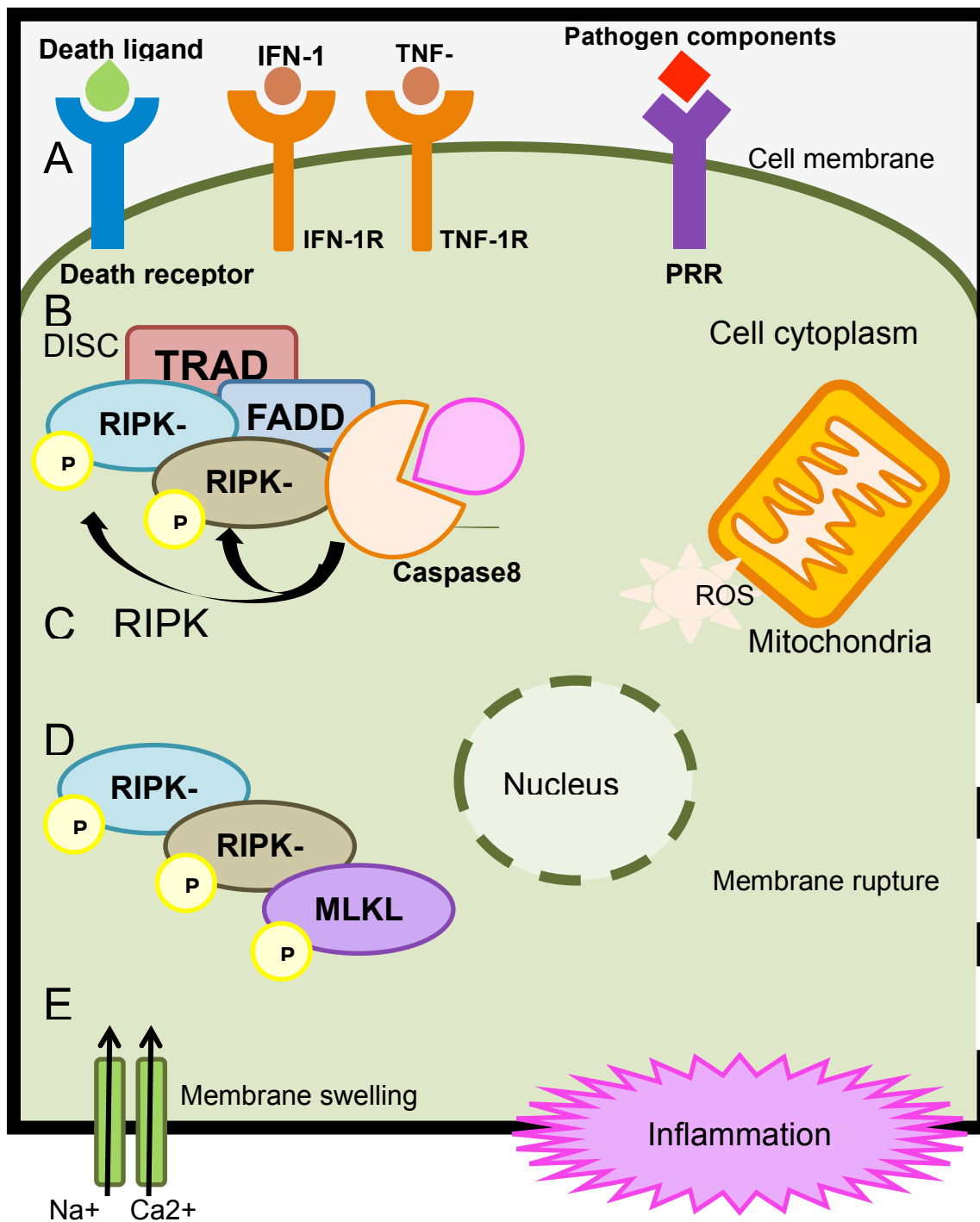


Figure 9. Schematic representation of the necroptotic signaling pathway. **A** Necroptosis can be triggered upon stimulation of death receptors or toll-like receptors when caspase-8 is inhibited. **B** Binding of TNF to TNFR-1 or IFN-1 to IFN-1R results in the recruitment of TRADD, FADD, RIPK-1, and RIPK-3. **C** The inactivation of caspase-8 enables RIPK-1 and RIPK-3 to auto-phosphorylate forming the necroptosome. **D** RIPK-3 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. **E** Cell swelling ensures with membrane rupture, releasing intracellular contents into the extracellular milieu.

1.5.2 ADAPTIVE IMMUNE RESPONSE TO *SALMONELLA*-INFECTION

Although the innate immune system can restrict replication of *Salmonella* to a certain degree, for effective control and eradication of *Salmonella*, the adaptive immune response is essential. APCs, such as macrophages, are capable of destroying bacteria to process and present antigen in the context of MHC to T cells. Recognition of peptide-MHC on APCs by T cell receptor (TCR)-expressing naïve T cells leads to activation and expansion of antigen-specific effector T cells. CD4⁺ T cells have been shown to play a prominent role in immunity to *Salmonella* (Albaghdadi et al. 2009); CD4⁺ helper T cells (T_H) are divided into two types depending on the profile of cytokines they secrete. T_H1 cells produce IFN- γ and TNF- α and activate cellular immunity and inflammation, while T_H2 cells produce IL-4, IL-5, and IL-13 and induce B cell activation and differentiation (Charles A Janeway et al. 2001). A number of studies have shown that *Salmonella* infection results in the induction of a T_H1 response. Nevertheless, the CD8⁺ T cell activation is delayed during infection with virulent *S.Tm*, therefore the phagosomal lifestyle allows escape for the host CD8⁺ T cell recognition, conferring a survival advantage (Sad et al. 2008; Luu et al. 2006).

1.5.3 IMMUNE RESPONSE TO *SALMONELLA* IN PREGNANCY

S.Tm-infection in non-pregnant 129X1Sv/J and C57129F1 mice is characterized by an increase in numbers of macrophages, dendritic cells, neutrophils and NK cells by 48 h, whereas this response is abolished in pregnant infected mice. Furthermore, splenic expansion and activation of NK cells post-infection seen in non-pregnant mice were lacking in pregnancy (Pejicic-Karapetrovic et al. 2007). Moreover, pregnant infected mice had decreased production of serum IL-12 and increased IL-6 and IL-10 levels. In another study, *S.Tm* was shown to

colonize the placenta and to cause widespread placental necrosis accompanied by neutrophil infiltration (Chattopadhyay et al. 2010).

The genetic background of mice influences outcome of infection. Mice of the 129X1/SvJ strain carry a functional *Slc11a1* gene, more commonly known as *Nramp-1*, 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 cells. Nramp-1 functions as an antagonist of bacterial metal acquisition within the phagosome, thereby depleting divalent metal ions required for successful bacterial replication. C57BL/6J mice have a single glycine to aspartic acid substitution at position 169 of the *Nramp-1* gene, rendering Nramp-1 protein non-functional. Studies in *Nramp-1*^{+/+} C57BL/6J pregnant mice, or C57BL/6J with functional Nramp-1, that are systemically infected with *S.Tm*, showed higher splenic neutrophil numbers compared to non-pregnant mice. In placental tissues, neutrophils, T lymphocytes, and NK cells in infected placenta were higher compared to pregnant non-infected tissue. Infection of *S.Tm* in pregnant pyroptosis inhibited, *Caspase-1*^{-/-}*Nramp-1*^{+/+} C57BL/6J mice and pregnant *Nramp-1*^{+/+} C57BL/6J mice resulted in no significant differences in bacterial burden within the spleen, liver, or placentas and there was no difference in the percent resorptions. However, *S.Tm*-infected pregnant necroptosis inhibited, *Ripk-3*^{-/-}*Nramp-1*^{+/+} C57BL/6J and pregnant *Nramp-1*^{+/+} C57BL/6J mice bacterial burden revealed no differences in spleen, liver or placentas, however the percent resorption was lower in *S.Tm*-infected pregnant *Ripk-3*^{-/-} *Nramp-1*^{+/+} mice, and inhibition of necroptosis in mice resulted in the reduction of damage by *S.Tm* to the placental-fetal unit (Wachholz, 2016 M.Sc Thesis).

1.6 *SALMONELLA* VACCINES

Currently, doctors prescribe antibiotics to alleviate *Salmonella* infection symptoms and to fight *Salmonella* infection; however, the selection of effective antibiotics has become more difficult as antibiotic resistance has increased. Antibiotic-resistant *Salmonella* have been isolated from various food products, and have been a causative agent in several foodborne disease outbreaks in the United States and worldwide (Su et al. 2004; Threlfall 2002). There are three vaccines against typhoid fever that are licensed for use which offer moderate protection against typhoid fever caused by serotype Typhi (Garmory, Brown, and Titball 2002). As a preventative measure, vaccination of chickens, along with other intervention measures, is an important strategy that is currently being used to reduce the levels of *Salmonella* in poultry flocks, which will ultimately lead to lower rates of human *Salmonella* infections (Dórea et al. 2010; Revollo and Ferreira 2012). Although vaccination against *Salmonella* Typhi infection is available, there are currently no licensed paratyphoid or non-typhoidal vaccines.

CHAPTER 2:
RATIONALE, HYPOTHESIS
AND OBJECTIVES

2.0 RATIONALE

In mice, susceptible strains (C57BL6) die of *Salmonella enterica* serovar Typhimurium infection to a low dose I.V. of 10^2 CFU within 7 days whereas resistant mice (129x1/SvJ) infected with 10^3 CFUs develop a chronic infection lasting 60-90 days, which is eventually cleared. While 129X1SvJ mice are resistant in a non-pregnant state, mid-pregnant (days 10-12) 129X1SvJ mice infected with 10^3 *S.Tm* CFUs die within 7 days (Luu et al. 2006). *S.Tm* infection of mid-pregnant 129X1/SvJ mice with 10^3 colony forming units (CFU) results in fatality within 7 days. This was correlated with ~1000 fold increase in bacteria burden in spleens of pregnant mice compared to non-pregnant controls. 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 ~100% fetal loss with a median survival of 6 days (Pejicic-Karapetrovic et al. 2007).

Furthermore, studies involving intravenous infection with a high dose (10^6 CFU) of an auxotrophic mutant *Salmonella* Typhimurium strain (*S.Tm-ΔaroA*) resulted in 100% of both pregnant and non-pregnant surviving infection. Growth kinetics indicated that while there was an increase in wild-type *S.Tm* splenic bacterial burden between 24 to 48 h, little growth was seen after 24 h with *S.Tm-ΔaroA* infection. Meanwhile, placental bacteria burden in which both wild-type *S.Tm* and *S.Tm-ΔaroA* reached a titer of $\sim 10^8$ bacteria by 72 h, indicating that *S.Tm* preferentially replicates within placental tissues compared to other systemic tissues. Systemic organs showed a steady increase in bacterial burden over time post-infection, however, there was no significant difference between non-pregnant and pregnant groups. In pregnant mice infected systemically with *S.Tm*, however, exhibited a rapid increase in bacterial burden in the placenta over time, which shows a preference of *S.Tm* to survive and proliferate in the placenta.

Moreover, intracellular *S.Tm* infection and intracellular bacteria were found in large numbers across all regions of the placenta. *In vitro*, *S.Tm* grew rapidly in choriocarcinoma JEG-3 cells. (Chattopadhyay et al. 2010).

These results highlight the need to study *Salmonella* infection during pregnancy in order to develop therapies to control *Salmonella* infection in the immunocompromised hosts.

It was unclear if the placenta, specifically trophoblast cells, was more permissive to *S.Tm* entry compared to other cell types. Furthermore, It is unknown if the mechanism(s) of entry of *S.Tm* into placental cells differ from that of entry into macrophages. It has been shown that upon entry into cells such as macrophages, *S.Tm* survives within phagosomes-like vesicles called SCV. In macrophages, *S.Tm* is known to alter the biogenesis of this compartment to favour its survival and proliferation. Nevertheless, immune cells such as macrophages are capable of restricting profound replication of the bacteria. The first question we addressed was if bacterial entry and phagosome maturation is differentially altered upon entry of *S.Tm* into placental trophoblasts. The ability of immune cells to control intracellular bacterial infection is attributed to the production of inflammatory cytokines and many bacteria have evolved mechanisms to alter this activation process in host immune cells.

More recently, *S.Tm*-infected pregnant necroptosis inhibited, *RIPK-3^{-/-}nramp-1^{+/+}* C57BL/6J and pregnant *nramp-1^{+/+}* C57BL/6J mice revealed no differences in bacterial burden found in the spleen, liver or placentas. The percent resorption, however, was lower in *S.Tm*-infected pregnant *necroptosis* inhibited mice. This indicates that, inhibition of necroptosis in the mice resulted in the reduction of damage by *S.Tm* to the placental-fetal unit (Wachholz, 2016 M.Sc. Thesis). Thus, specific inflammatory pathways may be evoked including leading to the

induction of specific cell death pathways of infected cells. Bacterial virulence factors may also modulate intracellular survival and *S.Tm* induced inflammation. Since *S.Tm* is a chronic pathogen that devoted a significant proportion of its genome to evade host immunity, the second question we addressed is the alteration of inflammatory responses by trophoblast cells and the mechanism of infection-induced cell death.

Majority of the previous studies were completed using a murine model. Here, we characterized *S.Tm* infection in human primary trophoblast cells from the placenta. Overall, there is a considerable gap in our understanding of the modulation of host immunity to *S.Tm* during pregnancy and a strong need to address these inquiries as food-borne infections such as Salmonella are considered a serious threat to pregnant women in Canada and abroad.

2.1 HYPOTHESIS

We hypothesized that placental trophoblast cells are more infected than other tissues and provide a unique intracellular niche that permits uncontrolled *S.Tm* replication due to an ineffective maternal innate immune response to the virulent bacterium resulting in placental death.

2.2 OBJECTIVES

The objectives were:

1. To characterize the specific susceptibility of trophoblast cells to *S.Tm*.
 - a. Determine the mechanisms employed by *S.Tm* to colonize trophoblast cells and the mechanism by which *S.Tm* gain entry into trophoblast cells.

- b. Characterize the intracellular niche within trophoblast cells that allow for uncontrolled proliferation.
2. To determine the mechanism of *S.Tm*-induced trophoblast cell death and inflammation.
 - a. Examine the extent of cell death in *S.Tm*-infected trophoblast cells.
 - b. Delineate the role of specific cell death pathways in *S.Tm*-infected trophoblast cells.
 - c. Identify strategies for averting death of *S.Tm* infected trophoblast cells.
3. To examine the role of cytokine signaling in modulating growth of *S.Tm* in trophoblast cells.
 - a. Determine the role of cytokines in modulating phagosome maturation in trophoblast cells.
 - b. Assess cytokine production in human trophoblast cells infected with *S.Tm*.
 - c. Map the cytokine signaling pathways in trophoblast cells.

CHAPTER 3:
MATERIALS AND
METHODS

3.0 MATERIALS AND METHODS

3.1 BACTERIA CULTURE

Salmonella Typhimurium (Wild-type SL1344, $\Delta invA$ SL1344, Δrck SL1344, and $\Delta rck\Delta invA$ SL1344) were grown in liquid culture containing brain-heart infusion (BHI) (Becton Dickinson Biosciences (BD), Franklin Lakes, NJ) with 50 $\mu\text{g}/\text{ml}$ streptomycin (Sigma-Aldrich, St. Louis, MO). These strains were provided by Dr. Brett Finlay (University of British Columbia) and Dr. Brian Coombes (McMaster University). At mid-log phase ($\text{OD}_{600} = 0.8$), bacteria were harvested and frozen at -80°C in BHI medium containing 20% glycerol until use. CFUs were determined by performing serial dilutions in saline (0.9% NaCl in H_2O) which were spread on BHI agar plates. In some experiments, frozen bacteria were thawed and directly used for infection. In such cases, *S.Tm* was thawed and centrifuged at 8000 x g for 5 minutes (min) for removal of glycerol. Bacteria were then washed and re-suspended in Roswell Park Memorial Institute (RPMI) medium (Gibco Life Technologies, ThermoFisher Scientific, Waltham, MA). *S.Tm* was added to the cells at a multiplicity of infection (MOI) of 10:1. In certain experiments, a live culture was used for infections: an *S.Tm* colony from a freshly streaked plate was cultured in BHI broth (5 mL) containing 50 $\mu\text{g}/\text{ml}$ of streptomycin at 37°C overnight at 200 rotations per min (rpm) in a shaking incubator. The next morning, *S.Tm* was sub-cultured in fresh BHI liquid culture containing 50 $\mu\text{g}/\text{ml}$ of streptomycin at a ratio of 1:40 (500 μl overnight culture in 20 mL BHI liquid culture containing streptomycin). The bacterial culture was grown to a standardized OD_{600} of 0.8 within 2.5 to 3 h. The culture was spun down at 8000 x g for 5 min and re-suspended in saline to achieve an approximate final concentration of 1×10^8 *S.Tm*/1000 μL . The exact bacterial count in an aliquot of the inoculum was retrospectively determined after use for infection studies.

3.2 *SALMONELLA* TYPHIMURIUM INFECTION AND INTRACELLULAR GROWTH IN CELLS

All infections were performed in a Containment Level 2 laboratory at the National Research Council, Ottawa, Ontario, Canada. HeLa, human epithelial carcinoma (American Type Culture Collection (ATCC), Manassas, VA); THP-1, human pro-monocytic leukemia cells (ATCC); THP-1 + 1 nM phorbol-12-myristate-13-acetate (PMA)-activated human monocytes differentiated into macrophages; JEG-3, human placental choriocarcinoma (ATCC); BeWo, human placenta choriocarcinoma (ATCC), and HTR-8/SVneo, human extravillous trophoblast (a gift from Dr. Andrée Gruslin, Ottawa Hospital Research Institute), were grown in RPMI medium containing 10 % fetal bovine serum (FBS, North Bio, Toronto, ON), known as R10, at 37°C and 5% carbon dioxide (CO₂), in T75 tissue culture flasks (Falcon™, BD Biosciences). Cells (5 x 10⁵ cells/ml R10) were seeded in 24-well plates (Falcon™, BD Biosciences) 24 h prior to infection. Cells were then infected for 30 min (at a MOI of 10:1; *S.Tm* was reconstituted to 5 x 10⁶ *S.Tm*/1mL) with wild-type SL1344 thawed from frozen stock. Bacteria and cells were centrifuged at 500 x g for 10 min to promote contact between bacteria and cells and incubated at 37°C for 30 min. After the 30 min infection, cells were washed thrice with 2 mL/well RPMI medium containing 50 µg/ml gentamicin (Sigma-Aldrich) and then incubated for 2 h in R10 medium containing 50 µg/ml gentamicin to remove extracellular *S.Tm*. For assessment of intracellular growth, cells in 3 representative cell culture wells were lysed at each time point between 2-24 h post-infection with 0.1% Triton X-100 (Sigma-Aldrich) and 0.01% sodium dodecyl sulfate (SDS, Sigma-Aldrich) in phosphate buffered saline (PBS, Gibco, Life Technologies). Serial dilutions were spread onto BHI agar plates to enumerate the number of internalized bacteria. For the remaining culture wells (post 2 h time points), R10 media

containing 50 µg/ml gentamicin was removed and replaced with fresh R10 medium containing 10 µg/ml gentamicin until lysed for determination of CFU. Plates were incubated at 37°C overnight, and colonies were counted.

3.3 MECHANISM OF *S.TM* ENTRY INTO THE CELLS

To determine the mechanism of *S.Tm* entry into the cells, 5×10^5 HeLa, PMA-activated THP-1, and JEG-3 cells were infected with an MOI of 10:1 wild-type *S.Tm* SL1344 (*S.Tm*-WT), SL1344 SPI-1 T3SS mutant (*S.Tm*- $\Delta invA$), receptor mediated entry mutant (*S.Tm*- Δrck), and a double mutant (*S.Tm*- $\Delta rck\Delta invA$). The number of internalized *S.Tm* was determined at the first time point, 2 h post-infection.

To determine the cell mediated uptake of *S.Tm*, various inhibitors were added to the cells 1 h before infection with *S.Tm*-WT (10 MOI) and during infection. Inhibitors used included: cytochalasin B and D [2 and 20 µM] for arrest of actin polymerization, inhibitors of phosphoinositide 3-kinase (LY294002 [3 and 30 µM] and Wortmannin [2 and 20 µM]) for arrest of PI3K, inhibitor of SR-A (fucoidan [0.5, 5, and 25 nM]), and inhibitor of the mannose receptor (mannan [10, 100 and 500 µg/mL]). All inhibitors were purchased from Sigma-Aldrich. Infection protocol used was similar to that described above. The percentage entry of *S.Tm* was calculated by dividing the number of CFU of infected cells in the presence of inhibitor by the number of CFU of infected cells without inhibitor (dimethyl sulfoxide (DMSO) control, Sigma-Aldrich) at 2 h post infection.

3.4 DOUBLING TIME

To determine the intracellular doubling time of *S.Tm*, HeLa, PMA-activated THP-1 and JEG-3 cells (5×10^5) were infected with *S.Tm*-WT at 10 MOI and cell lysates were plated on BHI-agar plates at various times (2, 4, 6, 8 and 24 h) after infection as described above. The intracellular doubling time was calculated using the formula $G = t / (3.3 \log b / B)$, where G is the generation time, t is the time elapsed, B is the CFU at the start time, and b is the CFU at the end time.

3.5 PHAGOSOMAL UPTAKE OF BEADS BY CELL LINES

THP-1, PMA-activated THP-1, JEG-3, and PMA-activated JEG-3 cells (3×10^6 /ml) were seeded onto 60 mm culture dishes (Falcon™, BD Biosciences). Dynabeads M-280 Tosylactivated magnetic beads (cat#14203, Invitrogen, ThermoFisher Scientific) (4 mg) was added to the cells for 30 min. Dynabeads M-280 Tosylactivated magnetic beads were washed using a magnetic block for 2 min, buffer was removed, and beads were re-suspended in 0.1M sodium bicarbonate buffer (NaHCO₃, Sigma-Aldrich). Beads (1 mg) in 100 μl 0.1 M NaHCO₃ buffer were added to each cell type. Beads were co-cultured with cells for 30 min. Extracellular beads were removed by washing the cells 3 times with 5 mL of RPMI medium each time. For phagosome isolation: after 5 and 60 min post co-incubation, 5 mL of PBS was used to wash the cells and then 5 mL was added to the cells after washing. A cell scraper (BD Biosciences) was used to scrape the cells. Cells were collected in a 15 mL tube and centrifuged at 500 x g for 10 min. Buffer (1.5 mL) containing 50 mM piperazine-*N,N'* bis [2-ethanesulfonic acid] (PIPES) buffer pH 7.0 (Sigma-Aldrich), 50 mM potassium chloride (KCl, Sigma-Aldrich), 2 mM

magnesium chloride (MgCl₂, Sigma-Aldrich), 5 mM ethylene glycol-bis (β-aminoethyl ethyl)-N,N,N',N'- tetraacetic acid (EGTA, Sigma-Aldrich), 220 mM mannitol (Sigma-Aldrich), 1 mM dithiothreitol (DTT, Sigma-Aldrich), and 10 μM cytochalasin B (Sigma-Aldrich) was added to the cell pellet and incubated on ice for 20 min. For lysis of the cells, buffer containing 50 mM PIPES buffer pH 7.0, 50 mM KCl, 2 mM MgCl₂, 5 mM EGTA, 220 mM mannitol, 68 mM sucrose (Sigma-Aldrich), 1 mM DTT, and 10 μM cytochalasin B) was added to the cells. Cells were then passed through a needle (3 mL 22G3/4 syringe, BD Medical) then centrifuged for 5 min at 400 x g. Pellets were stored in -20°C for loading control for Western blot. Following syringe passage, phagosomes in the supernatant were transferred into a 14 mL polystyrene tube (BD Biosciences) and were subjected to magnetic field for 10 min. Phagosomes were collected in Radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (Thermo Scientific) containing a Complete™ Roche Protease Inhibitor Cocktail Tablet (Hoffmann-La Roche, Basel, Switzerland) and 1:100 phosphatase inhibitor cocktail (Thermo Scientific) for Western blot stored at -20°C until used and supernatant was discarded.

3.6 PHAGOSOME ISOLATION OF *S. TM* INFECTED CELL LINES

THP-1, PMA-activated THP-1, JEG-3, and PMA-activated JEG-3 (3x10⁶/ml) were seeded onto 60 mm culture dishes overnight. Cells were infected with *S. Tm* and extracellular bacteria were removed as described above. Phagosome isolation protocol is also described above, however, for phagosome isolation following *S. Tm* infection, instead of using a magnetic block, 10 μg benzonase nuclease was added to the supernatant containing the phagosomes (2 mL in volume) following syringe passage and incubated for 5 min at 37°C. 1.05 mL of 65% sucrose was added to the supernatant for a final concentration of 39%. A sucrose gradient (10%, 32.5%,

phagosomes at 39%, 55%, and 65%) was prepared and ultra-centrifuged at 74,000 x g for 1 h at 4°C. Phagosomes in the cloudy middle phase were collected in RIPA lysis and extraction buffer and stored at -20°C until use. RIPA buffer was added to the pelleted cell debris and stored at -20°C until use to serve as loading control for Western blot.

3.7 PHAGOSOME MATURATION BY WESTERN BLOT

Phagosomes were isolated by swelling the cells and disrupting the cell membrane as described above. Proteins were quantified using Bradford protein assay (Bio-Rad, Hercules, California, United States). Bovine serum albumin (2 mg/ml, BSA) for the standard and proteins were serially diluted and equal amounts of 1X Bradford reagent (cat#B6916, Bio-Rad) was added. Absorbance at 595 nm was recorded and the protein concentration was determined by comparison to the BSA standard curve. After quantifying protein, an equal concentration (2 µg/µl) of each sample was prepared and diluted in PBS. The protein sample was mixed with Laemmli Sample Buffer (Bio-Rad) which contains 4% SDS, 20% glycerol, 0.0004% bromophenol blue, 0.125 M tris(hydroxymethyl)aminomethane-hydrochloric (Tris-HCl) and 10% β-mercaptoethanol (β-ME) to give a final protein concentration of 1 µg/µl. The samples in Laemmli buffer were heated on a heat block at 90°C for 10 min. An equal amount of each phagosomal protein sample (10 µg/well) was separated on 8% SDS-polyacrylamide gel and the proteins were transferred to a polyvinyl difluoride (PVDF) membrane (BioRad). The membrane was blocked in TRIS-buffered saline (TBS) containing 5% nonfat dried milk powder (w/v) (Blotting grade blocker, BioRad) and 0.1% Tween-20 (Sigma-Aldrich), for 1 h at room temperature. After three washes with TBS containing 0.1% Tween 20, the PDVF membranes were incubated with primary antibodies against Rab5 (cat# 2143 Cell Signaling Technology),

Rab7 (cat# 9367 Cell Signaling Technology), and or Cathepsin D (cat# 2284 Cell Signaling Technology). The primary antibody was used at 1:1000 dilution overnight at 4°C on a shaker. The membranes were washed thoroughly for 30 min with TBS containing 0.1% Tween-20 before incubation with 1:2000 dilution of the secondary horseradish peroxidase (HRP)-conjugated IgG for 1 h at room temperature and further washing for 30 min with TBS containing 0.1% Tween-20 followed by development using SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific). Cell pellets from phagosome isolation served as a loading control and were probed with β -actin (mouse IgG2b anti- β -actin, cat#3700, Cell Signaling Technology, Danvers, MA) 1:1000 as primary antibody with goat anti-mouse secondary HRP-conjugated immunoglobulin 1:2000 and developed using Enhanced Chemiluminescence (ECL) Western blotting substrate (cat#32106 ThermoFisher Scientific).

3.8 IMMUNOFLUORESCENCE ASSAYS FOR PHAGOSOME MATURATION

MARKERS

Cells (3×10^6), PMA-activated THP-1 and JEG-3 were seeded onto glass coverslips coated with 0.02 mg poly-L-lysine solution (Sigma-Aldrich)/coverslip 20 mm x 20 mm and were infected with *S.Tm* at 10:1 MOI as described above. Cells were fixed at early (5 min) and late (60 min) time points post-infection with freshly prepared 4% paraformaldehyde (PFA, Sigma-Aldrich) for 15 min and then washed three times in PBS. The cells were blocked and permeabilized with PBS containing 5% normal goat serum and 0.3% Triton X-100 for 1 h. The cells were then incubated with primary antibodies overnight at 4°C. Primary antibodies used to stain *S.Tm* were mouse anti-*Salmonella* Typhimurium LPS (cat#GWB-115B3D, GenWay

Biotech, Inc., San Diego, CA). Cells were also incubated with rabbit anti-phagosomal markers: anti-LAMP1 (cat#9091 Cell Signaling Technology, Danvers, MA); anti-Rab5 (cat#3547 Cell Signaling Technology), and anti-Rab7 (cat#9367 Cell signalling Technology) all diluted 1:100 in PBS containing 0.3% Triton X-100. Cells were washed with PBS three times and incubated with Alexa Fluor 488 (Rab5, Rab7, and LAMP1)-coupled goat anti-rabbit and Alexa Fluor 594 (*Salmonella* Typhimurium LPS)-coupled goat anti-rabbit secondary antibodies (1:200) for 1 h. Cells were washed three times with PBS containing 0.03% Triton X-100, stained with 1 µg/mL Hoechst (cat#62249, ThermoFisher Scientific), mounted with fluorescence mounting medium (Dako, Denmark) and imaged using 63x immersion oil confocal microscope (Olympus Fluoview FV1000, Richmond Hill, ON)

3.9 PHAGOSOME KINETICS USING C₁₂FDG BEADS OR C₁₂FDG *S.TM*

Dynabeads M-280 Beads (3 mg) were subjected to a magnetic field for 2 min, liquid was removed, and beads were resuspended in 0.1M NaHCO₃ buffer. Beads were incubated with 100 µg β-galactosidase substrate 5-Dodecanoylamino fluorescein Di-β-D-Galactopyranoside (FDG, cat# D2893 ThermoFisher Scientific), for 2 h and intermittently sonicated in a sonicator bath. *S.Tm*-WT were coated with FDG at 37°C and washed by centrifugation at 1200 x g for 15 min with PBS instead of magnetic separation. For positive control, β-galactosidase (1 µg/ml) (Sigma Aldrich) was added to the beads or bacteria that were coated with FDG for 15 min and acquired by flow cytometry. 10⁶ PMA-activated THP-1 and JEG-3 cells were infected with FDG coated *S.Tm*-WT and extracellular bacteria was removed as previously described or FDG Dynabeads M-280 was added to 10⁶ cells and extracellular beads were removed. At every 15 min up to 1 h, and at 22 h, 1 mL of PBS was added to the cells and cells were scraped using a cell scraper.

Samples were acquired immediately by Flow Cytometry. Flow cytometry was performed on fluorescence-activated cell sorting (FACS) Canto (BD Biosciences) instrumentation and data were analyzed using FlowJo software (FlowJo LLC, Ashland, OR). The gate was fixed on the region that was positive for Alexa 488.

3.10 MRSI ANALYSIS BY RT-PCR

MRSI expression was determined by semi-quantitative polymerase chain reaction (RT-PCR) of cells. Total ribonucleic acid (RNA) was isolated from PMA-activated THP-1 and JEG-3 cells (3×10^6) using the RNeasy Mini Kit (cat#74104 Qiagen, Hilden, Germany). Briefly, cells were washed with PBS and then disrupted in RNeasy lysis buffer (Buffer RLT) containing β -ME. Cells were scraped using a cell scraper and passed through a blunt 20-gauge needle (BD Biosciences) fitted to a syringe 5 times. 70% ethanol (Sigma-Aldrich) was added to the lysate to promote binding of the RNA to the RNeasy membrane. The sample was then applied to the RNeasy Mini spin column and centrifuged for 15 s at 8000 x g. The flow through was discarded. Wash buffer (Buffer RW1) was added to the RNeasy spin column and centrifuged for 15 s at 8000 x g to wash the spin column membrane. Wash buffer for washing membrane-bound RNA (Buffer RPE) was added to the RNeasy spin column and centrifuged for 15 s for 8000 x g to wash the spin column membrane two times. RNA was eluted in RNase-free H₂O by centrifugation for 1 min at 8000 x g. RNA was then quantified using a NanoDrop Spectrophotometer (ThermoFisher Scientific). 500 ng of RNA was reverse transcribed with 0.2 μ g oligo(dT) primer (Invitrogen). Transcription levels of target genes were assayed in triplicate, expression levels was normalized to β -actin and the messenger RNA (mRNA) quantified by $\Delta\Delta$ Ct method. The expression of macrophage scavenger receptor 1 (MSR1) (5'

TCCCCTGGAGAAGTGGTC; CTCCCCGATCACCTTTAAGAC 3') with homosapian-specific primers for β -actin (5' ATGGATGATGATATCGCCGCGC; TGAGGATGCCTCTCTCTTGCTCTGG 3') was evaluated by semi-quantitative PCR. For complementary DNA (cDNA) production, equal amounts of RNA were reverse-transcribed with the following primer pairs for MSR1 and expression at thermocycles 20-30 were compared with β -actin. Either a water control for the PCR or a reaction using RNA without the reverse transcriptase (to check for genomic DNA contamination) were performed as controls.

3.11 PROTEOME ARRAY FOR CYTOKINE EXPRESSION

Cell supernatant from non-infected and *S.Tm*-WT infected (MOI 10:1) cells was collected at 24 h post-infection. Relative levels of 40 different human cytokines/chemokines were detected using Proteome Profiler™ (R&D Systems, Minneapolis, MN). Membranes were incubated with 500 μ L supernatant, washed thrice with wash buffer and treated with Super Signal West Femto Chemiluminiscent detection reagent and imaged using FluorChem IS-8900 (Alpha Innotech) imager. Pixel intensity was calculated using ImageJ®.

3.12 CYTOKINE ANALYSIS BY CYTOMETRIC BEAD ANALYSIS

Culture supernatants (50 μ l) were collected from 5×10^5 human primary macrophages and human primary cTBCs 24h after they were infected with *S.Tm*-WT (MOI 3:1). Supernatants were assayed for cytokines (IL-12p70, TNF, IL-6, and IL-10) using commercially available BD™ Cytometric Bead Array human inflammatory cytokines kit (cat# 551811, BD Biosciences). The Human Inflammation Standards were serially diluted in assay diluent (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256). Human Inflammation Capture Bead suspension (10

µl/test of each) was mixed and 50µl of the mixed beads were transferred to each assay tube. phycoerythrin (PE) Detection Reagent (50 µl) was added to each assay tube. Samples were incubated in the dark at room temperature and then were washed with 1 mL Wash Buffer. Samples were re-suspended in 300 µL Wash Buffer for analysis and were acquired using BD Biosciences LSR Fortessa® Flow Cytometer.

3.13 HUMAN PRIMARY CYTOTROPHOBLAST ISOLATION

Human placentas from healthy women immediately after elected caesarean delivery were collected following written informed consent at The Ottawa General Hospital, Ottawa, Canada, and approved by the Ottawa Hospital Research Ethics Board. Cotyledons (80 g) were removed from the underlying tissue and vessels. Tissues were rinsed 5 times with 0.9% NaCl and were minced with scissors. Minced tissues were rinsed with 0.9% NaCl and transferred to stainless steel strainer with coarse screen to remove as much blood as possible. Minced tissues were then prepared for enzymatic digestion with 0.25% trypsin (Sigma-Aldrich) and 300 U/mL DNase (Sigma-Aldrich) in Hank's Balance Salt Solution (HBSS, Sigma-Aldrich) containing 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma-Aldrich) and placed in a 37°C shaking incubator for 30 min. After the incubation, tissue fragments were allowed to settle; the supernatant was collected and layered on FBS. Digestion of the minced tissues was repeated 3 times. Supernatant from the digestion layered on FBS was centrifuged at 1000 x g. Supernatant was removed and the white layer containing the trophoblast cells was collected and resuspended in Percoll® Density Gradient Media (GE Healthcare, VWR International, Radnor, PA) for gradient centrifugation at 1200 x g for 20 min. The interphase was then collected and cryopreserved until use. Before *in vitro* infection (12 h), the villous mononuclear cells were

thawed and then labelled with W6/32 (anti-human HLA-ABC Purified, cat#14-9983, Affymetrix eBiosciences, San Diego, CA) at 4°C for 30 min and then tagged with anti-mouse IgG microbeads (cat#130-048-401, Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 15 min . Cells were then placed into Stemcell Easysep magnetic separation (Stemcell Technologies, Vancouver, BC) for 5 min at room temperature. The magnet was then inverted and the fraction not adherent to the magnet was poured off and the desired fraction remained in the tube. The desired fraction was then centrifuged at 500 x g for 10 min at 4°C. Purity was assessed by flow cytometry using cytokeratin 7-fluorescein isothiocyanate (FITC) (cat# sc-23876, Santa Cruz Biotech) to identify cTBCs.

3.14 DERIVATION OF MACROPHAGES FROM PERIPHERAL BLOOD

MONONUCLEAR CELLS

For generation of macrophages from monocytes, blood was collected from healthy volunteer donors by phlebotomy (after informed consent under the protocol approved by NRC and Ottawa Hospital Research Institute (OHRI) Research Ethics Board, Ottawa, ON) and diluted 1:1 ratio with 0.9% NaCl in H₂O at the Canadian Blood Services in Vancouver, BC and was air shipped over night. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation over Ficoll®-Hypaque (Sigma-Aldrich) gradient centrifugation from whole blood 900 x g for 30 min with no brake. The upper layer containing the plasma and the platelets were removed. The mononuclear cell layer was transferred to another tube, and the cells were washed in PBS at 3 times the volume of the mononuclear cell layer and centrifuged for 10 min at 400 x g. Supernatant was removed and cells were resuspended in PBS. After 2 washes with PBS, cells were resuspended in R10 medium. CD14⁺ cells were isolated from PBMCs using

MACS Miltenyi Biotech CD14 positive selection kit (cat#130-050-201, Miltenyi Biotec). CD14⁺ cells were magnetically labeled with CD14 MicroBeads and the cell suspension was loaded onto a MACS® Separator column. The magnetically labeled CD14⁺ cells were retained within the column and the unlabeled cells were eluted from the Separator column using a plunger. The CD14⁺ cells were cultured in R10 with 100 ng/mL macrophage colony-stimulating factor (M-CSF, R&D Systems) for 7 days to obtain a macrophage-like morphology. To achieve a population of 5×10^5 macrophages after 7 days in culture, 25×10^5 monocytes were plated per well of a 24 well plate at day 0. Media (R10 with 100 ng/mL M-CSF) was changed at day 2, 4, and 6. Medium with cells in the wells were centrifuged at 500 x g for 10 min at day 2 and 4, and the cells were collected and re-cultured into the plate, whereas on day 6, medium was directly aspirated and fresh R10 with 100 ng/mL M-CSF medium was added.

3.15 PRE-ACTIVATION OF MACROPHAGES AND CTBCS

Human primary macrophages and cTBCs (5×10^5 cells/per well/1 ml) were cultured for 16 h with 0.1 µg/mL LPS (Sigma-Aldrich) in 1 mL R10 before infection to produce pro-caspase-1, pro-IL-1β, and pro-IL-18 transcripts in culture for cell death experiments.

3.16 CYTOTOXICITY ASSAYS

Cells (10^4 /well/ml) were seeded in a 96-well plate overnight and stimulated with 0.1 µg/mL LPS, prior to infection with *S.Tm*-WT. At 1,4, 8 and 24 h post infection, supernatants were collected for lactate dehydrogenase (LDH) release assay (cat#TOX7-1KT, *In vitro* toxicology assay kit, Lactic Dehydrogenase based, Sigma-Aldrich) and the cell lysates were used for Neutral Red uptake assay. For the Neutral Red uptake assay, (cat#TOX4-1KT, *In vitro*

Toxicology Assay Kit, Neutral Red based, Sigma-Aldrich), Neutral Red dye was added to a final concentration of 1% and incubated for 1 h. Cells were then fixed with 0.1% calcium chloride (CaCl_2) in 0.5% formaldehyde and solubilized with 1% acetic acid in 50% ethanol, and absorbance of converted dye was measured at a wavelength of 540 nm for spectrophotometric measurement of viable cells. For LDH release assay, the total LDH was measured to determine the absorbance of non-infected cells for calculations. 1/10 volume of LDH assay lysis solution was added per well to the non-infected wells and the plate was incubated for 45 min at 37°C. The plate was centrifuged at 250 x g for 4 min to pellet debris. The supernatant was transferred to a clean flat-bottom plate for enzymatic analysis. For the infected samples, the plate was centrifuged at 250 x g for 4 min and supernatant collected and transferred to a clean flat-bottom plate. LDH assay mixture was prepared by mixing equal volumes of LDH assay substrate solution, LDH assay dye solution, and 1x LDH assay cofactor preparation. The LDH assay mixture was added to each sample in a volume equal to twice the volume of supernatant removed from the samples to assay. The samples were protected from light and incubated at room temperature for 30 min. The absorbance was measured spectrophotometrically at a wavelength of 490 nm with the background at 690 nm. For both LDH release assay and neutral red uptake assay, percent cell viability was calculated by dividing non-infected by infected absorbancies x 100. In order to elucidate the mechanism of cell death, cTBCs were treated with 20 μM Z-YVAD-FMK (Sigma-Aldrich) 1 h before infection to inhibit caspase-1; 10Mm necrostatin-1, 10 μM dabrafenib, and 10 μM necro-sulfonamide (Sigma-Aldrich) to inhibit necroptosis. Primary human macrophages and cTBCs (10^4 cells/1 ml) in 96 well plates were then infected with live culture of *S.Tm*-WT at a MOI of 3:1 as described above. Supernatant was collected and LDH

release and Neutral red dye uptake was determined to estimate the extent of cell death as described above.

3.17 WESTERN BLOT FOR CELL DEATH MARKERS

Human primary macrophages and cTBCs (5×10^5 cells/ml/well) were infected with live culture of *S.Tm*-WT at MOI of 3:1. Protein from the cell lysate were lysed in RIPA buffer and collected and were quantified by Bradford protein assay. An equal amount of protein sample was denatured with heat and disulphide bonds reduced with β -ME (Sigma-Aldrich). Proteins were then separated on 8% SDS-polyacrylamide gel and the proteins were transferred to a PVDF membrane. The membrane was incubated with primary antibodies 1:1000 dilution against caspase-1 (rabbit anti-caspase-1, cat#2225, Cell Signaling Technology), caspase-3 (rabbit anti-caspase-3, cat#9662, Cell Signaling Technology), caspase-8 (mouse IgG1, anti-caspase-8, cat#9746, Cell Signaling Technology), RIPK-1 (rabbit anti-rip-1, cat#4926, Cell Signaling Technology), phosphorylated-RIPK-1 (rabbit IgG anti-phospho-RIP-1, cat#65746, Cell Signaling Technology) RIPK-3 (rabbit IgG anti-RIP-3, cat#13526, Cell Signaling Technology), MLKL (rabbit IgG anti-MLKL, cat#14993, Cell Signaling Technology), phosphorylated-MLKL (rabbit IgG anti-phospho-MLKL, cat#91689, Cell Signaling Technology), NLRP3 (rabbit IgG anti-NLRP3, cat#13158, Cell Signaling Technology), NLRC4 (rabbit IgG anti-NLRC4, cat#12421, Cell Signaling Technology) and β -actin (mouse IgG2b anti- β -actin, cat#3700, Cell Signaling Technology). For the caspase-1 p10 (rabbit IgG anti-caspase-1 p10, cat#4199, Cell Signaling Technology) subunit released into the supernatant, the supernatant was collected and precipitated with 10% trichloroacetic acid (TCA) (vol/vol) for 1 h on ice. Precipitated proteins were pelleted at 20,000g for 30 min at 4°C, washed with ice-cold acetone, air-dried, resuspended

in SDS-PAGE sample buffer, and heated to 95°C for 10 min. After the primary antibody incubation, membranes were washed 3 times with TBS-0.1% Tween-20. Goat anti-rabbit IgG-HRP secondary antibody (cat#sc-2030, Santa Cruz Biotechnology) or goat anti-mouse IgG-HRP secondary antibody (cat#sc-2031, Santa Cruz Biotechnology) were used. The proteins were visualized by enhanced chemiluminescence. Membranes were stripped and re-probed with anti- β -actin for loading control.

3.18 WESTERN BLOT FOR CELL SIGNALING MARKERS

Human primary macrophages and cTBCs (5×10^5) were infected with *S.Tm*-WT at MOI of 3:1. Protein from cell lysates were collected 0.5, 2, and 4 h after infection and separated on 8% SDS-polyacrylamide gel. The proteins were then transferred to a PVDF membrane that was blocked with 5% BSA and 0.1% Tween-20, for 1 h at room temperature. Primary antibodies for Jak1 (rabbit anti-Jak1, cat#3332, Cell Signaling Technology), p-Jak1 (rabbit anti-phospho-Jak1, cat#3331, Cell Signaling Technology), Tyk2 (rabbit anti-Tyk2, cat#9312, Cell Signaling Technology), p-Tyk2 (rabbit anti-phospho-Tyk2, cat#9321, Cell Signaling Technology), STAT-1 (rabbit anti-STAT-1, cat#9172, Cell Signaling Technology), phosphorylated-STAT-1 (rabbit IgG anti-phospho-STAT-1, cat#9167, Cell Signaling Technology), STAT-3 (mouse IgG2a anti-STAT-3, cat#9139, Cell Signaling Technology), phosphorylated-STAT-3 (rabbit IgG anti-phospho-STAT-3, cat#9145, Cell Signaling Technology) at 1:2000 dilution were used. Goat anti-rabbit IgG-HRP secondary antibody (cat#sc-2030, Santa Cruz Biotechnology) or goat anti-mouse IgG-HRP secondary antibody (cat#sc-2031, Santa Cruz Biotechnology) were used. The proteins were visualized by enhanced chemiluminescence. Membranes were stripped and re-probed with anti-actin for loading control.

3.19 INTRACELLULAR PHOSPHO-STAIN BY FLOW CYTOMETRY

Cells (1×10^6 per 3 ml/well) were seeded overnight onto 35 mm petri dishes before infection. Cells were infected with *S.Tm* at 3 MOI and fixed in 0.01% formaldehyde at 30 min post infection and permeabilized with 0.25% Triton x-100 at 4 h post infection. Non-infected cells served as control. Cells were stained with phospho-STAT-1 (rabbit IgG anti-Phospho-STAT-1, cat#8062, Cell Signaling Technology) and phospho-STAT-3 (rabbit IgG anti-Phospho-STAT-3, cat#7119, Cell Signaling Technology) conjugated with PE. Cells were washed twice in PBS + 2 % BSA (Sigma-Aldrich) and acquired using BD LSR Fortessa® analyzer flow cytometer.

3.20 STATISTICS

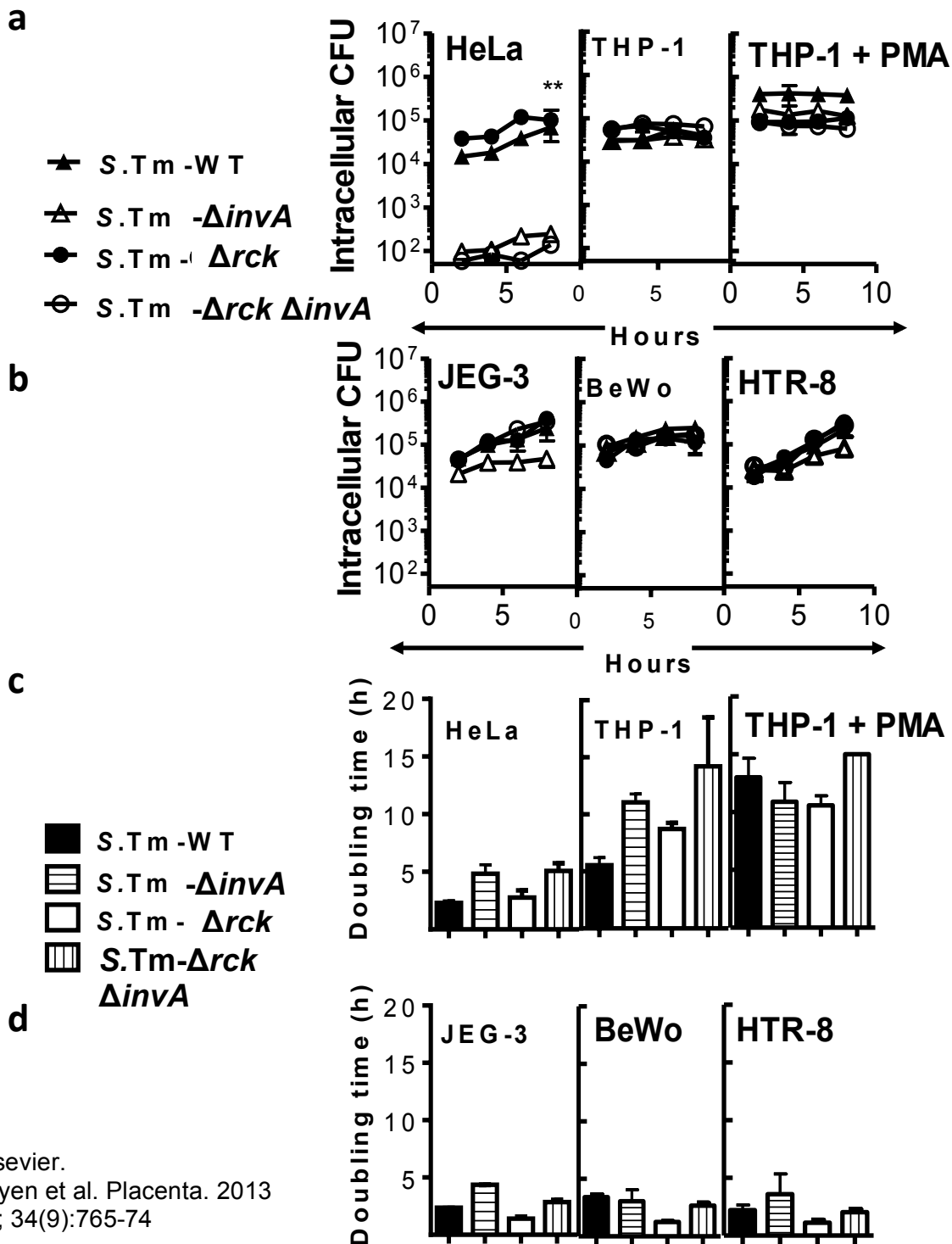
Statistical analyses were performed using GraphPad Prism® software. All results are expressed as means +/- standard deviation (SD). Groups were compared by paired Student's *t*-test or 2-way analysis of variance (ANOVA) as appropriate. A value of $p < 0.05$ was considered significant.

CHAPTER 4: **RESULTS**

4.0 RESULTS

4.1 *SALMONELLA* TYPHIMURIUM PROLIFERATES IN TROPHOBLAST CELLS

The intracellular growth of *S.Tm*-WT and mutant strains (*S.Tm- Δ invA*, *S.Tm- Δ rck*, *S.Tm- Δ rck Δ invA*) was first characterized in various cell lines: HeLa (human uterine epithelial cells), THP-1 (human monocytes), and THP-1 cells differentiated to macrophages with PMA, JEG-3 and BeWo (human placental choriocarcinoma), and HTR-8/SVneo (human extravillous trophoblast cells). Fig. 10a shows infection rates and intracellular bacterial growth in HeLa, and THP-1 cells (non-activated monocytes and PMA activated macrophages). Following exposure, at 2 h, wild-type (WT) bacteria were efficiently internalized within both cell types. In contrast, *S.Tm- Δ invA*, a SPI-1 mutant, lacked the ability to invade epithelial HeLa cells, but was able to gain entry into THP-1 cells, indicating that monocytes/macrophages utilized a TTSS-independent mode of entry such as phagocytosis to internalize bacteria. Enumeration of intracellular bacteria was carried out kinetically at various time points after infection to ascertain replication rate. The doubling time was calculated based on the intracellular CFU at various time points (Fig. 10c). The doubling time of *S.Tm*-WT in HeLa cells was rapid (2 h), compared to THP-1 cells (5 h), whereas in PMA-activated THP-1 cells, *S.Tm*-WT exhibited a much slower doubling time of nearly 13 h, indicating that macrophages were able to curtail rapid intracellular bacterial proliferation. Furthermore, the doubling time of *S.Tm- Δ invA* was similar to *S.Tm*-WT in the THP-1 cells. We next compared the entry and growth of *S.Tm* in trophoblast cells; *S.Tm*-WT efficiently entered JEG-3 cells and unexpectedly, *S.Tm- Δ invA* also efficiently entered JEG-3 cells (Fig. 10b). This suggested that trophoblast cells were similar to macrophages and a TTSS-independent mode of bacterial internalization was occurring. More markedly, *S.Tm*-WT exhibited profound intracellular proliferation within the trophoblast cells, with a doubling time of



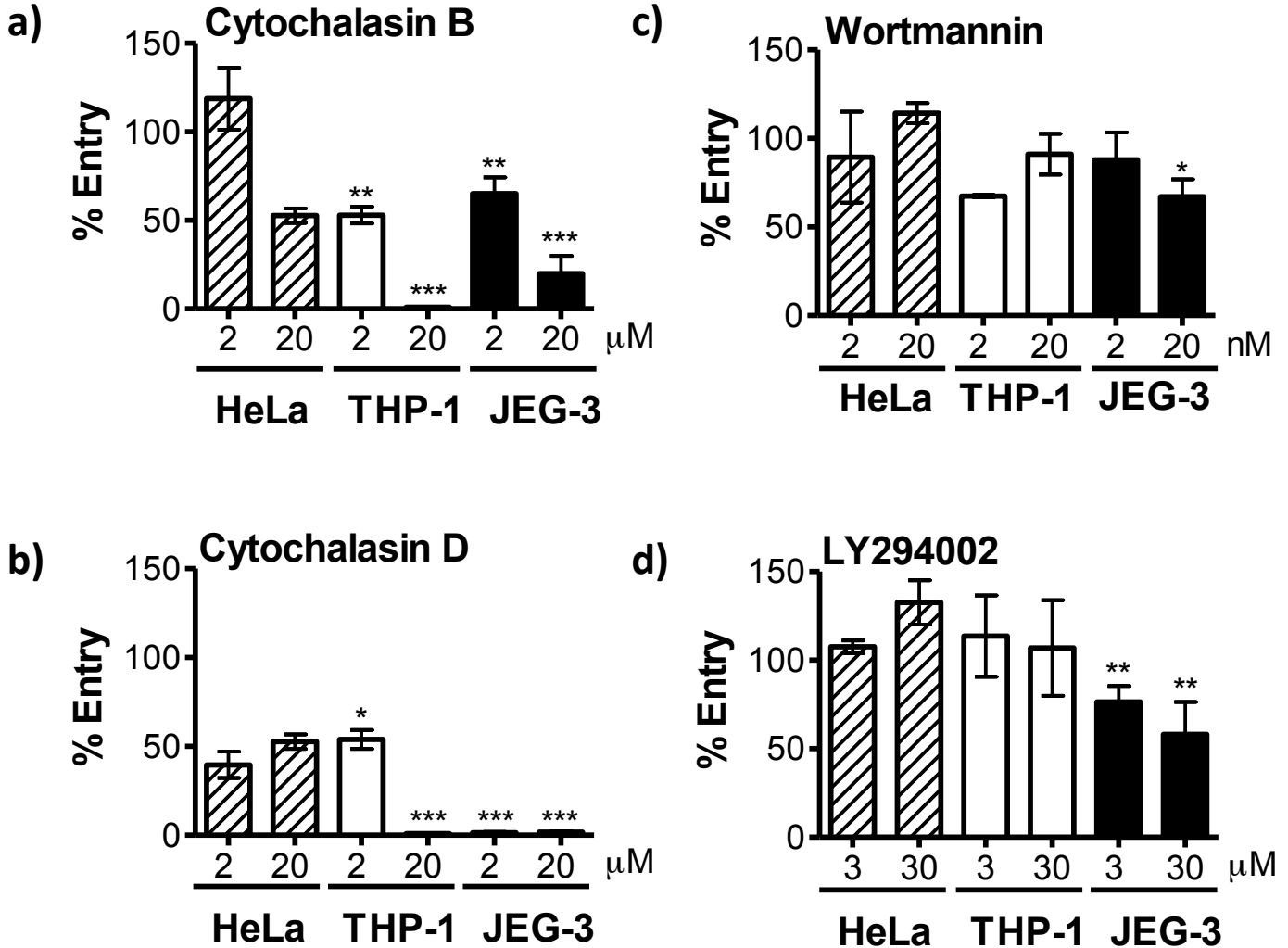
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Figure 10. Intracellular entry and growth in vitro of *S.Tm*. HeLa, THP-1, PMA-activated THP-1 (THP-1 + PMA) (a), JEG-3, BeWo and HTR-8 (b) cells were infected with wild-type *S.Tm* (WT-*S.Tm*), SPI-1 TTSS mutant, *S.Tm*- $\Delta invA$, receptor mediated entry mutant, *S.Tm*- Δrck , and a double mutant, *S.Tm*- $\Delta rck \Delta invA$ at a MOI of 10 and intracellular replication of the bacteria at various times was enumerated. The intracellular bacterial burden at various times (a, b) was determined based on CFU after cell lysis and doubling time was calculated (c, d). Data indicate mean \pm SD of triplicate infected cultures. **, Data in panel (a) for HeLa cells infected with *S.Tm*-WT is significantly different from *S.Tm*- $\Delta rck \Delta invA$ by two-way ANOVA for both bacterial strain ($p < 0.01$) and time ($p < 0.0001$). The data are representative of three independent experiments.

<2 h (Fig. 10d). Both *S.Tm*-WT and *S.Tm-ΔinvA* strains also efficiently entered and proliferated within BeWo and HTR-8 cells (Fig. 10d). Overall, these results indicated that trophoblast cells can internalize *S.Tm* by a TTSS-independent mechanism and provide a supportive environment for profound bacterial proliferation.

4.2 TROPHOBLAST CELLS INTERNALIZE *S.TM* BY SCAVENGER RECEPTOR A MEDIATED ENDOCYTOSIS

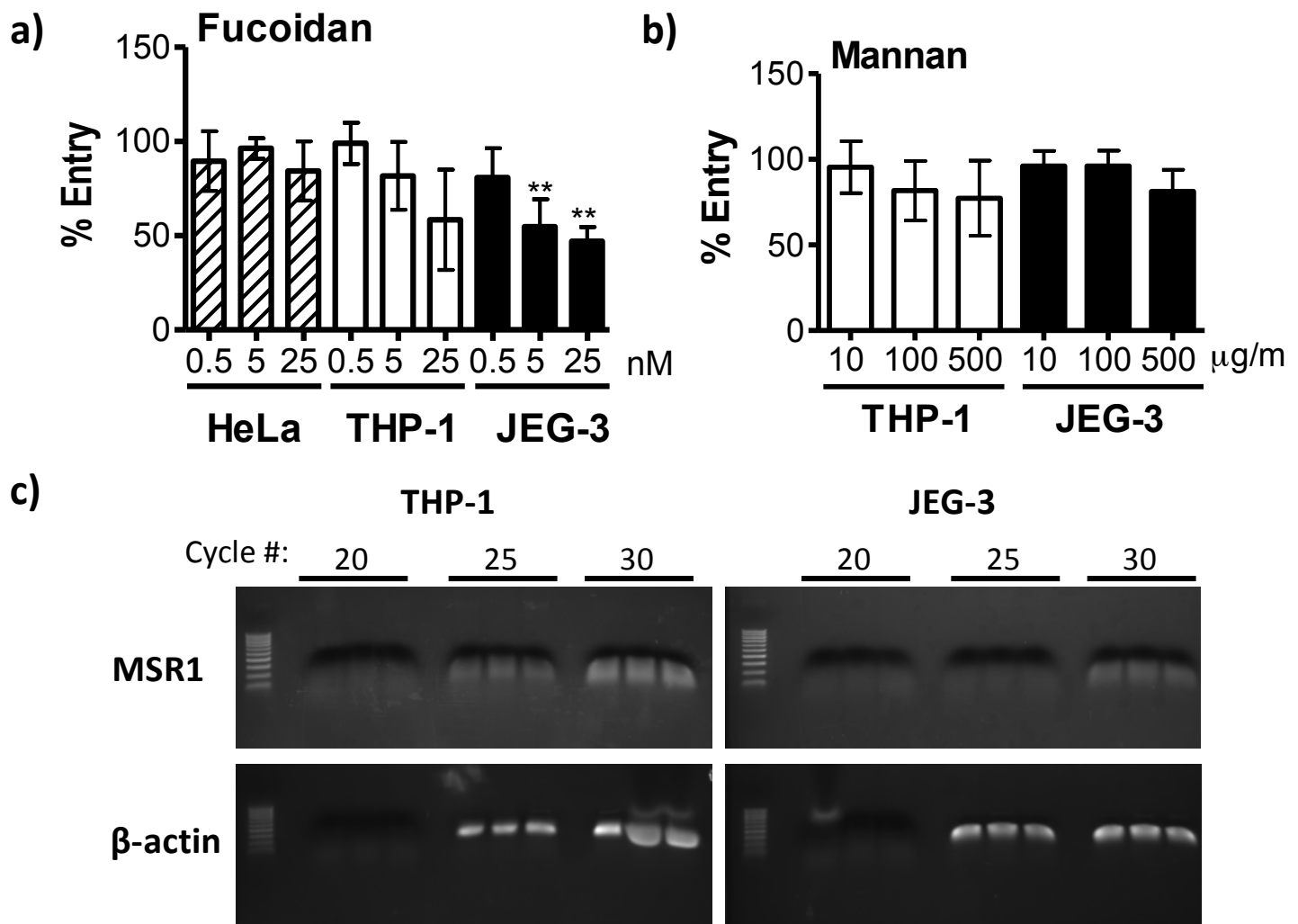
To determine the mechanism of *S.Tm* entry into trophoblast cells, the effect of various inhibitors that block specific pathways of receptor-mediated entry and phagosomal processing was assessed for their ability to block uptake of *S.Tm* by cells. Firstly, cytochalasin B and D, inhibitors of actin filament rearrangement, strongly inhibited the entry of *S.Tm* into PMA-activated THP-1 cells and JEG-3 (Fig. 11a and 11b). At a dose of 20 μ M, almost complete inhibition of *S.Tm* entry into PMA-activated THP-1 and JEG-3 cells was observed, whereas >50% of *S.Tm* entry was still evident in HeLa cells. The high sensitivity of PMA-activated THP-1 and JEG-3 to cytochalasin B and D suggested phagocytosis as a mode of entry of *S.Tm* into these cells. Wortmannin and LY294002, inhibitors of Pi3 kinase activity respectively, blocked entry of *S.Tm* into JEG-3 cells in a dose dependent manner, but had little effect on HeLa cells (Fig. 11c and 11d). Finally, fucoidan, a scavenger receptor inhibitor (Fig. 12a), but not mannan, a mannose receptor inhibitor (Fig. 12b), blocked entry of *S.Tm* into JEG-3. Taken together, it appears that JEG-3 cells, similar to PMA-activated THP-1, internalized *S.Tm* via a scavenger receptor-mediated endocytosis. Both, JEG-3 and PMA-activated THP-1 cells expressed the macrophage scavenger receptor 1 (MSR1) (Fig. 12c). Thus, this receptor may be implicated in facilitating the entry to *S.Tm* into the cells.



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Figure 11. Mechanism of *S.Tm* entry into trophoblast cells. HeLa, PMA-activated THP-1 (THP-1) and JEG-3 cells were treated with inhibitors; cytochalasin B (a), cytochalasin D (b), Wortmannin (c), and LY294002 (d) before infection as described in the methods. The control non-inhibitor treated cultures were treated with DMSO at the same concentration utilized for solubilizing the inhibitors. The number of internalized bacteria was determined at 2 h after exposure of infected cells to high dose gentamycin (to remove extracellular bacteria). The number of bacteria internalized in control cultures was calculated as 100% and the relative percentage of internalized bacteria in inhibitor-treated cultures is shown. Data indicate mean \pm SD of triplicate infected cultures. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.0001$ in comparison to entry of *S.Tm* into HeLa cells in the presence of the same concentration of inhibitor as calculated by Student's t test. The data are representative of three independent experiments conducted.



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Figure 12. Internalization of *S.Tm* by scavenger receptor A mediated endocytosis.

HeLa, PMA-activated THP-1 (THP-1) and JEG-3 cells were treated with inhibitors: fucoïdan (a) or mannan (b) prior to infection pulse of 30 min and the number of internalized bacteria at 2 h after exposure to high gentamycin was enumerated. The number of bacteria internalized in control cultures was calculated as 100% and the relative percentage of internalized bacteria in inhibitor-treated cultures is shown. The expression of MSR1 was evaluated by semi-quantitative PCR (c). Agarose gel (1%) of PCR products is shown in triplicates resulting from amplification of 20, 25, and 30 number of cycles using human-specific primers for MSR-1 (200 bp) and β-actin (200 bp). Directload® PCR 100 bp ladder was used for molecular weight marker. Data indicate mean ± SD of triplicate infected cultures. **, p<0.01 in comparison to entry of *S.Tm* into HeLa cells in the presence of the same concentration of inhibitor as calculated by Student's t test. The data are representative of three independent experiments conducted.

4.3 *SALMONELLA* TYPHIMURIUM THRIVES WITHIN CONTRASTING VACUOLAR NICHE IN MACROPHAGES AND TROPHOBLAST CELLS

Next, the vacuolar biogenesis pathway following entry of *S.Tm* into trophoblast cells by immunofluorescence staining was examined. Cells were infected with *S.Tm*-WT, and fixed after either 5 min (early) or 60 min (late) time points for assessment of intracellular co-localization of *S.Tm* with marker proteins of phagosomal maturation. Fig. 13 is a representative confocal images showing the internalization of *S.Tm*-WT in PMA-activated THP-1 and JEG-3 cells at 60 min post-infection. In PMA-activated THP-1 cells at 60 min, *S.Tm* containing vacuoles exhibited weak Rab5 staining, a marker of early endosome, whereas co-localization of *S.Tm* with Rab7 and to a stronger degree with LAMP-1 containing late endosomal vacuoles was clearly evident (Fig. 13a). In contrast, in JEG-3 cells, *S.Tm* co-localized primarily with Rab5 containing vacuoles, with poor LAMP-1 co-localization even after 60 min post-infection (Fig. 13b). By enumerating 100 fluorescent intracellular bacteria and their co-association with various markers of the endocytic pathway, the percentage of *S.Tm* containing vacuoles that also expressed specific endosomal proteins was quantified. Based on such analysis, it was deduced that in PMA-activated THP-1 macrophages, early on after infection (at 5 min), ~80% of early endosomal vacuoles expressing Rab5 also co-localized with *S.Tm* and this was reduced to <50% by 60 min. Furthermore, in accordance with phagosome maturation, by 60 min of infection, 75% of *S.Tm* containing vacuoles exhibited the expression of LAMP-1 (Fig. 13c). In contrast, in JEG-3 cells, early on after infection only ~15% of *S.Tm* containing vacuoles expressed Rab5 and this increased to ~75% by 60 min post-infection. Very little co-localization of *S.Tm* with LAMP-1 was seen in JEG-3 cells even after 60 min post-infection. These data suggest that *S.Tm* is

localized in contrasting early and late vacuoles within JEG-3 and PMA-activated THP-1 cells respectively.

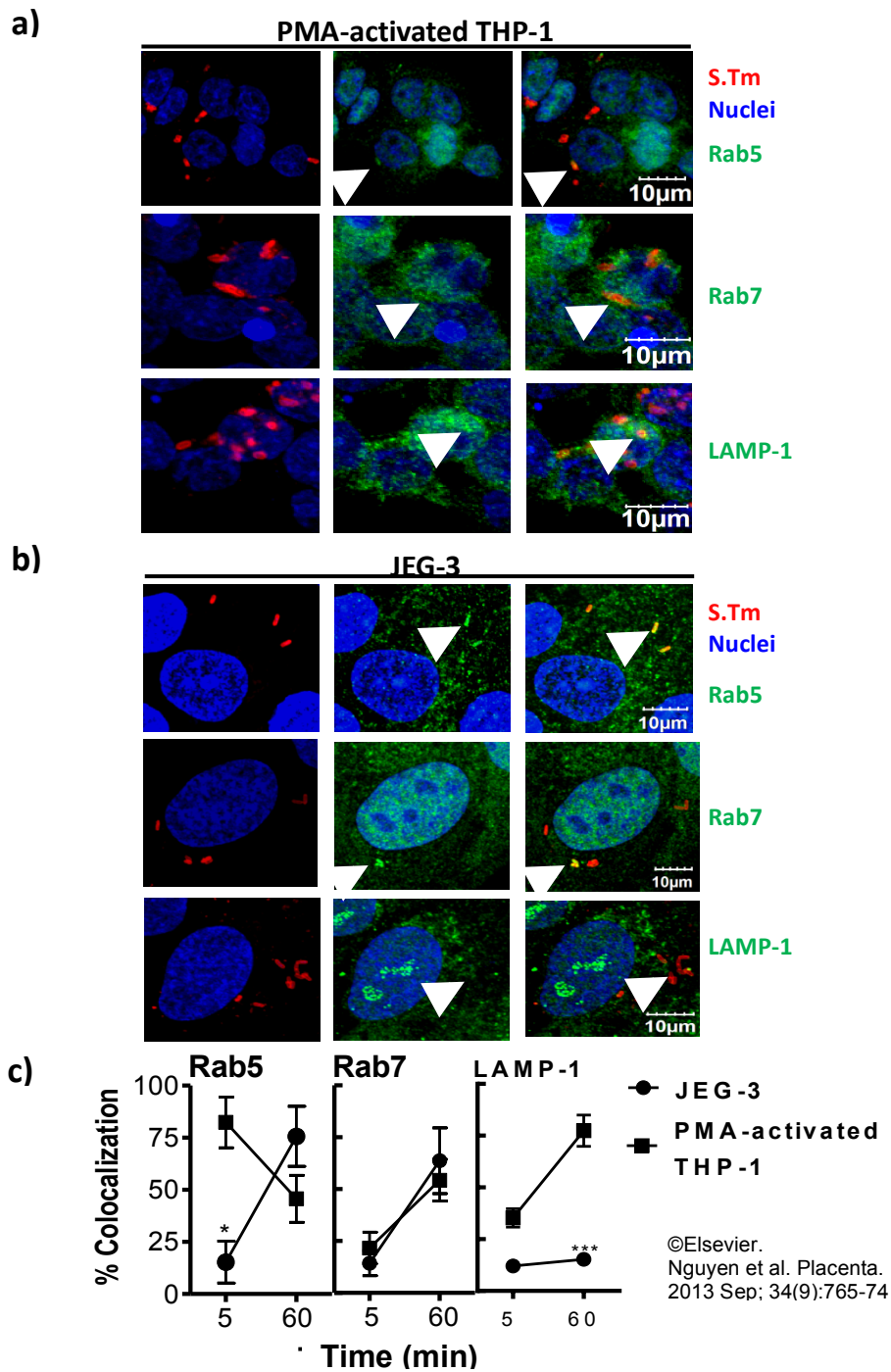


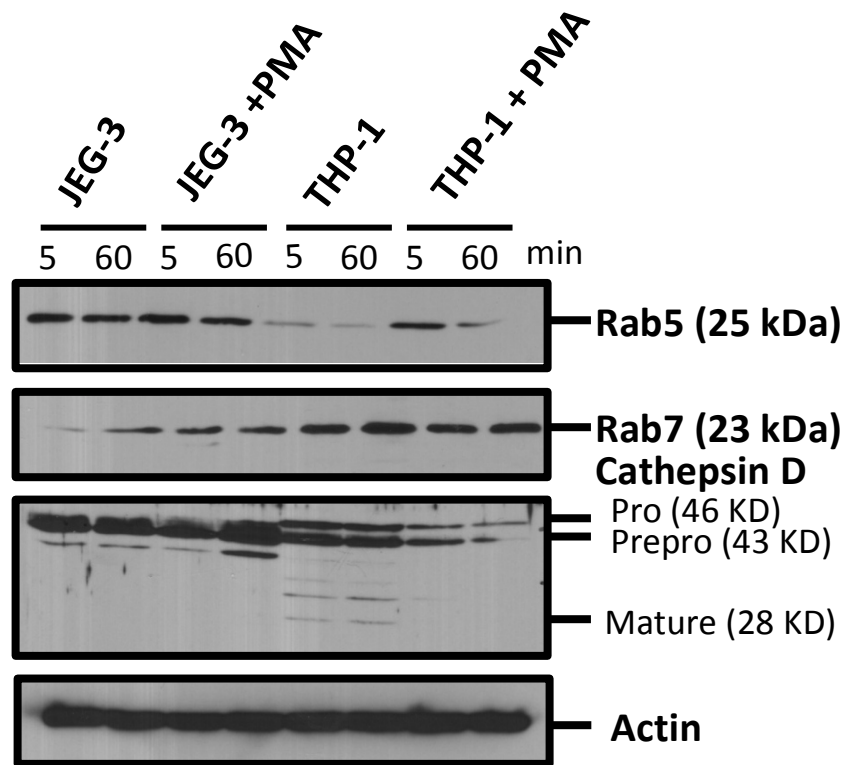
Figure 13. Characterization of *S.Tm* containing vacuoles by confocal microscopy.

Monolayers of PMA-activated THP-1 (a) and JEG-3 (b) were infected with *S.Tm*-WT (10 MOI) and the immunofluorescence for endosomal proteins and *S.Tm* lipopolysaccharide (*S.Tm*-LPS) was carried out by confocal microscopy. Representative confocal image at 60 min post-*S.Tm* infection of THP-1 and JEG-3 cells indicating the staining of the endosomal markers Rab5, Rab7, and LAMP-1 (green) with *S.Tm*-LPS (red), and nuclei (blue). Similar images were analyzed for 5 min as well. Enumeration of 100 intracellular *S.Tm* with the specific endosomal marker at 5 and 60 min post-infection based on overlapping yellow staining was used to determine % co-localization (c) for both cell types. Composite data (mean +/- SD) quantified from images produced in 3 different experiments. ***, $p < 0.0001$ for both cell type and time for expression of LAMP-1; *, $p < 0.01$ for expression of Rab5 at 5 minutes in JEG-3 compared to THP-1 cells by two way ANOVA. Data are representative of 3 independent experiments.

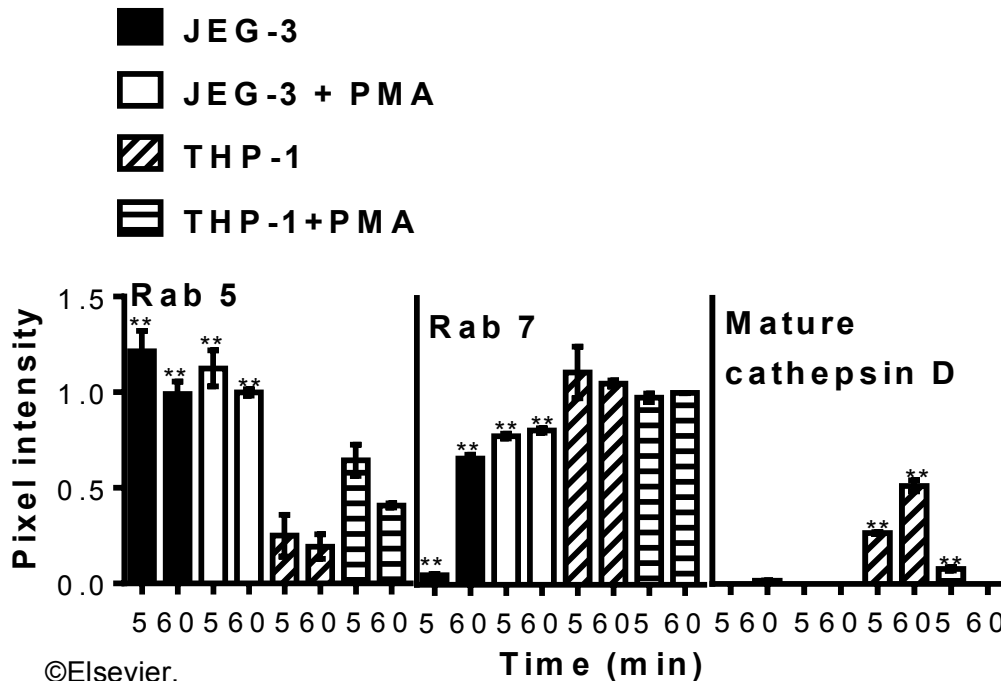
4.4 *SALMONELLA* TYPHIMURIUM INHIBITS PHAGOSOMAL MATURATION IN TROPHOBLAST CELLS

An alternate approach to study phagosomal maturation following *S.Tm* infection included studying the expression of proteins in isolated phagosomes by Western blotting. Phagosomes isolated from JEG-3 cells infected with *S.Tm*-WT exhibited strong expression of Rab5 at 5 and 60 min (Fig. 14a). Similar levels of expression of Rab5 at 60 min was also seen when JEG-3 cells were activated with PMA prior to infection. In contrast, phagosomes isolated from PMA-activated THP-1 cells infected with *S.Tm* expressed Rab5 only at 5 min post-infection, whereas there was more co-localization with the intermediate endosomal protein Rab7. Finally, the phagosomes of *S.Tm*-WT-infected JEG-3 cells expressed primarily pro-cathepsin D at both 5 and 60 min post infection. Activation of JEG-3 cells with PMA did not result in cleavage of pro-cathepsin D to its active form. In contrast, THP-1 cells phagosomes showed degradation of pro-cathepsin D to its active form, suggesting progression to phagolysosome formation. THP-1 cells that were activated showed diminished expression of pro-cathepsin D and active cathepsin D could not be detected. The quantification of these results confirmed that phagosomal maturation is delayed in trophoblast cells following *S.Tm* infection and this could not be rescued by pre-activation with PMA (Fig. 14b).

a)



b)



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Figure 14. Western blot analysis of isolated phagosomes from *S.Tm* infected cells.

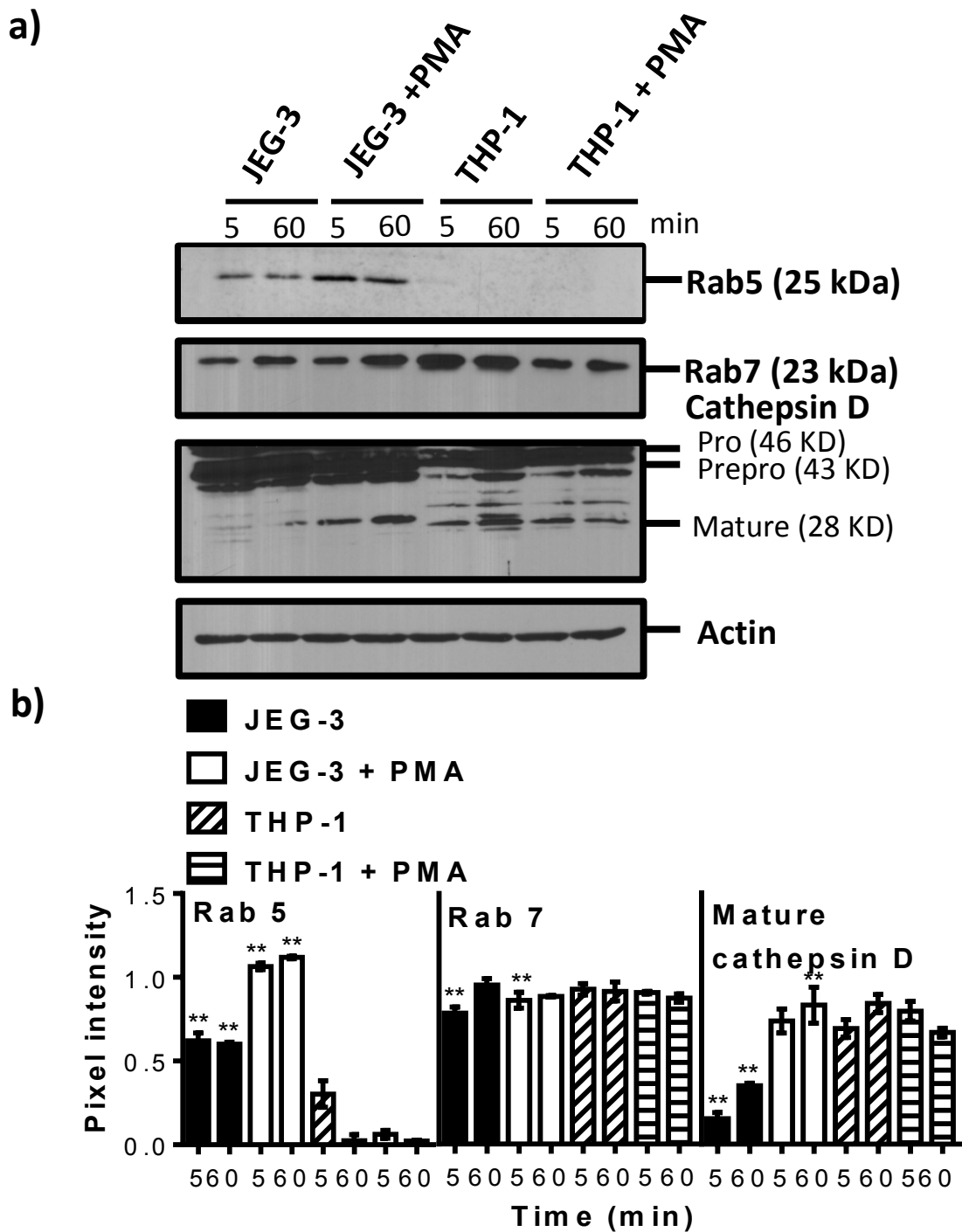
Cells were infected with 10 MOI of *S.Tm*-WT and phagosomes were isolated at 5 and 60 min post infection and equal amount of phagosomal protein was loaded into each well. The cell lysate was used as a loading control for actin. The expression of Rab5, Rab7, and cathepsin D in JEG-3 and THP-1 cells is indicated (a). Cells were also exposed to PMA as described in the materials and methods. Western blots were quantified based on intensity using ImageJ normalized to actin and data from 3 independent experiments were pooled and graphed (b). **, $p < 0.01$ by Student *t* test for JEG-3 cells in comparison to THP-1 + PMA cells at the specific time indicated. Data are representative of 3 independent experiments conducted.

4.5 TROPHOBLAST CELLS INHERENTLY EXHIBIT WEAK PHAGOSOMAL MATURATION PROCESS

S.Tm can manipulate the phagosomes maturation process consequently reducing the acquisition of cathepsin D by employing its SPI2-TTSS. Therefore; I next addressed the inherent ability of trophoblast cells to phagocytose inert beads and elucidated the phagosomal maturation process. The expression of endosomal proteins in isolated phagosomes by Western blot following uptake of beads demonstrated expression of Rab5 in JEG-3 cells (Fig. 15a). In contrast, phagosomes from PMA-activated THP-1 cells lacked expression of this early endosomal marker. The active form of cathepsin D which is seen in late phagosomes after cleavage from pro-cathepsin D was evident in the phagosomes of PMA-activated THP-1 cells but not JEG-3 cells. However, when JEG-3 cells were activated with PMA prior to uptake of beads, cleavage of procathepsin D to its active form was evident. The quantification of the Western blot data reiterates the reduced ability of JEG-3 cells to mature phagosomes towards acquisition of cathepsin D in the absence of pre-activation with PMA (Fig. 15b). Additionally, even pre-activation of JEG-3 cells with PMA was not sufficient to promote phagosomal maturation following *S.Tm*-WT infection (Fig. 14). Thus, it appears that trophoblast cells are inherently less competent in activating the phagosomal maturation machinery.

4.6 TROPHOBLAST CELLS FAIL TO ACHIEVE PHAGOSOMAL-LYSOSOMAL FUSION

A novel methodology was utilized to study phagosomal-lysosomal fusion following incubation with tosyl-activated beads-coated with fluorescein C₁₂-di-β-D-galactopyranoside



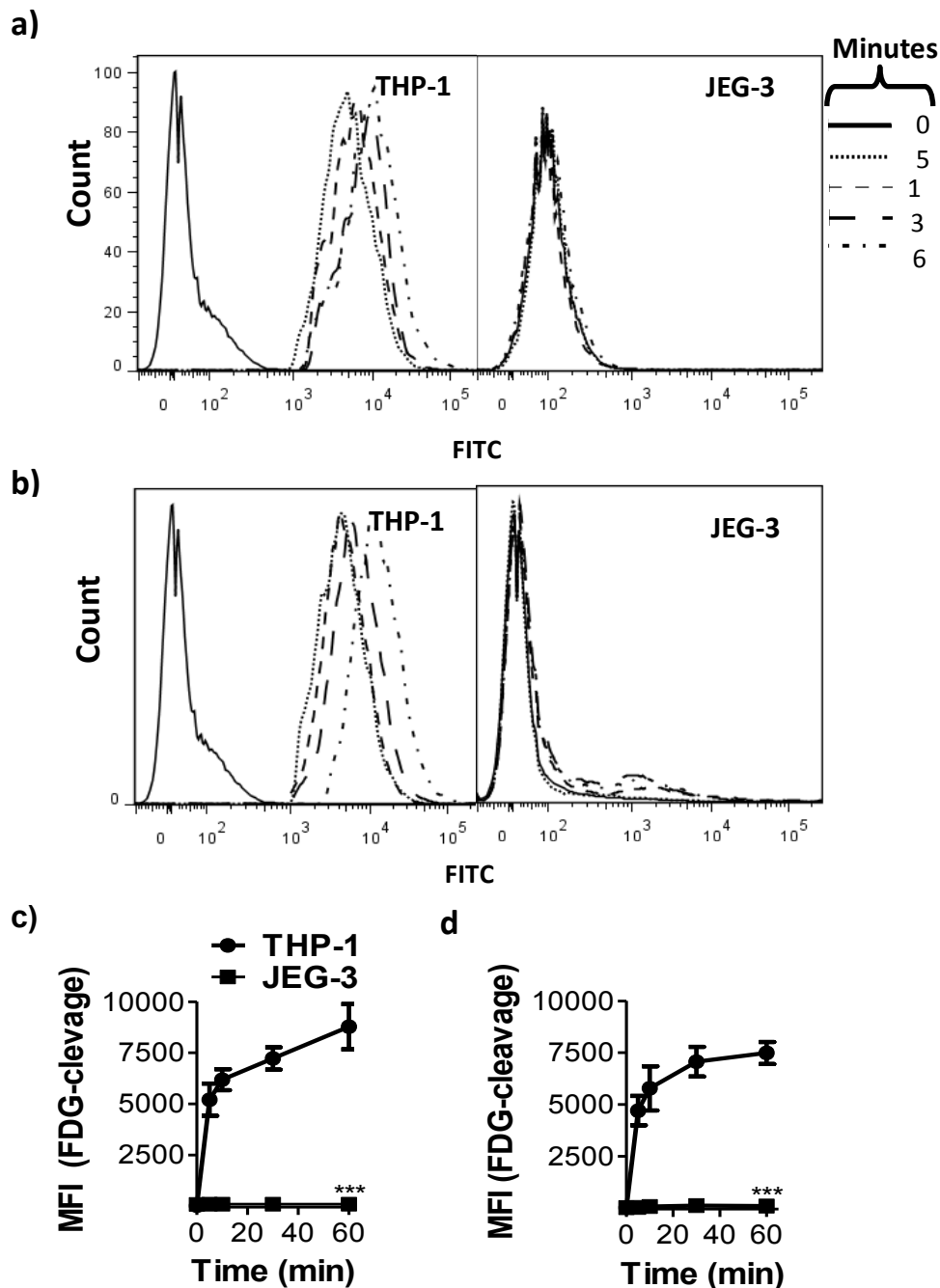
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Figure 15. Western blot analysis of isolated phagosomes from cells incubated with beads. Cells were allowed to internalize inert beads and phagosomes were isolated after 5 and 60 min. Equal amount of phagosomal protein was loaded into each well and the cell lysate was used as a protein loading control for actin. The expression of Rab5, Rab7, and cathepsin D in JEG-3 and THP-1 cells is indicated (a). Cells were also exposed to PMA as described in the materials and methods. Western blots were quantified based on intensity using ImageJ normalized to actin and data from 3 independent experiments were pooled and graphed (b). **, $p < 0.01$ by Student t test for JEG-3 cells in comparison to THP-1+ PMA cells at the specific time indicated. Data are representative of 3 independent experiments conducted.

(FDG) or infection with FDG coated *S.Tm*. FDG is a self-quenched non-fluorescent molecule that is hydrolyzed by β -galactosidase (β -gal) found in the lysosomes. As a result, fluorescein is formed only upon phagosomal-lysosomal fusion, the ultimate event in phagosomal maturation. The flow cytometric profile of cells at various time points after internalization of C₁₂FDG beads or *S.Tm* is shown in Fig. 16a and 16b. Based on the increase in mean fluorescent intensity (MFI), phagosomal maturation in PMA-activated THP-1 cells following uptake of beads or FDG coated *S.Tm*-WT occurred rapidly (Fig. 16c and 16d). In contrast, JEG-3 cells did not exhibit increased MFI even after 17 h of infection with *S.Tm*-WT or uptake of beads coated with FDG, suggesting lack of phagolysosomal fusion (Fig. 16b and 16d). These results corroborate previous observations of significantly limited phagosomal maturation in trophoblast cells.

4.7 ISOLATION OF HUMAN PRIMARY CYTOTROPHOBLAST CELLS LEAD TO CULTURES WITH OVER 95% PURITY

Choriocarcinoma cell lines constitute a convenient model to study trophoblast infection by *S.Tm*. To confirm that human primary trophoblast cells were also permissive to *S.Tm* infection, Human trophoblast cells were purified from term placentas by enzymatic dissociation of villous placental tissue, followed by gradient centrifugation and immunomagnetic bead purification. Purity was assessed by flow cytometry using cytokeratin 7 to identify cTBCs (Fig. 17a). Generally, the cell preparations contained over 95% cTBCs. Macrophages differentiated from monocytes of human peripheral blood mononuclear cell (PBMC) was studied in comparison.



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Figure 16. Flow cytometric analysis of phagolysosome fusion. THP-1 + PMA and JEG-3 cells were infected with FDG coated *S.Tm* or incubated with FDG coated beads. Cells were fixed after 5, 10, 30, or 60 min after incubation with beads or *S.Tm* and acquired by flow cytometry to measure the release of green fluorescence which was correlated to phagosome-lysosome formation. Data were analyzed by FlowJo. Representative histogram plot showing fluorescence intensity of cells prior to and at various times after internalization of *S.Tm* or beads is indicated (a *S.Tm*, b beads). Mean +/- SD of MFI from three independent experiments of *S.Tm* or beads (c *S.Tm*, d beads). ***, $p < 0.001$ by two-way ANOVA for JEG-3 cells over time in comparison to THP-1 cells.

4.8 *SALMONELLA* TYPHIMURIUM PROLIFERATES IN HUMAN PRIMARY CYTOTROPHOBLAST CELLS

Next, I characterized the intracellular growth of *S.Tm*-WT in isolated human term primary cTBCs in comparison to primary monocyte-derived macrophages was characterized. Cells were infected *in vitro* with a live culture of *S.Tm*-WT at a MOI of 3:1; after a 30 min pulse of infection, media were changed and gentamycin added to remove extracellular bacteria. In cTBCs, from 2 to 10 h post-infection, there was ~ 10-fold increase in number of intracellular *S.Tm*, resulting in a doubling time of approximately 1.5 h (Fig. 17b). In contrast, the doubling time was approximately 10 h in human primary monocyte-derived macrophages (Fig. 17b). These results indicate that *S.Tm* productively infects and proliferates efficiently within human primary term cTBCs, whereas intracellular bacterial replication is curbed in human primary macrophages.

4.9 *SALMONELLA* TYPHIMURIUM INDUCES HUMAN PRIMARY TERM CTBC DEATH

Profound intracellular *S.Tm* replication can ultimately evoke lysis of infected cells by various inflammatory cell death mechanisms. I therefore sought to characterize *S.Tm*-WT-infection induced cell death in trophoblast cells. At 4 h post-infection, ~40% cell death was observed by neutral red release assay amongst the cTBC population infected with *S.Tm*-WT. In contrast, the extent of cell death in *S.Tm* infected macrophages was <20 % (Fig. 18a). Even at 8 h post-infection, only ~25% of infected macrophages succumbed to *S.Tm* induced cell death. By this time point of infection, >50% of cTBCs succumbed to cell death. Similar results were

observed using LDH release assay (Fig. 18b). These results indicate the rapid vulnerability of primary cTBCs to *S.Tm*-WT intracellular infection.

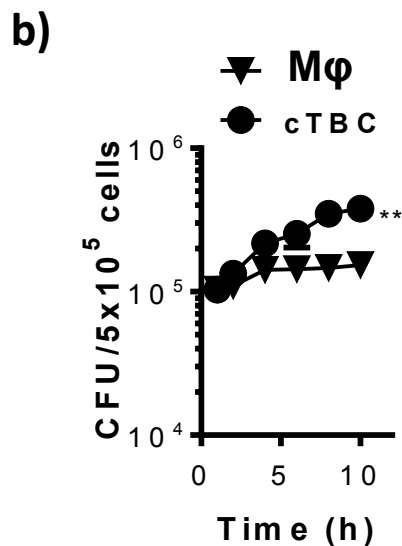
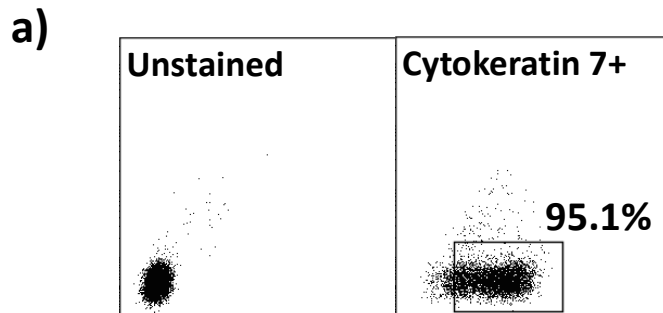
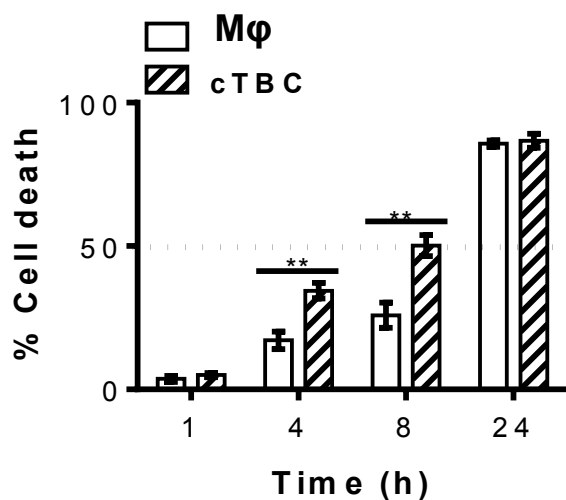


Figure 17. Salmonella Typhimurium proliferates in human primary cytotrophoblast cells. Cytotrophoblast purity from isolated trophoblast cells (a). Primary human cytotrophoblast cells (cTBCs) were isolated from term placentas by enzymatic digestion, gradient density centrifugation and subsequent negative immunoselection using MHC-class I antibody-coated magnetic beads. Purity of the cTBCs was assessed by flow cytometry using antibody to cytokeratin-7. Intracellular *S.Tm* growth kinetics of infected purified cTBCs (b). Human primary macrophages (mφ) and cTBCs were cultured in a 24 well plate at a cell density of 5×10^5 . Cells were exposed to 5×10^6 *S.Tm* and exposed to gentamicin to kill extracellular bacteria after infection period. The rates of intracellular bacterial proliferation were enumerated by plating cell lysates. Data indicate mean \pm SD of triplicate infected cultures. **, $p < 0.01$ by two-way ANOVA Data in panel (b) for cTBCs infected with *S.T* is significantly different overtime in comparison to THP-1 cells. The data are representative three independent experiments.

a) Neutral Red Assay



b) LDH Release Assay

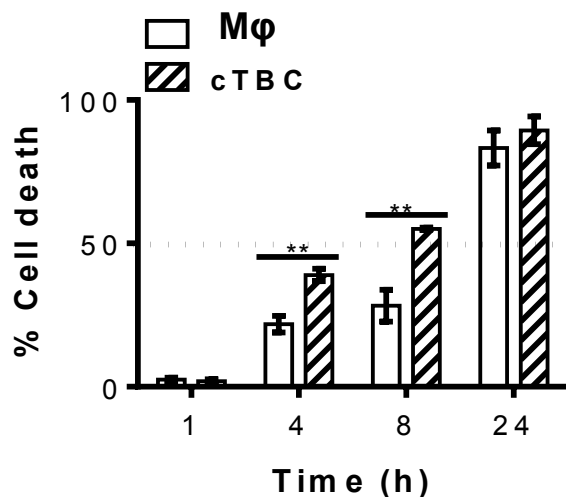


Figure 18. The extent of cell death in *S.Tm*-infected human primary term cTBCs. The extent of death in *S.Tm* infected human primary term cTBCs and human primary mφ derived from monocytes of PBMC was determined by neutral red assay (a) and LDH release assay (b) at 1, 4, 8, and 24 hours post-infection. The absorbance at a wavelength of 540 nm of non-infected cells was calculated as 0% death and the relative percentage of cell death post *S.Tm*-infection is shown. Each experiment was carried out 4 times using different placentas, and statistical significance was determined by **, $p < 0.01$ by Student *t* test for cTBCs in comparison to mφ cells at the specific time indicated.

4.10 CYTOTROPHOBLAST CELLS INFECTED WITH *S.TM* DO NOT UNDERGO AN APOPTOTIC FORM OF CELL DEATH

Cell death may occur by various mechanisms, some of which are more damaging to the surrounding host tissue due to release of various inflammatory and toxic signals.

Morphologically, apoptotic cells initially maintain plasma membrane integrity and do not rapidly release their intracellular contents. Before apoptotic cells disintegrate, they are usually ingested by resident phagocytes. Thus, this form of cell death is quiescent with minimal collateral damage to surrounding tissue. Caspases are cysteine proteases that are known to function as key executors of apoptotic cell death. Caspase-8, the initiator caspase acts by cleaving and activating the downstream executioner, caspase-3. The target, PARP, is cleaved by caspase-3. Therefore, active caspase-3, -8 and cleaved PARP serve as markers for apoptosis. Following *S.Tm*-WT infection of primary macrophages and cTBCs, the expression of pro-caspase-8 and -3, and uncleaved PARP was observed, however, there was no expression of the active form of caspases or cleaved PARP (Fig. 19). This suggested that *S.Tm* infected cTBCs and macrophages do not succumb to apoptosis, but to another cell death pathway.

4.11 CYTOTROPHOBLAST CELLS INFECTED WITH *S.TM* UNDERGO A NECROPTOTIC FORM OF CELL DEATH

Cell death that occurs through inflammatory pathways such as pyroptosis or necroptosis can induce severe collateral damage resulting in loss of integrity of the surrounding tissue.

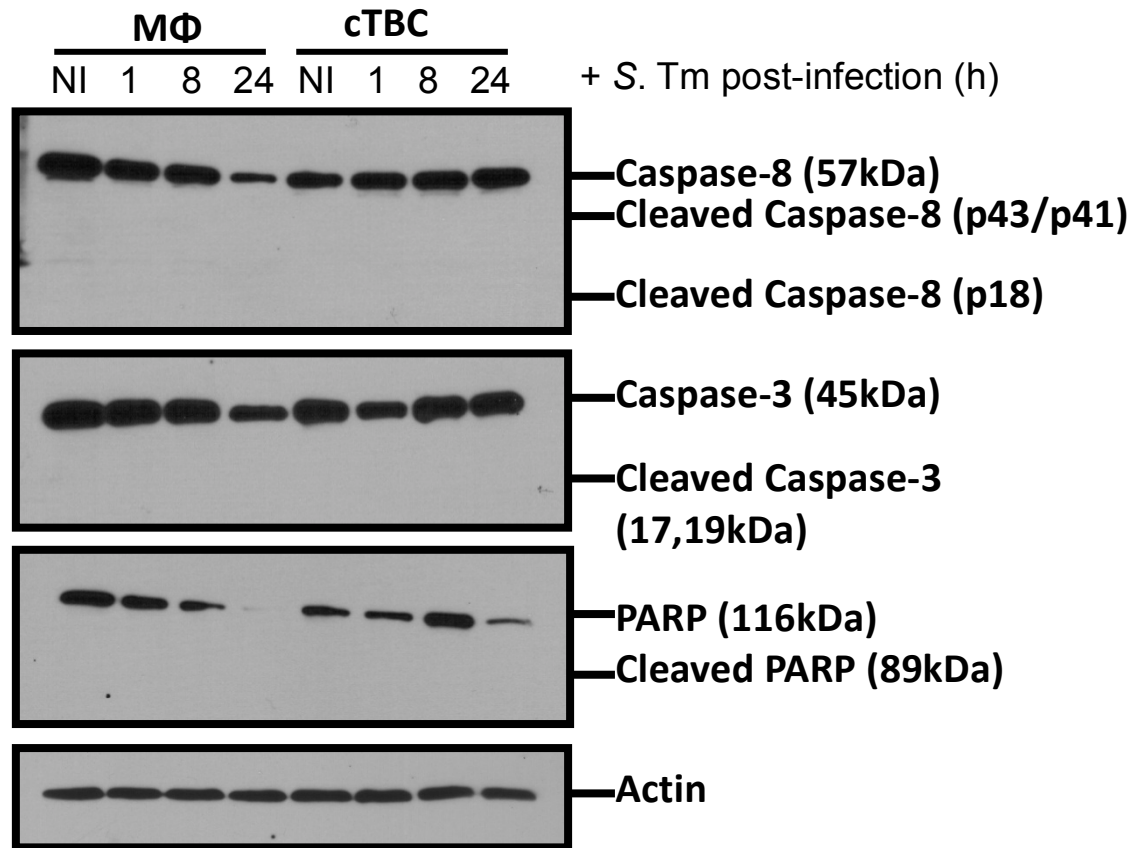
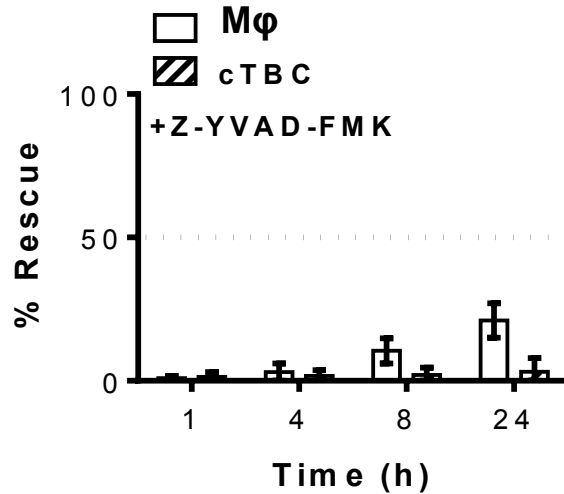


Figure 19. Apoptosis marker expressions of *S.Tm*-infected human primary term cytotrophoblast cells. Protein expression of markers of apoptosis (Caspase-8, Caspase-3, and PARP) by Western blot. Human primary mφ and term cTBC cultures were infected with *S.Tm*. Whole cell extracts at 1, 8, and 24 hours post infection and non-infected (NI) samples were prepared for Western blot analysis and an equal amount of protein was loaded into each well. The data are representative three independent experiments.

Induction of cell death by such inflammatory pathways in cells infected with *S.Tm* may contribute significantly to the pathogenesis in the host. In order to elucidate the pathway of inflammatory cell death, I treated cells in culture with various inhibitors of pyroptosis and necroptosis and determined the percent cell death using neutral red assay and LDH release assay. Figure 20 shows the % rescue from both the neutral red assay and the LDH release assay in the presence of a pyroptosis inhibitor. Z-YVAD-FMK, an inhibitor of caspase-1 in the pyroptosis pathway, resulted in approximately 20% inhibition of the death of macrophages at 24 h post infection and no inhibition of death in cTBCs when assessed by neutral red uptake assay (Fig. 20a) and LDH release assay (Fig. 20b).

Inhibition of necroptosis was achieved by using three inhibitors: necrostatin-1, which binds RIP1 kinase and stabilizes its inactive conformation; necrosulfonamide, an inhibitor of MLKL-RIP1-RIP3 necrosome complex formation, and debrafenib, a RIP-3 inhibitor. At 4 h post-infection, all three necroptosis inhibitors failed to rescue macrophages from cell death (Fig.21), whereas 20% of cTBCs escaped cell death (Fig. 21). However, at 24 h post *S.Tm*-WT infection, approximately 50% of the cells, macrophages and cTBCs, were rescued. Overall, these results indicate that cell death in cTBCs could be averted even at earlier time points through inhibition of necroptosis. Necrostatin-1, debrafenib, and necro-sulfonamide rescued cTBCs at early time points of infection indicating that cTBCs die of necroptosis at early time points post *S.Tm* infection.

a) Neutral Red Assay



b) LDH Release Assay

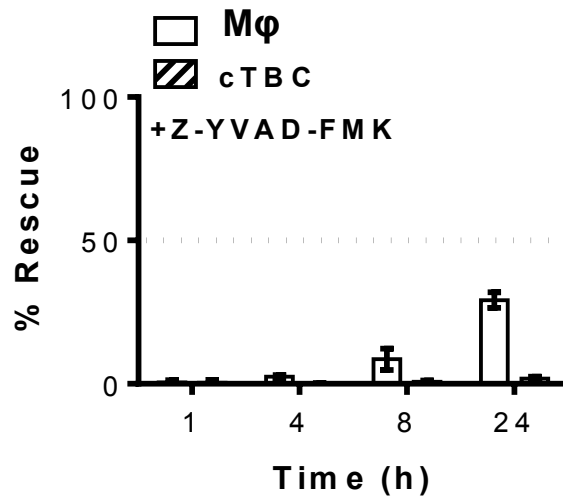
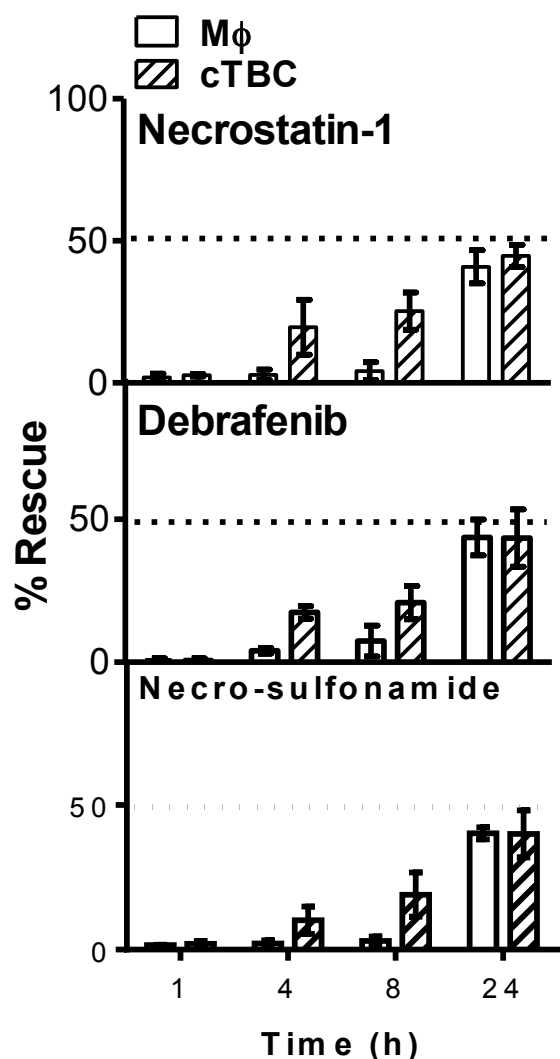


Figure 20. The extent of cell death in *S.Tm*-infected human primary cells in the presence of a pyroptosis inhibitor. Human primary m ϕ and term cTBC cultures were treated with an inhibitor of pyroptosis (Z-YVAD-FMK) and then infected with *S.Tm*. At 1, 4, 8, and 24 hours post-infection, the relative percent rescue was calculated based on *S.Tm*-infected treated cells and *S.Tm*-infected non-treated cells, and the absorbance at a wavelength of 540 nm from neutral red assay (a) and LDH release assay (b). The relative percent rescue is shown. Each experiment was carried out 4 times, and statistical significance was determined by the ANOVA test.

a) Neutral Red Assay



b) LDH Release Assay

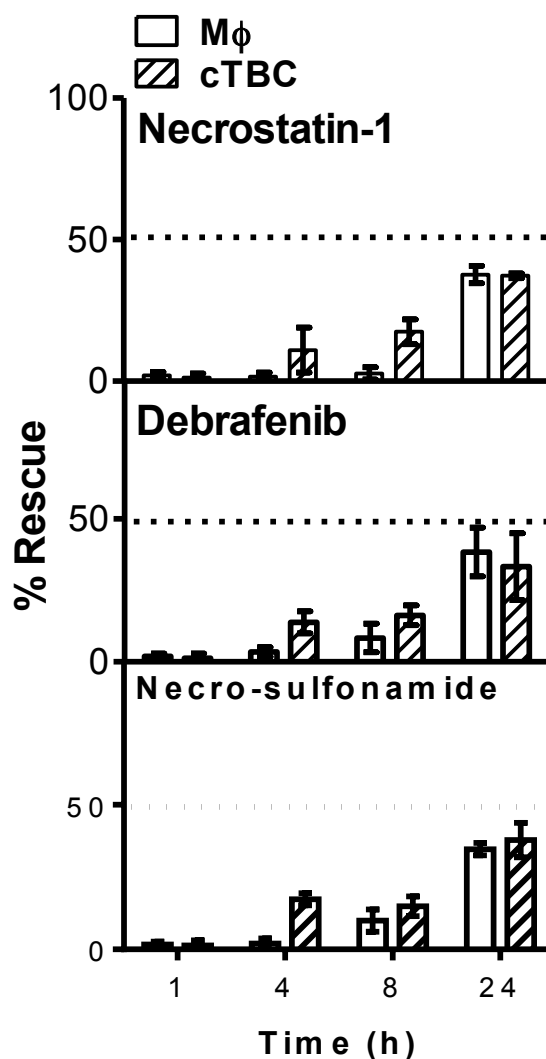


Figure 21. The extent of cell death in *S.Tm*-infected human primary in the presence of necroptosis inhibitors. Human primary mφ and term cTBC cultures were treated with inhibitors of necroptosis (Necrostatin-1, Debrafenib and Necro-sulfonamide) and then infected with *S.Tm*. At 1, 4, 8, and 24 hours post-infection, the relative percent rescue was calculated based on *S.Tm*-infected treated cells and *S.Tm*-infected non-treated cells, and the absorbance at a wavelength of 540 nm from neutral red assay (a) and LDH release assay (b). The relative percent rescue is shown. Each experiment was carried out 4 times, each with different placenta, and statistical significance was determined by the ANOVA test.

4.12 *SALMONELLA* TYPHIMURIUM INFECTED CYTOTROPHOBLAST CELL DEATH IS NON-PYROPTOTIC

Intracellular pathogens can be recognized by cell intrinsic receptors such as the NLRs which recruit caspase-1 to an intracellular multi-protein PRR complex known as the inflammasome. Activated caspase-1 processes pro-IL-1 β and pro-IL-18 into their biologically active secreted forms, resulting in recruitment of other inflammatory leucocytes that can clear the pathogen. Human macrophages expressed both nod-like receptors, NLRP3 and NLRC4, while cTBCs lacked expression of NLRP3, but expressed NLRC4 (Fig. 22a) Expression of the p20 form of caspase-1 was observed at 1, 8 and 24 h post infection in macrophages but not in cTBCs. The quantification of these results confirmed that *S.Tm*-infected cytotrophoblast cells do not succumb to pyroptosis (Fig. 22b). Furthermore, macrophages secreted copious amounts of IL-1 β following *S.Tm*-WT infection, whereas this was significantly lower in cTBCs (Fig.22c). These results indicate that while macrophages infected with *S.Tm* assemble and activate the pyroptosis machinery, cTBCs do not undergo this form of cell death.

4.13 CYTOTROPHOBLAST CELLS EXPRESS PHOSPHORYLATED-RIP1 AND PHOSPHORYLATED-MLKL

Necroptosis is a recently delineated cell death pathway that is caspase-independent, unlike apoptosis, but has an inflammatory outcome and exhibits a unique signaling pathway that requires the involvement of receptors RIPK-1 and RIPK-3. MLKL interacts with RIPK-3 and is essential to induce necroptosis. To map the mechanism of cell death in cTBCs, I collected

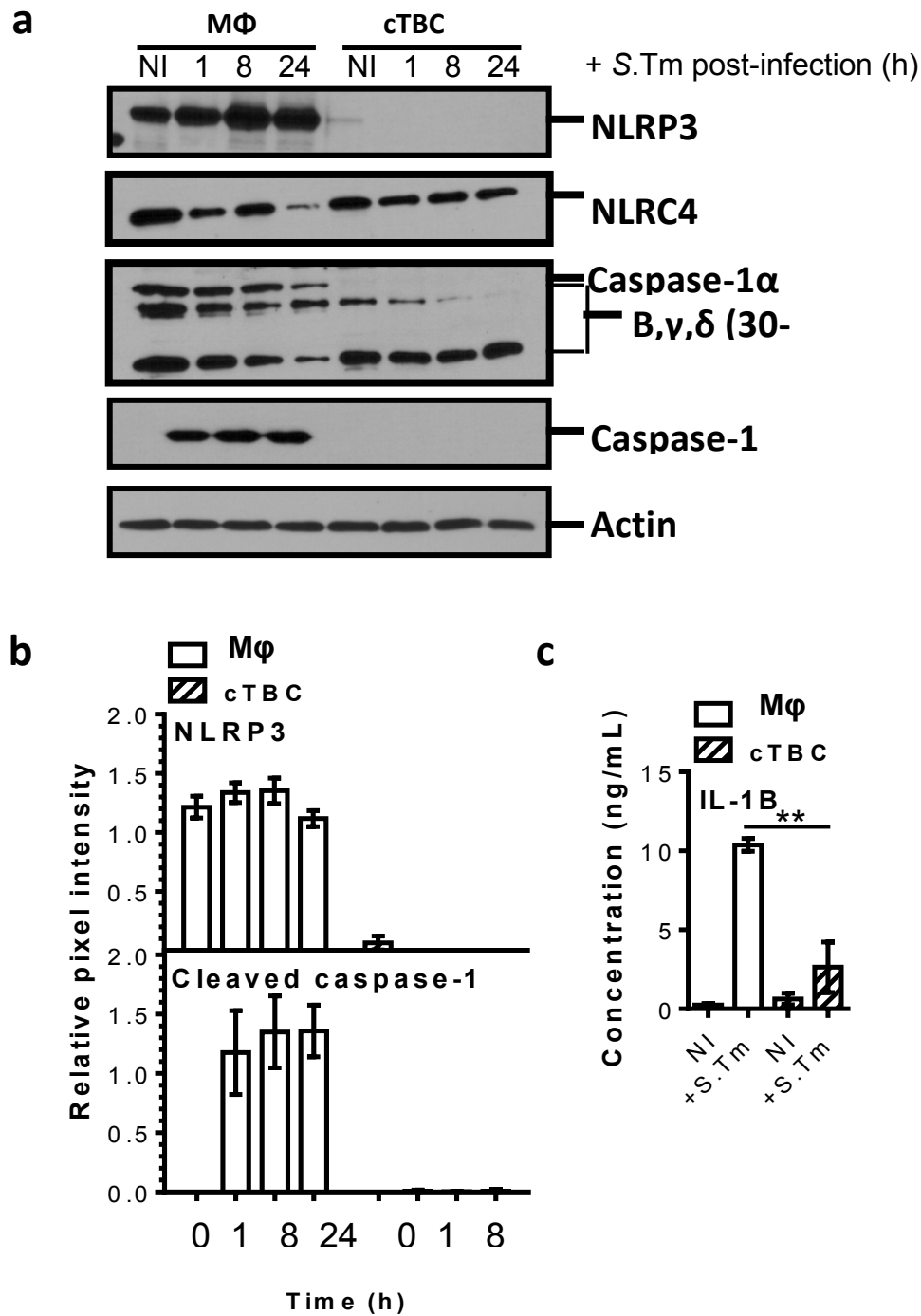


Figure 22. Pyroptosis marker expressions of *S.Tm*-infected human primary cTBCs. Human primary m ϕ and cTBC cultures were infected with *S. Tm*. Whole cell extracts at 1, 8, and 24 hours post infection and NI samples were prepared for Western blot analysis and an equal amount of protein was loaded into each well. Protein expression for nod-like receptors NLRP3, NLRC4, and Caspase-1. After blotting for each specific protein, the same blot was stripped and reprobed with β -actin to confirm equal loading of cell lysate among different samples. Western blots were quantified based on intensity using ImageJ (b). Secretion of pro-inflammatory cytokine, IL-1 β (c). Supernatant from non-infected and infected human primary m ϕ and cTBC cultures were collected for ELISA. **, $p < 0.01$ by Student *t* test for m ϕ in comparison to cTBC. The data are representative of three independent experiments.

proteins of *S.Tm*-WT infected cTBCs and of *S.Tm*-WT infected macrophages. Phospho-RIPK-1 and phospho-MLKL were observed only after 24 h of infection in macrophages, but in infected cTBCs, expression of phospho-RIPK-1 and phospho-MLKL was seen as early as 1 h post-infection (Fig. 23). The quantification of these results is shown in (Fig. 23b). These data reiterated that *S.Tm*-WT-infected cTBCs succumbed to necroptosis, whereas *S.Tm*-WT-infected macrophages succumbed to pyroptosis at early time points post-infection.

4.14 TROPHOBLAST CELLS FAIL TO EVOKE INFLAMMATORY CYTOKINE PRODUCTION FOLLOWING *S.TM* INFECTION

The secretion of cytokines and chemokines was determined in non-infected and *S.Tm*-infected cultures of THP-1 macrophages (following activation with PMA), HeLa, and JEG-3 cells. Cytokines can promote pathogen persistence. Infection with *Salmonella* induces expression of multiple chemokines and proinflammatory cytokines in cultured intestinal epithelial cells and macrophages. In placenta, cytokines are essential but can be detrimental for successful establishment and maintenance of a healthy pregnancy, regulating interactions between the semi-allogenic placenta and maternal immune system. *Salmonella* has immunomodulatory properties that alter the host immune response to infection which may affect cytokine secretion within *Salmonella*-infected placenta and at the materno-fetal interface. The cytokines secreted by cells of the placenta post *Salmonella* infection are not known. Fig. 24a shows a representative proteome[®] blot indicating the signals for various cytokines in supernatants obtained from non-infected or *S.Tm*-infected cells. Fig. 24b shows the relative amount of cytokines produced upon *S.Tm*-infection relative to non-infected cells. After 24 h of infection with *S.Tm*, PMA-activated

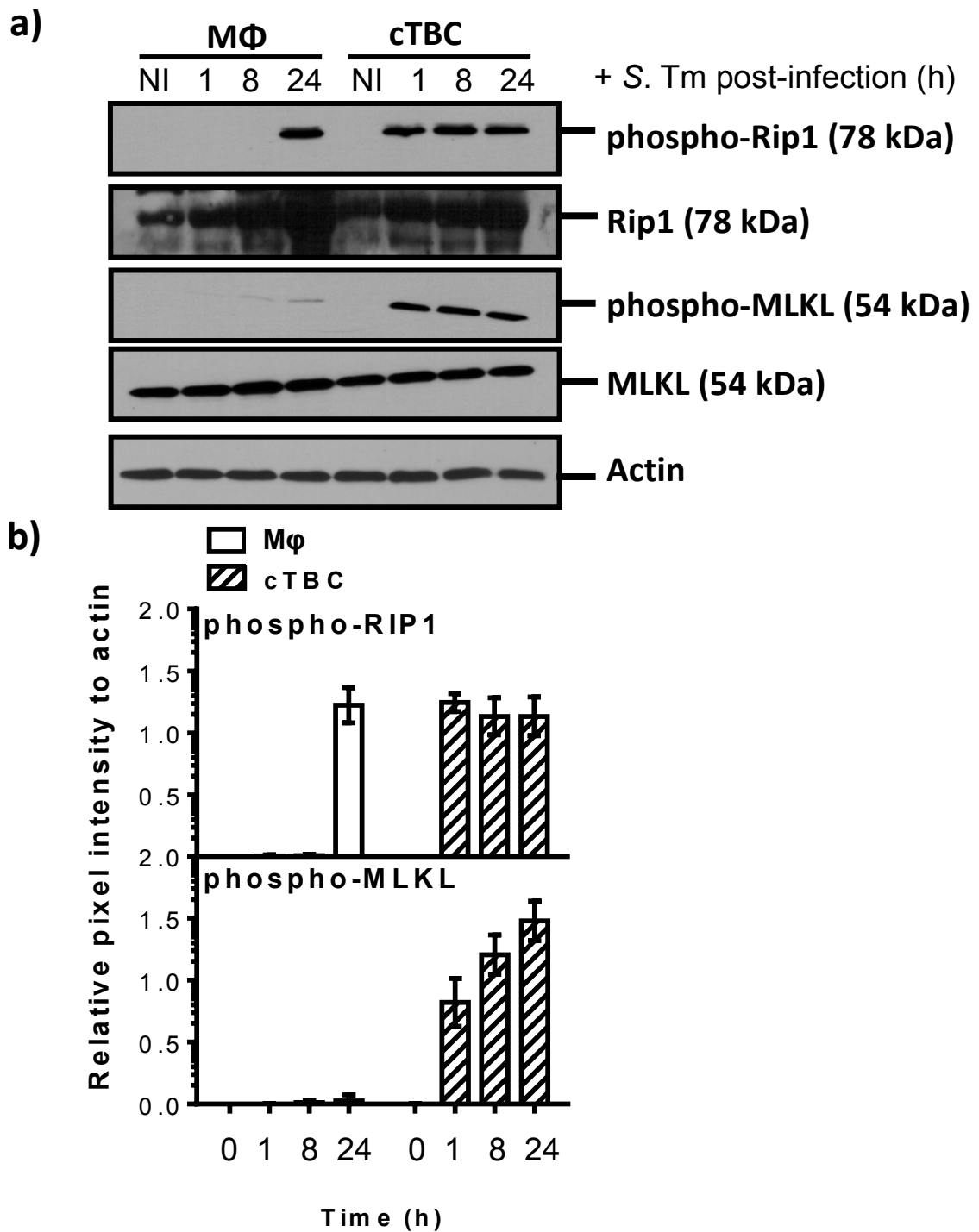


Figure 23. Necroptosis protein expression of *S. Tm*-infected human primary cTBCs. Protein Human primary mφ and cTBC cultures were infected with *S. Tm*. Whole cell extracts at 1, 8, and 24 h post infection and NI samples were prepared for Western blot analysis and an equal amount of protein was loaded into each well. Markers for necroptosis include: phospho-RIPK-1, RIPK-1, phospho-MLKL, and MLKL and are indicated. After blotting for each specific protein, the same blot was stripped and reprobed with β -actin to confirm equal loading of cell lysate among different samples. Western blots were quantified based on intensity using ImageJ (b). The data are representative of three independent experiments.

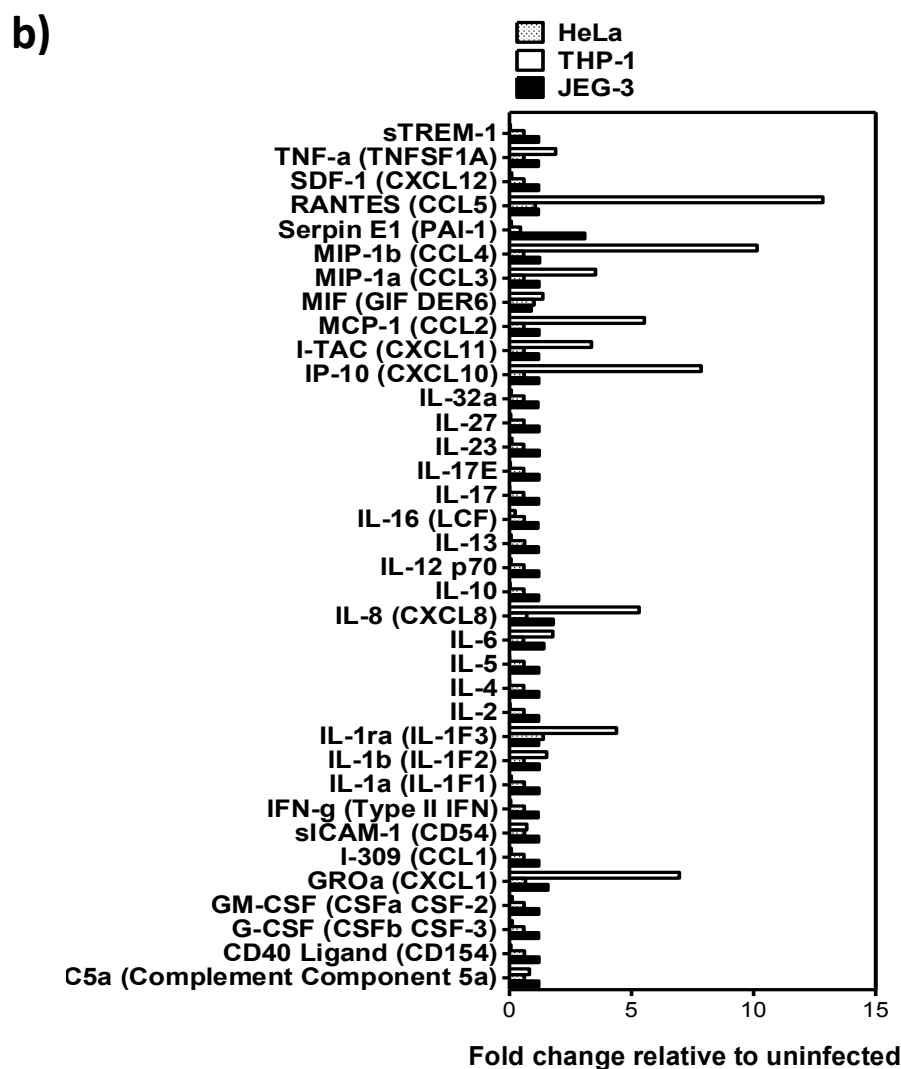
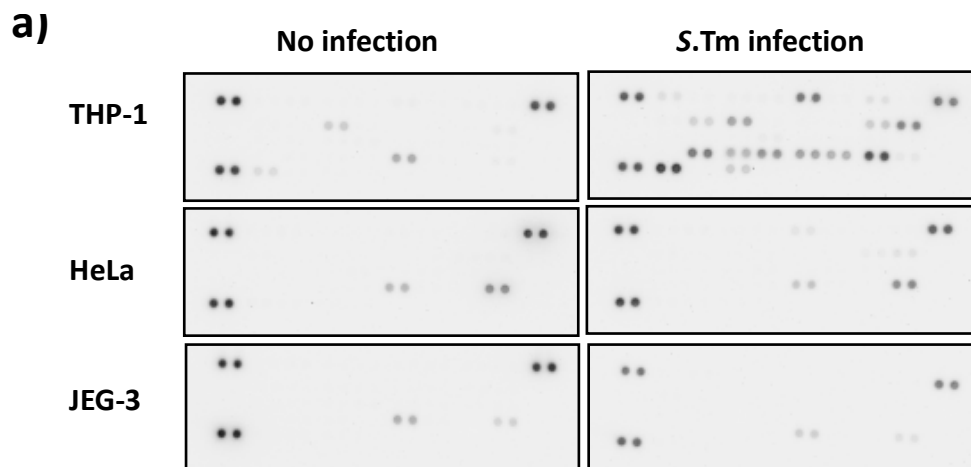


Figure 24. *Salmonella* Typhimurium-infection did not induced cell cytokine production. HeLa, PMA-activated THP-1, and JEG-3 cells were infected with 10 MOI of *S.Tm* and 24 h later the production of cytokines in the supernatant was analyzed by Proteome Profiler Human Cytokine Array®. (a) Representative Proteome Profiler Human Cytokine Array® image of supernatant derived from non-infected and infected cells. Blots were quantified using ImageJ® software (b).

THP-1 secreted abundant quantities of many inflammatory cytokines and chemokines. In contrast, HeLa and JEG-3 cells remained non-responsive to *S.Tm* infection despite the high bacterial burden within these cells. While the proteome blot described above allows for pan-analysis of cytokine production, it has low sensitivity (>1 ng/ml). I therefore utilized an alternate more sensitive method (cytometric bead array) to quantify the expression of the pro-inflammatory cytokines IL-12, TNF- α , and IL-6 and the anti-inflammatory cytokine, interleukin-10 (IL-10) in the culture supernatants of infected and non-infected cells. In primary macrophages, 24 h post *S.Tm*-WT-infection, pro-inflammatory cytokines IL-12, TNF, and IL-6 levels were copiously secreted (Fig. 25a), whereas primary cTBCs, produced only the anti-inflammatory cytokine, IL-10 (Fig. 25b). *S.Tm* infected primary human cTBCs secrete anti-inflammatory cytokine, IL-10.

4.15 IL-10 PRODUCED BY JEG-3 INHIBITS PHAGOSOMAL MATURATION AND PROMOTES INTRACELLULAR PROLIFERATION OF *S.TM*

Trophoblast cells are known to secrete type 2 cytokines such as IL-10 that are known to have anti-inflammatory effects. Increased IL-10 production in response to infection with many intracellular pathogens is associated with decreased resistance to infection. I investigated the role of IL-10 on *Salmonella*-phagosome maturation. JEG-3 cells were pre-treated with anti-IL-10 antibody prior to infection with *S.Tm*-coated C₁₂FDG. Phagosome maturation based on release of fluorescein was followed by flow cytometry. Treatment of JEG-3 cells with anti-IL-10 antibody increased phagosomal maturation following *S.Tm* infection (Fig. 26a and b). Additionally, anti-IL-10 antibody pre-treated JEG-3 cells were more able to curtail *S.Tm* growth as evidenced by increased doubling time (Fig. 26c and d).

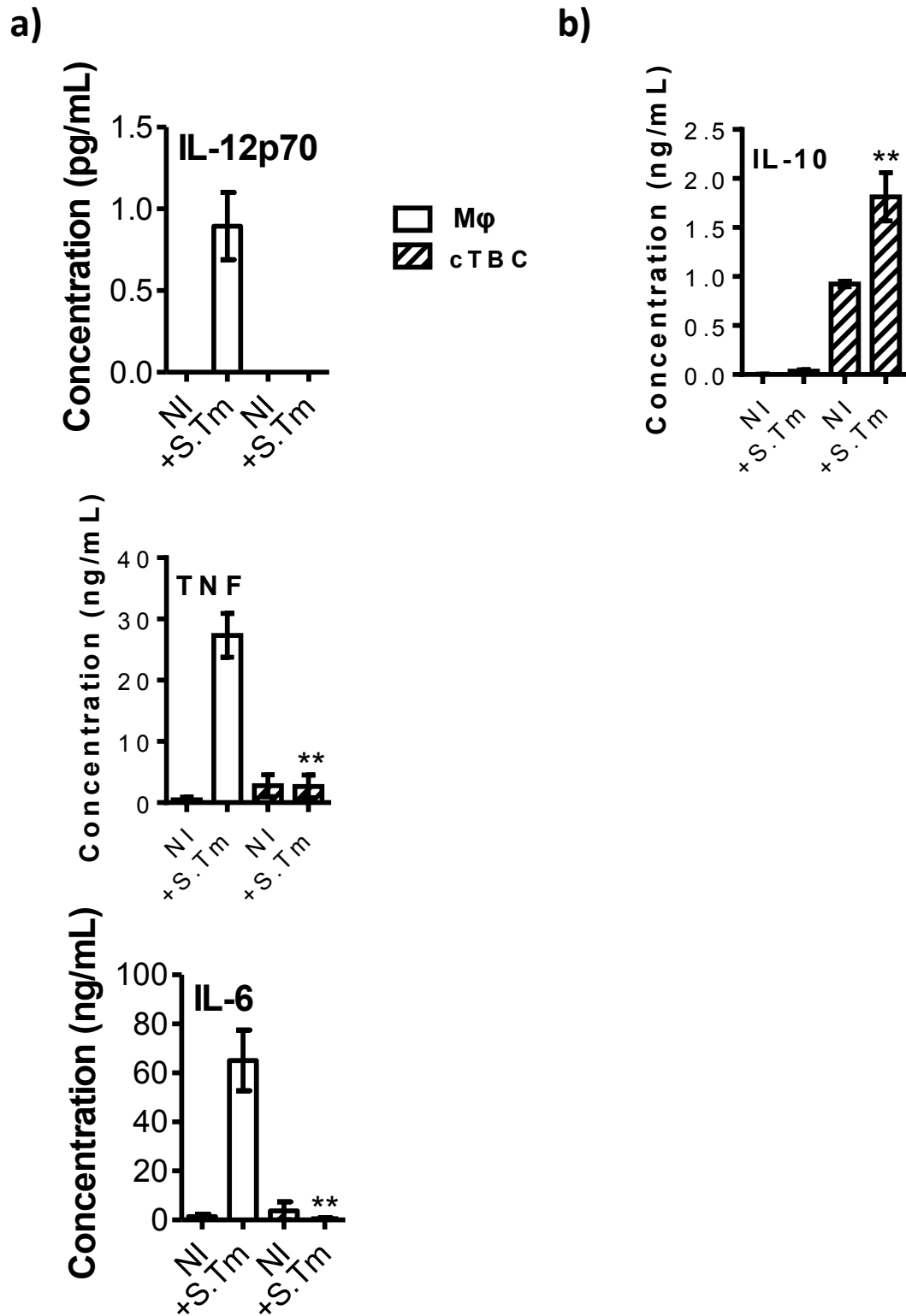
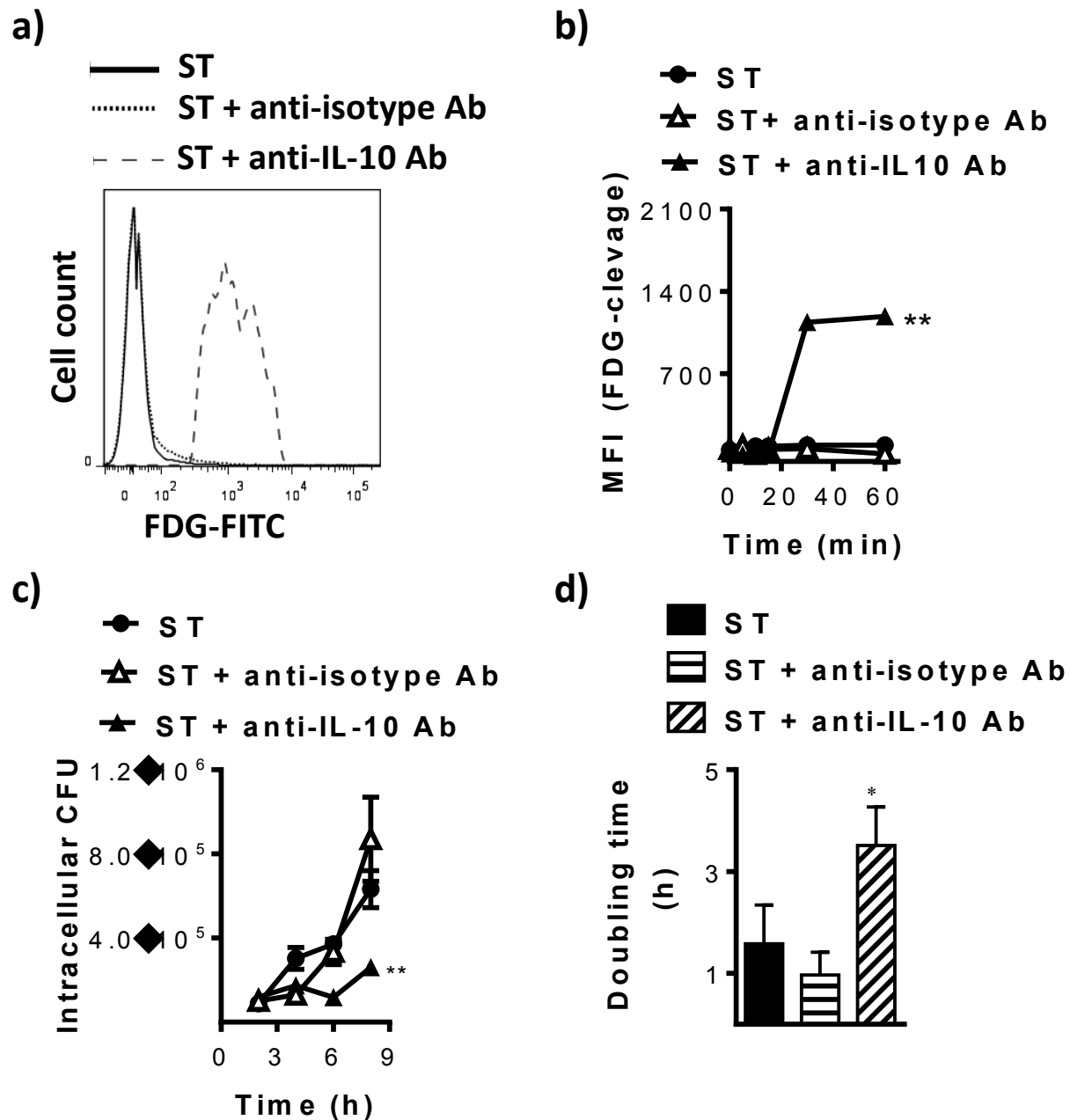


Figure 25. Modulation of cytokine secretion from *S.Tm* infected primary human cytotrophoblast cells and macrophages. Supernatants of *S.tm* infected primary human cTBC and mφ were collected at 24 h after infection. Cytokine concentrations were measured by a human pro-inflammatory cytokine bead array assay and are expressed as mean + SD of n = 3 samples/replicates. **, p<0.01 by Student *t* test for cTBC in comparison to mφ. The data are representative three independent experiments.



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Figure 26. Role of IL-10 produced by JEG-3 in modulating *S.Tm* infection. JEG-3 cells were treated with an isotype or anti-IL-10 Ab (50 µg/ml) before and during infection with FDG-*S.Tm*. Phagosome maturation kinetics were determined based on FDG cleavage. Representative histogram demonstrating the release of FDG, 30 minutes after infection (a). The increase in MFI over time indicative of release of FDG (b) **, $p < 0.01$ by two-way ANOVA for JEG-3 cells over time. Intracellular bacterial burden (Mean +/- SEM) of triplicate infected JEG-3 cells after treatment with anti-IL-10 Ab (c). Treatment ($p = 0.0073$) and time ($p < 0.0001$) are significantly different by two-way ANOVA. The doubling time of *S.Tm* was then calculated (d). *, $p < 0.05$ by Student's *t* test for doubling time in the presence of anti-IL-10 antibody in comparison to *S.Tm*-infected control JEG-3 cells.

4.16 INTERLEUKIN-10 PRODUCED BY HUMAN PRIMARY cTBCs INHIBITS PHAGOSOMAL MATURATION AND PROMOTES INTRACELLULAR PROLIFERATION OF *S.Tm* SIMILAR TO JEG-3

To determine if the same trend is seen in human primary cTBCs, phagosomes were isolated from non-treated, anti-IL-10 pre-treated, and the isotype control pre-treated human primary macrophages and cTBCs infected with *S.Tm*-WT to study the phagosome maturation pathway within these cells by Western blot (Fig. 27a). Macrophages express Rab5 at 5 min post-infection and Rab7 and LAMP1 at 60 min post infection. This is in accordance to phagosome maturation. Untreated infected cTBCs as well as the isotype control pre-treatment express Rab5 at both 5 and 60 min and no expression of Rab7 or LAMP1 was observed. This trend is similar to the infected JEG-3 as previously observed. However, when cTBCs were treated with anti-IL-10 before infection, Rab7 and LAMP1 was observed at 60 min similar to infected macrophages, indicating that *S.Tm* containing phagosomes mature when anti-IL-10 receptors are blocked. Using flow cytometry to determine the phagosome maturation kinetics, there is a shift in MFI which indicates that anti-IL-10 pre-treated cTBCs infected with *S.Tm* allowed for phagosome maturation to occur (Fig. 27b and c) in association with the suppression of *S.Tm* growth (Fig. 27d and e). Thus, trophoblast cells appeared to respond inappropriately to *S.Tm* infection by secretion of unopposed IL-10, which in turn inhibited phagosomal maturation necessary for bacterial growth curtailment.

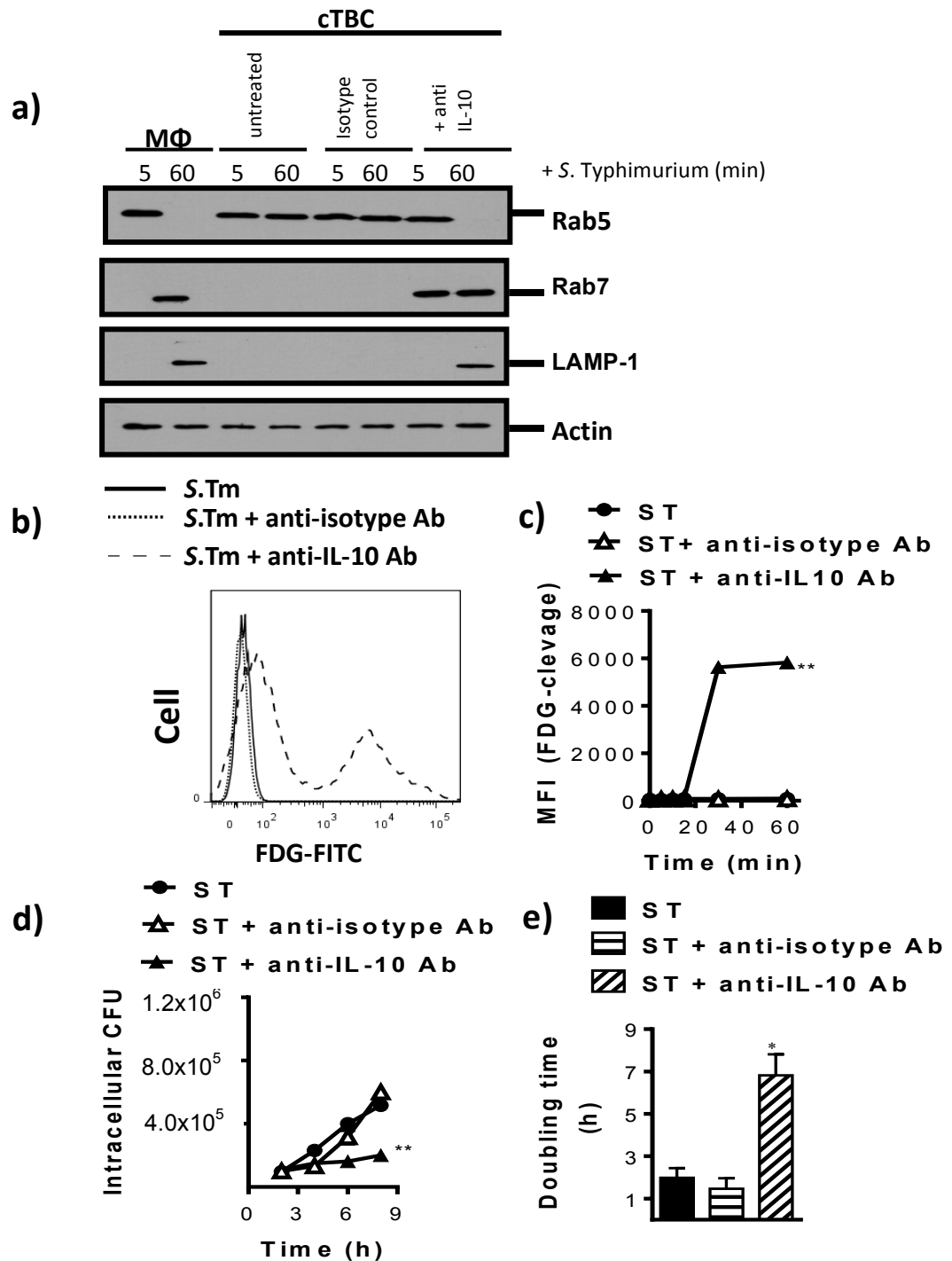


Figure 27. Role of IL-10 produced by human primary cTBCs in modulating *S.Tm* infection. cTBCs were treated with an isotype or anti-IL-10 Ab (50 µg/ml) before infection with *S.Tm*. Phagosome maturation markers were examined by Western blot for expressions of Rab5, Rab7, and LAMP-1 (a). Phagosome maturation kinetics was determined based on FDG cleavage post infection with FDG-*S.Tm* (b). **, $p < 0.01$ by two-way ANOVA for cTBCs over time in comparison to mφ. The increase in MFI over time indicative of release of FDG (c). Intracellular bacterial burden (Mean +/- SEM) of triplicate infected cTBCs after treatment with anti-IL-10 Ab (d). The doubling time was then calculated (e). *, $p < 0.05$ by one way ANOVA for doubling time in the presence of anti-IL-10 antibody in comparison to *S.Tm*-infected control cTBCs. Experiments were performed 3 times.

4.17 SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3 IS PHOSPHORYLATED IN CYTROTROPHOBLAST CELLS POST *S.TM* INFECTION

Salmonella can manipulate the pathways critical for the immune response and host defenses. IL-10 has been shown to block phagosome maturation in mycobacterium tuberculosis-infected human alveolar macrophages by exerting effects on STAT-3. To determine if IL-10 exerts its effect on STAT-3 in trophoblast cells which allows for *Salmonella* growth, I mapped the signaling pathway in human primary macrophages and cTBCs. Analyzing the expression patterns of signaling proteins, both cell types were found to express Jak1 and Tyk2, and upon infection, phosphorylation was observed (Fig. 28a). STAT-1 signaling augments production of inflammatory cytokines such as IFN- γ by the cells. Phospho-STAT-1 (Fig. 28) was expressed immediately upon infection of primary macrophages with *S.Tm* which increased to substantial levels by 6 h post-infection. In contrast, phospho-STAT-1 expression was very low in cTBCs, even at 6h post-infection. Increased activation of STAT-3 was observed at 0.5 h post-infection in the cTBCs compared to non-infected cells. To confirm the Western blot results, flow cytometry was used to detect intracellular phospho-STAT-1 and phospho-STAT-3 before and after infection in macrophages (Fig. 29a) and in cTBCs (Fig. 29b). In macrophages, at 1 h post infection, there is detection of phospho-STAT-1 as shown by the shift in MFI and only a slight shift was observed in phospho-STAT-3. These results indicate that inhibition of phagosome maturation in *Salmonella* infected trophoblast cells by IL-10 is mediated by STAT-3, as IL-10 engages with its receptor and activates Jak1 and Tyk2, thereby mediating its anti-inflammatory properties on cTBCs.

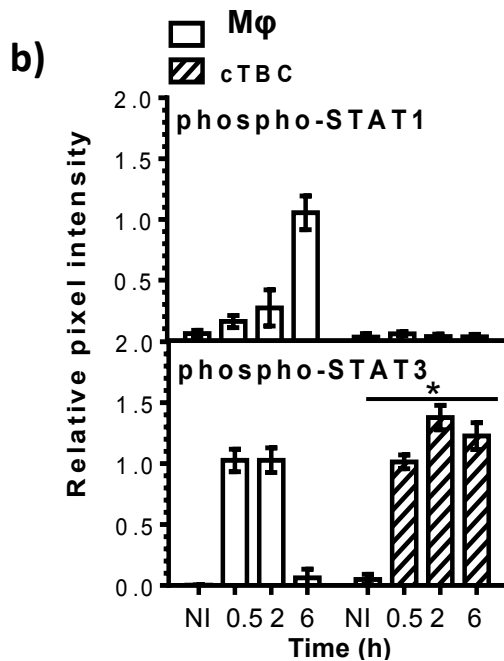
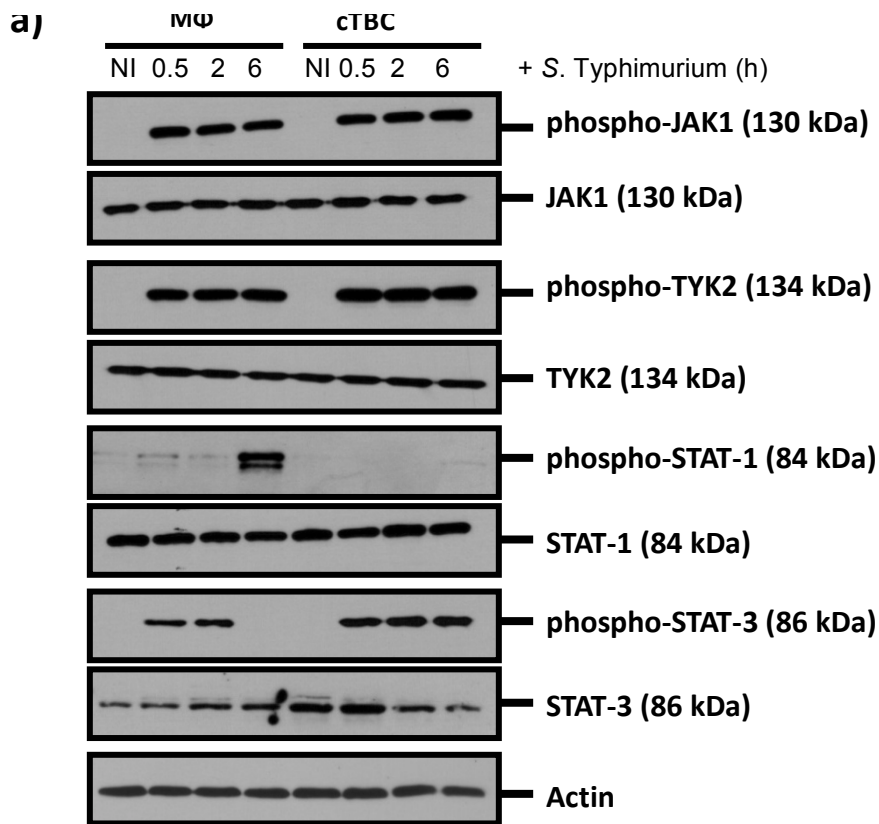


Figure 28. Cell signaling pathway protein expression of *S.Tm*-infected human primary cTBCs. Human primary mφ and cTBC cultures were infected with *S.Tm*. Whole cell extracts at 0.5, 2, and 6 h post infection and NI samples were prepared for Western blot analysis and an equal amount of protein was loaded into each well. Markers for cell signaling pathway include: phospho-Jak1, Jak1, phospho-Tyk2, Tyk2, phospho-STAT-1, STAT-1, phospho-STAT-3, and STAT-3. After blotting for each specific protein, the same blot was stripped and reprobbed with β -actin to confirm equal loading of cell lysate among different samples. Western blots were quantified based on intensity using ImageJ (b). **, $p < 0.01$ by two-way ANOVA for cTBCs over time in comparison to mφ.

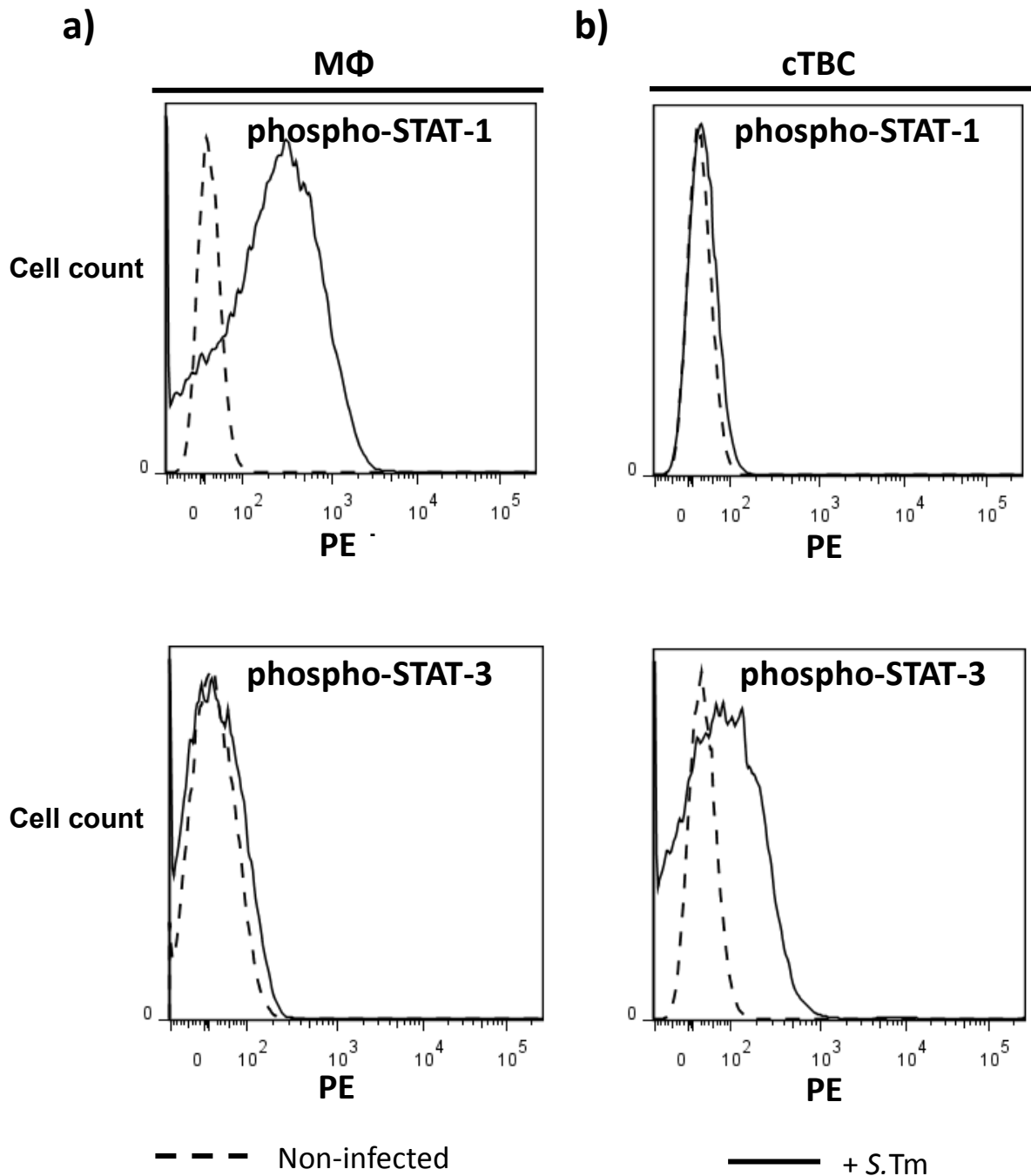


Figure 29. Intracellular phosphorylation of STAT-1 and STAT-3 post *S.Tm* infection in human primary cTBCs. Human primary m ϕ (a) and cTBC cultures (b) were infected with *S.Tm*. Cells were fixed 4 h post-infection, permeabilize and stained for phospho-STAT-1, and phospho-STAT-3. Samples were then acquired by flow cytometry and analyzed by FlowJo. Flow cytometry histograms are representative of 3 independent experiments.

CHAPTER 5: **DISCUSSION**

5.0 DISCUSSION

5.1 PRELUDE

The placenta is an immunoprivileged organ that has a dual physiological role: permits transmission of nutrients for nourishment of the fetus while providing an effective barrier against invading pathogens. There are only a few pathogens that can invade the placenta, and of these pathogens, the majority has either facultative or obligate intracellular life cycles. Examples of pathogens that can infect the placenta are: viruses such as cytomegalovirus (Schleiss, Aronow, and Handwerger 2007), varicella-zoster (Balducci et al. 1992), and hepatitis E (Chaudhry, Verma, and Koren 2015); parasites such as *Taxoplasma gondii* (Abbasi et al. 2003), *Plasmodium falciparum* (Andrews and Lanzer 2002), and *Schistosome species* (Bittencourt et al. 1980; Schleenvoigt et al. 2014); and bacteria such as *Coxiella burnetti* (Jones et al. 2010), *Chlamydia trachomatis* (Rours et al. 2011), *Chlamydia abortus* (Longbottom et al. 2013), *Brucella abortus* (Tobias, Cordes, and Schurig 1993), *Porphyromonas gingivalis* (Vanterpool et al. 2016; D. Lin, Smith, Elter, et al. 2003; Katz et al. 2009), *Listeria monocytogenes*, (Robbins and Bakardjiev 2012) and *Salmonella species* (Chattopadhyay et al. 2010). Many of these infections can induce fetal adverse effects but are often not life-threatening to the mother. However, previous results in the laboratory first demonstrated that intracellular *S.Tm* infection in mice is detrimental to both the fetus and fatal to the mother (Pejic-Karapetrovic et al. 2007).

In humans, exposure to *Salmonella* during pregnancy has been reported to cause spontaneous abortions, stillbirths, and preterm births, however, the mechanisms are unknown. In this context, in this thesis, I have revealed the ability of *S.Tm* to thrive within placental trophoblast cells, and elucidated a mechanism for the increased susceptibility of the maternal host to infection.

5.2 PLACENTAL TROPHOBLAST CELL LINES AS *IN VITRO* MODELS FOR ELUCIDATING INFECTION SUSCEPTIBILITY

In the human placenta, maternal and fetal cells are juxtaposed to each other in two specific areas where pathogen invasion may potentially occur: the syncytiotrophoblast cell-blood interface or the extravillous trophoblast-uterine interface (trophoblast giant cell-uterine interface in mice). The syncytiotrophoblast cells in both human and mice are bathed in maternal blood to enable nutrient and gas exchange, while the extravillous trophoblast cells (giant trophoblast cells in mice) directly invade the maternal uterus and are capped with maternal decidua containing immune cells. In humans, the trophoblast layer cells differentiate into several trophoblast subsets in order to create the placenta. Of these subsets, the cTBC is considered a putative progenitor cell, which replenishes the outer layer of the villous syncytiotrophoblast, but which is also able to invade the decidua to develop into the extravillous trophoblast (Robbins and Bakardjiev 2012).

Primary trophoblast cells are the ideal choice for studies, but the availability of placenta tissue obtained from healthy women c-section placenta is often restricted, isolation of trophoblast cells is labour intensive, and the isolated cells have a restricted life span in culture. Cell lines are widely used and have a number of advantages. Cell lines can unlimitedly self-replicate providing large quantities, and exhibit high degree of homogeneity. Varieties of trophoblast cell lines are commonly used to overcome the limitations of primary trophoblast cells. Experiments using primary trophoblast were used to verify experiments done first using cell lines, and caution was used when interpreting results from trophoblast cell line experiments. Disadvantages of using cell lines include genotypic and phenotypic drifts during continual culture.

In the first part of this thesis, I examined the ability of *S.Tm* to infect and thrive within human placental choriocarcinoma cells such as JEG-3 and BeWo, which are derived from placental villous trophoblast and are considered to resemble the undifferentiated cTBCs (Orendi et al. 2011) were examined. BeWo is the first human trophoblastic endocrine cell type to be maintained in continuous culture and exhibits characteristics of villous trophoblast cells including ability to syncytialize. BeWo cells express certain differentiation markers associated with trophoblast syncytialization. Another placental cell line used in my thesis is HTR8/SVneo, which are transformed first trimester extravillous trophoblast cells often used to model the physiologically invasive extravillous trophoblast (Orendi et al. 2011). These three human placenta cell lines collectively comprise the various types of trophoblast cells and hence were convenient models to initially study the infection kinetics of *S.Tm*.

Fibroblast contamination is a frequent problem when culturing human primary cells. The primary cytotrophoblast cells were contaminated with about 5% fibroblast cells. Fibroblasts are non-phagocytic and have been shown to restrain intracellular replication of *S.Tm* (Núñez-Hernández et al. 2014), thus contaminated fibroblast cells in the culture may not alter the intracellular replication and the cellular death results, but precaution was taken when analyzing the results.

Many intracellular pathogens productively infect trophoblast cell lines. For example, BeWo cells are permissive to *Toxoplasma gondii* infections (Barbosa et al. 2008). The abortive pathogen *Coxiella burnetti* infects and replicates substantially in BeWo trophoblast cells, despite its inability to replicate in macrophages (Ben Amara et al. 2010). Furthermore, JEG-3 cells were

shown to be infected by *Listeria*, *Chlamydia*, CMV and HIV (Pizarro-Cerdá, Kühbacher, and Cossart 2012; Equils et al. 2006; Jun et al. 2000; Vidricaire, Tardif, and Tremblay 2003). My data demonstrate that similar to these various intracellular pathogens, *S.Tm* infection can be easily established in all trophoblast-derived choriocarcinoma cells as well as primary human cTBCs (Fig. 10). The primary human cTBCs infection results confirm the infected cell line results, thus primary human cTBCs provide a useful model for infection studies in the placenta.

5.3 MECHANISM OF *SALMONELLA* UPTAKE INTO PLACENTAL TROPHOBLAST CELLS

Salmonella can gain direct entry to the feto-maternal interface by escaping maternal blood and infecting trophoblast cells, or alternatively, they may establish infection by potentially reaching the placenta through infected macrophages or dendritic cells. The ability of *S.Tm* to invade specific cell types is attributed to the TTSS needle apparatus under the control of the SPI-1 locus (Coburn, Grassl, and Finlay 2007). More recently SPI-1 independent invasion such as Rck have been reported (Cirillo et al. 1996; Mijouin et al. 2012). The intracellular life cycle of *Salmonella* has been previously primarily studied in epithelial cells such as HeLa cells (Jones, Richardson, and Uhlman 1981). Salmonella gain entry into such non-phagocytic cells by employing one of the two different mechanisms, a TTSS dependent entry or a host cell receptor mediated entry (Steele-Mortimer et al. 2002; Cirillo et al. 1996; Mijouin et al. 2012). TTSS is found in several Gram-negative bacteria, such as *Shigella*, *E.coli*, *Chlamydia*, *Vibrio*, and *Pseudomonas* (Cornelis 2006; Coburn, Sekirov, and Finlay 2007). It remains possible that *S.Tm* utilizes these mechanisms to gain entry to trophoblast cells. Bacteria such as *Yersinia* and *Listeria* have evolved various other mechanisms to trigger rearrangement of host actin, enabling

their entry into the host cells. These bacteria express surface proteins that interact with cellular transmembrane receptors connected to the cytoskeleton known as the Zipper mechanism (Ke, Chen, and Yang 2013; Pizarro-Cerdá, Kühbacher, and Cossart 2012). For example, another intracellular bacterium known to preferentially infect trophoblast cells is *Listeria monocytogenes*. Internalization of *Listeria monocytogenes* into epithelial cells occurs through the interaction of the bacterial surface molecules InIA and InIB with the cellular receptors E-cadherin and Met, respectively (Bonazzi, Lecuit, and Cossart 2009; Pizarro-Cerdá, Kühbacher, and Cossart 2012). The interaction triggers the recruitment of endocytic effectors, the subversion of the phosphoinositide metabolism, and the remodeling of the actin cytoskeleton that leads to bacterial engulfment. *Listeria monocytogenes* can also infect the cells by spreading from cell to cell utilizing its virulence protein ActA that facilitates spread from infected host cells to neighbouring cells without bacterial exposure to the extracellular milieu. Bacterial ActA interacts with the Arp2/3 complex and actin monomers to induce actin polymerization on the bacterial surface generating an actin comet tail (Pizarro-Cerdá, Kühbacher, and Cossart 2012; Ireton 2013). While *S.Tm* does not possess these other entry mechanisms, the results show TTSS-independent mode of entry of *S.Tm* into trophoblast cells.

In addition to the TTSS mediated entry, *Salmonella* may enter a cell using a TTSS-independent mechanism, by utilizing the cell's receptor mediated by Rck protein found on the bacteria's membrane which alone is able to promote adhesion and internalization of *Salmonella* into epithelial cells. Binding of Rck to its receptor, allows microvillus-like extensions to form a zipper-like structure that engulfs the adherent bacteria (Mijouin et al. 2012; Cirillo et al. 1996). Previous work demonstrated that *S.Tm* invades not only HeLa human epithelial cells and IC-21 murine macrophages, but also human choriocarcinoma cells, such as JEG-3 (Chattopadhyay et

al. 2010). However, it was unclear if *S.Tm* infection of the trophoblast cells occurred through similar mechanisms identified for epithelial cells. Results indicated that only the *S.Tm-ΔinvA* strains were unable to infect HeLa epithelial cells suggesting primarily TTSS-dependent invasion. Trophoblast cells actively internalized *S.Tm*, *S.Tm-ΔinvA* and *S.Tm-Δrck* strains suggesting a highly active TTSS-independent mode of entry in these cells. Indeed, the initial uptake of *S.Tm* at 2 h into JEG-3, BeWo and HTR-8 was 2-fold higher than in HeLa cells (Fig. 10). Thus, active phagocytosis by trophoblast cells may trump the need for bacterial mediated invasion. Subsequent experiments using inhibitors of phagocytosis confirmed that similar to macrophages, trophoblast cells are able to actively uptake the bacteria (Fig. 11). Indeed trophoblast cells have been reported previously to possess phagocytic capacity (Bevilacqua et al. 2010).

5.4 SALMONELLA INFECTION DURING PREGNANCY IN OTHER SPECIES

Salmonella enterica serovar Abortusovis (*S.Abortusovis*) is naturally restricted to ovine species, primarily infects sheep, and does not infect humans (Habrún et al. 2006). Like *S.Typhi*, *S.Abortusovis* causes systemic infection in which, under natural conditions, animals are not able to raise a rapid immune response. Failure to induce the appropriate response allows pathogens to reach the placenta and results in an abortion in sheeps. TLR4 pathway engaged by *S.Tm* is not triggered by *S.Abortusovis*, suggesting that *S.Abortusovis* LPS might not be recognized by TLR4 and is able to successfully evade the immune system whereas *S.Tm* and other serovars fail to do so (Chessa et al. 2014).

S.Tm LPS however, is mediated by TLR2 and TLR4 and results in production of pro-inflammatory cytokines such as IL-6 and TNF- α . Mice lacking TLR4 are more susceptible to *S.Tm* (Arpaia et al. 2011).

5.5 *SALMONELLA* IMMUNOGLOBULIN ANTIBODIES AND PRETERM BIRTH

Salmonella IgG antibodies are long-lasting while IgM and IgA antibodies decline 3 to 4 months after the onset of infection. Occupationally exposed women are frequently infected with *Salmonella* as revealed by the secondary antibody response. It is likely that many of these women have partial immunity, resulting in asymptomatic infections to these pathogens leading to a fast clearance of the pathogens by the IgG antibodies (Kantsø et al. 2014). In the study by Kantsø et al., correlation of Ig antibodies to adverse pregnancy outcomes, especially miscarriage, could not be calculated due to a small sample size.

5.6 SCAVENGER RECEPTOR A-MEDIATED *SALMONELLA* ENTRY INTO TROPHOBLAST CELLS

In rodent and humans with hemochorial placentation, phagocytic ability is a hall-mark of trophoblast differentiation and instructs invasive capacity for tissue remodelling of the decidua during implantation (Enders and Carter 2012). After placental maturation, trophoblast cells maintain phagocytic functions for purposes other than nutrition. Trophoblast cells participate in embryonic and fetal innate immune defense through elimination of microorganisms present at the maternal-fetal interface. Once phagocytic, trophoblast cells produce and release reactive oxygen and nitrogen species such as superoxide anion, hydrogen peroxide and nitric oxide, suggesting that trophoblast phagocytosis plays a role in microbial defense mechanisms (Amarante-Paffaro et al. 2004).

During phagocytosis in immune cells such as neutrophils and macrophages, receptors in the cell membrane first recognize antibodies on the target in a zipper-like mechanism. This is

followed by fusion with lysosomes, acidification of the phagosome, and degradation of the target.

Phagocytosis of JEG-3 and activated-THP-1 can be inhibited by cytochalasin B or D, which binds to actin filaments and blocks polymerization of the elongation of actin. PI3K participates in the signalling cascade of phagocytic receptors. PI3K catalyzes phosphorylation at the D-3 position of the inositol ring of phosphatidylinositol (PI), PI(4)P and PI(4,5)P₂ and is activated by many tyrosine kinase receptors that trigger the polymerization of actin. Fc receptor-mediated phagocytosis is prevented by Wortmannin or LY294002, which are specific inhibitors of PI3K.

The mannose receptor is involved in the phagocytosis of pathogenic microorganisms by recognizing mannose on the surfaces of pathogens and mediates phagocytosis of the organisms. The high affinity of this receptor for branched mannose makes the mannose receptor a phagocytic receptor with broad pathogen specificity. Mannan is an inhibitor of phagocytosis. Recognition of glycoconjugates on the surface of microorganisms by the mannose receptor leads to the phagocytosis of several pathogens such as *Candida albicans*, *Leishmania donovani*, and *Pneumocystis carinii*. Pathogenic microorganisms have developed several strategies to circumvent microbicidal responses of host cells. *Salmonella montevideo* has been shown to invade cells through complement receptor-1 instead of the mannose receptor on human erythrocytes (Joiner et al. 1986).

Collective observations with various inhibitors implicated scavenger receptor-mediated endocytosis as a mechanism of *S.Tm* internalization into JEG-3 cells (Fig. 12). Trophoblast cells are known to express scavenger receptor-A (Shi, Swan, and Henson 2003). Macrophages mediate clearance of many pathogens by utilizing Scavenger Receptor A-mediated phagocytosis

(Greaves and Gordon 2009). Furthermore, scavenger receptor mediated endocytosis is implicated in the internalization of *Clostridium sordellii* by decidual macrophages and *Brucella abortus* and *Listeria monocytogenes* by trophoblast giant cells (Thelen et al. 2010; Watanabe et al. 2008; Tachibana et al. 2015). THP-1 and JEG-3 cells strongly expressed scavenger receptor-A suggesting scavenger receptor-A mediated *S.Tm* internalization. However, as fucoidan induced only ~50% inhibition, other host cell receptors may also modulate *S.Tm* entry (Fig. 12). My data suggest that *S.Tm* may have evolved to utilize a ubiquitous phagocytosis receptor to gain entry into various cell types. In the case of trophoblast cells however, this relates to the undesirable consequence of uncontrolled bacterial replication resulting in fetal demise.

5.7 THE SYNCYTIOTROPHOBLAST CELL BARRIER

Studies involving *Listeria monocytogenes* have shown that human syncytium provides a formidable fortress and is often resistant to infection (Robbins et al. 2010). 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 spread (Zeldovich et al. 2013). Moreover, the actin cytoskeleton within syncytiotrophoblast cells prevented deformation compared to other trophoblast cell types (Ishikawa et al. 2014; Sartori et al. 2003). In contrast, syncytial damage has been shown to facilitate the spread of placenta infection of the underlying cTBCs (Zeldovich et al. 2013). For example, artificial enzymatic degradation of syncytiotrophoblast cells enables increased bacterial and parasitic colonization of the underlying cTBCs during *Listeria monocytogenes* and *Toxoplasma gondii* infection (Robbins et al. 2010, 2012). *Plasmodium falciparum* infection during pregnancy is associated with syncytial degradation and increased accumulation of

infected erythrocytes within the intervillous space of human placentas (Goldberg et al. 1990). Thus, the syncytium *in vivo* forms an effective barrier against pathogen invasion and breach of this barrier may only occur once a certain level of cellular damage has occurred. *In vitro*, I observed that syncytial cells derived from cTBC cultures could be infected with *S.Tm*, albeit to a lesser extent. However, my observation that cTBCs are highly susceptible to *S.Tm* replication suggests that any mechanism that may breach the syncytium *in vivo* will increase vulnerability of infection during pregnancy.

Extravillous trophoblast cells in humans are more susceptible to infection than syncytiotrophoblast cells. During *Listeria monocytogenes* or *Toxoplasma gondii* infection in human placental explants, the extravillous trophoblast cells are the preferred site of invasion (Zeldovich et al. 2011; Robbins et al. 2012). CMV efficiently infects the decidua and extravillous trophoblast cells in human term placentas (T. Liu et al. 2015). Murine studies have also revealed that trophoblast giant cells (extravillous trophoblast cells in humans) are the initial site of invasion and colonization during *Brucella abortus*, *Fusobacterium nucleatum*, *Coxiella burnetti*, *Listeria monocytogenes*, and *Toxoplasma gondii* infection (Watanabe et al. 2008; Tobias, Cordes, and Schurig 1993; Hu and Cross 2010; Ferro et al. 2002; Hashino et al. 2015; Monnier et al. 2006; Krishnan, Nguyen, and McComb 2013). It is thus possible that the junction of *S.Tm* entry is the extravillous trophoblast cells.

5.8 PMA ACTIVATION OF THP-1 AND JEG-3 CELLS

Treatment of cells with PMA activates protein kinase C. PMA treatment enhances the adherence of the THP-1 cells relative to untreated cells. Another feature of macrophage differentiation is enhanced granularity, as demonstrated by increase in side scatter on flow

cytometry, and reduction in nucleocytoplasmic ratio due to an increase in cytoplasmic volume. Enhanced granularity results from an increase in the number of mitochondria and lysosomes organelles. CD14 is a monocyte marker that is downregulated during differentiation. TLR2 is another surface marker that is down regulated with macrophage differentiation. PMA activated THP-1 cells phagocytose opsonized particles (Daigneault et al. 2010).

BeWo cells can be induced to fuse by treatment with PMA. This fusion is accompanied by increase expression of dysferlin, a syncytiotrophoblast membrane repair protein and increase in mRNA for the placenta fusion proteins syncytin1 and syncytin2 (Suzuki et al. 2002). Upon PMA treatment of JEG-3 cells, the ovine IFN_T gene is transactivated. PMA activates p42 MAPK and phospho-Elk1 in JEG-3 cells. MAP, also known as ERKs, is involved in a wide variety of cellular processes such as proliferation, differentiation, and transcription regulation and development. Elk1 functions as a transcription activator. PMA stimulates increase in hormone human chorionic gonadotropin, characteristic of syncytiotrophoblasts, secretion of JEG-3 cells (Tremblay and Beaudoin 1993).

PMA treated JEG-3, which allows differentiation, expressed mature cathepsin D in the isolated phagosomes, which can inhibit intracellular growth of the bacteria. Our collaborator's lab has shown that *S.Tm* infected syncytiotrophoblast layer of first trimester explant cultures does not allow of intracellular bacterial proliferation.

5.9 *SALMONELLA* INTRACELLULAR GROWTH IN PLACENTAL CELLS

Previous work in the laboratory demonstrated that after *S.Tm* entry into cells, *S.Tm* proliferated profoundly in JEG-3 human choriocarcinoma cells and slower in HeLa human epithelial cells, but did not grow in IC-21 murine macrophages (Chattopadhyay et al. 2010). In this study, I have demonstrated that even among primary cells, monocyte-derived macrophages

were more resistant to *S.Tm* growth relative to cTBCs that supported profound bacterial replication (Fig. 17). Various endosome proteins are gained and lost on the way to phagolysosomal maturation and constitute efficient trackers of the vacuolar biogenesis. Previous studies have mapped the biogenesis of SCVs in epithelial cells and macrophages (A. C. Smith et al. 2007). In general, SCVs associate early on with proteins such as Rab5, indicative of fusion with early endosomes. Later on, SCVs acquire and retain Rab7 and late lysosomal membrane proteins such as LAMP-1, but exclude lysosomal hydrolytic enzymes such as cathepsin D (Fairn and Grinstein 2012). In my study, I characterized the phagosomal maturation pathway in trophoblast cells using three different techniques: immunofluorescence, Western blotting and an assay for phagolysosomal fusion (Fig. 13, 14, 16). Similar to other studies, SCVs of PMA-activated THP-1 macrophages rapidly recruited late endosomal proteins Rab7 and LAMP-1, yet sequestered cathepsin D which is consistent with *Salmonella* induced evasion of fusion with trans-golgi-recycled endosomes. However, in trophoblast cells, internalized *S.Tm* remained co-localized with early endocytic protein Rab5, failed to recruit LAMP-1 and exhibited lack of lysosomal β -galactosidase activity. Thus, *Salmonella* are maintained in early phagosomes within trophoblast cells and phagolysosomal fusion is delayed if it takes place at all.

Similar to *Salmonella*, mycobacteria reside within phagosomes and can evade host adaptive immunity (Seto, Tsujimura, and Koide 2011). However, mycobacteria are extremely slow growing and remain quiescent in host cells. On the other hand, *Salmonella* devotes >4% of its genome to virulence mechanisms (Paesold et al. 2002). Thus, even in a competent host, adaptive immunity to *Salmonella* is substantially delayed, making it important for innate immunity to control infection (Luu et al. 2006). The inability of trophoblast cells to mount this initial innate immune control appears to contribute to overt bacterial proliferation and triggers a

cascade of downstream inflammatory events that result in serious consequences for the host. *Salmonella* expend substantial energy to regulate various virulence genes of the SPI-2 locus to survive within the hostile milieu of phagosomes in macrophages (Paesold et al. 2002).

5.10 PLACENTA ACTS AS A RESERVOIR FOR PATHOGENS

Placental infections are major causes of maternal and fetal disease. Many pathogens utilize the placenta as a reservoir. *Listeria monocytogenes* is an intracellular cytosolic pathogen which activates the toxin Listeriolysin O in the phagosomes, restricting its ability to survive (Vázquez-Boland et al. 2001). The ability of extravillous trophoblast cells and BeWo cells to prevent cytosolic escape of *Listeria monocytogenes* from LAMP-1 positive vesicles results in curtailment of bacterial replication (Zeldovich et al. 2011). In contrast, *Salmonella* are capable of surviving within harsh acidic phagosomes (Rathman, Sjaastad, and Falkow 1996). Moreover, trophoblast cells exhibited an inherent defect in phagosome biogenesis even after uptake of inert beads (Fig. 15). Thus trophoblast cells appear to conveniently provide a safe early endosomal niche for phagosomally adapted *Salmonella* wherein profound pathogen proliferation can occur under conditions of minimum stress.

Characterization of *Coxiella burnetii* containing endocytic compartment in BeWo cells indicated that initially the organisms co-localized with LAMP-1 but not cathepsin D whereas after 6 days post-infection, the organism co-localized with cathepsin D indicating their presence within phagolysosomes (Ghigo et al. 2002; Ben Amara et al. 2010). Although presence of *Coxiella burnetii* in phagolysosomes suggests that trophoblast cells may have the ability to fuse with lysosomes at later time points after infection, virulent pathogens such as *Salmonella* cause rapid trophoblast cell death (Fig. 18).

CMV has been shown to infect the placenta and spread of CMV from the mother to the placenta occurred early during the course of infection. Virus remains present in placental tissues long after CMV has been cleared from maternal blood. CMV replicates in placental tissues. Viral nucleocapsids were seen within nuclei of trophoblastic cells and virions were present surrounding infected cells. The placenta serves as a reservoir for CMV, while also limits transmission of the virus to the fetus (Griffith et al. 1985).

5.11 ROLE OF IL-10 IN BACTERIAL GROWTH

The production of IL-10 at the feto-maternal interface *in vivo* has pleiotropic effects for fetal survival (Chaouat et al. 1996). Human cTBCs produce IL-10 (Roth et al. 1996). Neutralizing IL-10 activity strikingly resulted in increased phagosome maturation and curtailment of *Salmonella* replication by JEG-3 and human primary cTBCs (Fig. 26 and 27). The production of IL-10 by macrophages correlated with increased replication of *Mycobacterium tuberculosis*. Moreover, IL-10 blocked phagosome maturation in *Mycobacterium tuberculosis*-infected human macrophages (O’Leary, O’Sullivan, and Keane 2011). Murine recombinant IL-10 treatment of RAW 264.7 macrophages was shown to increase internalization of *S.Tm* and bacterial survival (K.-S. Lee et al. 2011). The decrease in intracellular *S.Tm* numbers over time indicates that IL-10 production by trophoblast cells may support bacterial growth.

5.12 LACK OF NLRP3 EXPRESSION IN HUMAN TERM CTBCS

NLRP3 is expressed predominantly in macrophages and senses products of damaged cells such as extracellular ATP, crystalline uric acid, and diverse molecules originating from viruses such as Sendai, influenza, and adenoviral strains; fungi such as *Candida albicans* and

Saccharomyces cerevisiae; and bacteria such as *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Listeria monocytogenes* (Shao et al. 2015). The precise signal that is detected by NLRP3 remains unclear (Broz et al. 2010). Activated NLRP3 binds to adaptor ASC protein PYCARD to form the caspase-1 activating complex known as the NALP3 inflammasome, which in turn triggers an immune response (Abderrazak et al. 2015; Jo et al. 2016). *Listeria monocytogenes* virulence factor, listeriolysin O (LLO) hemolysin, dominantly activates the NLRP3 inflammasome to induce IL-1 β release in macrophages, which positively correlates with adverse pregnancy outcome in mice (Li et al. 2016; Kim et al. 2010; Bakardjiev, Stacy, and Portnoy 2005). Furthermore, conditioned medium from *Listeria monocytogenes*-infected-macrophages or the recombinant IL-1 β significantly up-regulated TNF- α , IL-6 and IL-8 production in SM9-1 mouse trophoblasts (Li et al. 2016). *Chlamydia trachomatis* infections of monocytes stimulates IL-1 β secretion through activation of NLRP3 inflammasome (Kim and Jo 2013; Abdul-Sater et al. 2010). Conversely, in THP-1 cells, Influenza A Virus NS1 protein was shown to inhibit the NLRP3 inflammasome by targeting NLRP3 and NF- κ β , leading to a reduction in levels of inflammatory cytokines as a viral immune evasion strategy (Cheong et al. 2015). Data have shown so far that H1N1 viremia is rare and that placental transmission of H1N1 does not occur, however preterm birth rates were 17-61% for women with influenza virus infection admitted to the intensive care unit (Kanmaz et al. 2011; Cetinkaya et al. 2011). Group B streptococci infection *in utero* of mice induced fetal injury and pre-term birth in both NLRP3 inflammasome-dependent and NLRP3 inflammasome-independent manners (Whidbey et al. 2015). Palmitic acid caused activation of NLRP3 inflammasomes and inflammatory responses, inducing IL-1 β , IL-6, and IL-8 secretion in human Sw71 first trimester immortalized trophoblast cells (Shirasuna et al. 2016). Studies using human first-trimester trophoblast cell lines, human

term placental explants and isolated human term cytotrophoblast cells showed that IL-1 β was produced in response to uric acid via the activation of the NLRP3 inflammasome (Mulla et al. 2011, 2013). The studies used an inhibitor of caspase-1 but did not show expression of NLRP3 within these cells. In my studies, I showed by Western blot that NLRP3 is not present in human primary cTBCs isolated from term pregnancies, suggesting that NLRC4 plays a more important role in sensing *S.Tm* (Fig. 22).

NLRC4 is essential for caspase-1 activation in macrophages infected with intracellular pathogens included *S.Tm* and *Pseudomonas aeruginosa* (Miao et al. 2010). Bacterial flagellin or components of the TTSS, such as PrgJ or needle protein, bind to neuronal apoptosis inhibitor proteins (NAIPs), and this promotes interactions between the NAIPs and NLRC4 (Kofoed and Vance 2011).

I have shown that cTBCs express NLRC4 but do not undergo pyroptosis. CTBCs are not the only cells that express NLRC4 and do not elicit pyroptosis. Neutrophil resistance to pyroptosis allows these cells to continue the production and secretion of mature-IL-1 β at the site of infection without dying.

5.13 SALMONELLA-INFECTION INDUCED CTBC DEATH MECHANISM

Successful pregnancy is dependent on normal placental function, commencing from implantation of the embryo, until delivery of the infant. Previous work in the laboratory using *in vivo* pregnant mice demonstrated that *S.Tm* infection adversely effects both maternal and fetal survival and that *S.Tm* grew profoundly in the labyrinthine trophoblast of the placenta leading to massive placental inflammation and maternal and fetal death (Pejcic-Karapetrovic et al. 2007; Chattopadhyay et al. 2010). The rapid loss of placental integrity observed *in vivo* in mice,

suggested that placental cell death might be crucial in *S.Tm* pathogenesis during pregnancy. Infection of invasive trophoblast cells by HPV induces apoptotic cell death and is associated with spontaneous preterm delivery and preeclampsia (Gómez and Parry 2009).

I showed that *S.Tm* infection in cTBCs succumb to necroptosis (Fig. 23). However, pathogens differ in their virulence, replication rate, intracellular habitats, and nature of inflammation evoked, thus these factors influence their interaction with the host. For example, *Listeria monocytogenes* proliferates in the cytoplasm allowing the host innate and adaptive immune response to rapidly control pathogen replication (Joseph and Goebel 2007). Placental cell death is increased upon *in vivo* exposure of both *Listeria monocytogenes* and *S.Tm* (Monnier et al. 2006; Chattopadhyay et al. 2010). However, studies have shown that necrosis in the placenta was not noted after maternal infection with *Listeria monocytogenes* in pregnant guinea pigs treated orally (Irvin et al. 2008; Bakardjiev, Stacy, and Portnoy 2005). Furthermore, another study has shown that cytosolic double stranded DNA from *Listeria monocytogenes* does not induce necroptotic cell death in trophoblast cells which could contribute to placental barrier against infection (Chu et al. 2014). Overall, relative to other intracellular pathogens that infect the trophoblast, the profound adverse effects evoked by *S.Tm* may be attributable to invocation of an inflammatory cell death mechanism, necroptosis, which may be expected to evoke significant collateral damage.

Type 1 IFN, tumor necrosis factor, and TLR3 and TLR4 agonists can trigger necroptosis (Christofferson and Yuan 2010). To protect against viral infection, trophoblast cells activate the TANK-binding kinase/ Interferon regulatory factor-3 (TBK-1/IRF-3) pathway leading to the expression of type I IFN (Bayer et al. 2016). However, human first-trimester trophoblast cells did not mount such a response when infected with Zika virus, suggesting that lack of IFN

response correlated with the lethal effect of Zika virus infection in the trophoblast cells. Mice lacking IFN signaling demonstrated high trophoblast Zika infectivity resulting in apoptosis of the cell and placental damage (Miner et al. 2016). In contrast, *S.Tm* has been shown to exploit host type I IFN signaling to kill macrophages through the induction of necroptosis (Robinson et al. 2012). TNF binding to TNF receptor 1 triggers a signaling reaction that culminates in binding of RIPK-3 with RIPK-1 forming a necroptosome which recruits MLKL. Phosphorylated MLKL forms oligomers that translocate to intracellular membranes and plasma membrane, which eventually leads to membrane rupture (Christofferson and Yuan 2010). Human primary cTBCs did not produce increases levels of TNF- α post *S.Tm* infection (Fig. 25), which indicates high bacterial burden within the cells. Necroptosis also occurs in mouse macrophages stimulated through TLR3 and TLR4. TLR3 and TLR4 respond to double-strand RNA and LPS respectively, by recruiting the RIP homotypic interaction motif (RHIM)-containing adaptor protein TRIF (TIR domain-containing adaptor-inducing interferon β). The RHIM in TRIF can engage the RHIM in RIPK-1 and RIPK-3 (Kaiser et al. 2013). Thus, in cTBCs infected with *S.Tm*, caspase-8 is inhibited and *S.Tm*-LPS induced necroptosis (Fig. 23). My results demonstrate that key players in the necroptotic pathway are activated upon *S.Tm*-infection in the cTBCs.

5.14 TROPHOBLAST CELL CYTOKINE RESPONSE TO *SALMONELLA* INFECTION

The innate immune system responds to invading pathogens by the activation of proinflammatory cascade aiming at eradication of the infectious agents. PRRs play a part of this innate immune reaction. Each PRR specifically recognizes conserved fragments of pathogens known as PAMPS. Upon recognition, a signalling cascade leads to the production of proinflammatory cytokines, type-1 IFNs and chemokines (Ospelt and Gay 2010).

TLRs have shown to play a role in preterm birth. TLR4 recognizes LPS which when LPS is delivered systemically, intrauterine, or intra-amniotically triggers preterm birth in animals. Moreover, mice deficient for TLR4 are protected against bacterial and LPS induced preterm birth (Elovitz et al. 2003; L. Li, Kang, and Lei 2010). TLR2-mediated preterm birth in response to Gram-positive bacterial peptidoglycan, has been found to be associated with excessive placental apoptosis (Ilievski, Lu, and Hirsch 2007; Jaiswal et al. 2013). The expression of all 10 TLRs has been described in the human placenta (Gonzalez et al. 2007). TLRs are highly expressed at the maternal-fetal interface on trophoblasts and uterine immune cells (Koga and Mor 2010). The expression pattern of TLR by trophoblast cells varies by gestational age as well as by the stage of trophoblast differentiation. In first trimester placentas, villous cTBCs and extravillous trophoblast cells express TLR2 and TLR4 (N. Kumar et al. 2016). It has been reported that TLR-4 signalling induces synthesis of cytokines in first-trimester cTBCs and TLR2 signaling promoted apoptosis (Tangerås et al. 2014). Syncytiotrophoblast cells do not express these receptors. The lack of TLR expression by the outer trophoblast layer suggests that early pregnancy placenta will only respond to a microbe that has breached the barrier (Beijar, Mallard, and Powell 2006). In term placenta, there is a different pattern of TLR expression. TLR2 and TLR4 are constitutively expressed on the surface of syncytiotrophoblast cells (Holmlund et al. 2002). Choriocarcinoma cell lines such as BeWo, JEG-3, JAR, AC1M-32 and ACH-3P broadly express TLR mRNA, but lacked functional cytokine responses to *in vitro* TLR ligand activation. This could be due to lack of TLR protein expression. TLR2 and TLR4 protein expression however, has been reported for JEG-3 and JAR cells (Gierman et al. 2015).

It is well established that TLR4 recognizes LPS motifs found on the majority of gram negative bacteria (Miller, Ernst, and Bader 2005; Akira and Takeda 2004). The TLR4 pathway

has been implicated in the pathogenesis of preterm births (L. Li, Kang, and Lei 2010; Pawelczyk et al. 2010). *Mycoplasma hominis* and *trachomonas vaginalis* infections are associated with preterm outcomes (Kataoka et al. 2006; Cotch et al. 1997). Both bacteria are gram negative and thus have a capacity to signal via TLR4 (Zuo, Wu, and You 2009; Zariffard et al. 2005; Thaxton, Nevers, and Sharma 2010). A number of animal models have established that gram-negative bacteria can trigger preterm labour, and this has been shown to be mediated by TLR4. Intrauterine injection of heat-killed *E.coli* into pregnant mice at gestational day 14 induced preterm delivery whereas in C3H/HeJ mice, which have a spontaneous mutation in TLR4, none of the mice underwent preterm delivery (Kaul et al. 1999). LPS administration has also been shown to change the cytokine profile by increasing maternal serum concentrations of TNF- α and IL-6, as well as placental expression of TNF- α , IL-6, and IL-1 α , which imply that systemic and local inflammatory responses induced by LPS administration can cause preterm labour (Salminen et al. 2008; Lin et al. 2006). Oral infection with human periodontal pathogens *Campylobacter rectus* and *Porphyromonas gingivalis*, causative agents of maternal periodontitis, is able to induce placental inflammation and TLR4 expression in pregnant mice (Arce et al. 2009). Furthermore, *Fusobacterium nucleatum* induces preterm and stillbirth in mice and activates both TLR2 and TLR4 *in vitro*. *In vivo*, the fetal death rate was reduced in TLR4 deficient mice, but not in TLR2-deficient mice, following *Fusobacterium nucleatum* infection. The reduced fetal death in TLR4 deficient mice was accompanied by decreased placental inflammatory responses. *Fusobacterium nucleatum* colonization in the mouse placenta was accompanied by inflammation, suggesting placental inflammatory response can be a factor in the pathogenesis of bacterial-induced pre-term birth (Han et al. 2004). TLR2 is required for optimal control of *Listeria monocytogenes* infection (Torres et al. 2004). Dephosphorylation of mitogen-

activated protein kinase (MAPK) signal transduction pathway by *Lm* LLO through the TLR2 signaling pathway induced placental immune cell, trophoblast giant cell death and caused abortion in mice (Hashino et al. 2015). Furthermore, infection of trophoblast cell lines with CMV evokes an inflammatory response that correlated to trophoblast cell death and preterm birth (Yamamoto-Tabata et al. 2004; Tabata et al. 2012). In human, studies support the association between elevated levels of circulating pro-inflammatory cytokines and preterm birth (Sata et al. 2009; Farina and Winkelman 2005). In the case of *S.Tm*-infection in trophoblast cells, anti-inflammatory cytokine IL-10 is produced and inflammatory cytokines such as IL-12, TNF- α , and IL-6 was not detected (Fig. 25).

5.15 THE ROLE OF STAT-3 IN CTBCS

IL-10 is noted for its abilities to inhibit macrophage proliferation by activating STAT-3 and to suppress IFN- γ -mediated functions of macrophages by blocking STAT-1 activation, thereby preventing the development of type-1 immune reactions that are deleterious for both the establishment and maintenance of pregnancy. In the pregnant human uterus, IL-10 is produced in large amounts by both the syncytiotrophoblast and the decidual macrophages. The IL-10 receptor is constitutively expressed on placental trophoblasts and the decidual macrophages are known to be highly responsive to IL-10 (Thaxton and Sharma 2010).

I have shown that *S.Tm* exerts its anti-inflammatory effect in association with activation of the JAK/STAT-3 pathway, with the increased expression of phosphorylated-STAT-3 (Fig. 28). *S.Tm* induces IL-10 dependent STAT-3 activation in cTBC cells which lead to proliferation of *S.Tm* in the phagosomes. Placental malaria caused by *Plasmodium falciparum*-infection increases placental plasma IL-10 (Kabyemela et al. 2008). CMV-infection leads to upregulation

of expression of IL-10 which impaired cTBC invasion in *in vitro* explant cultures. When IL-10 was added to *Listeria monocytogenes*-infected macrophages, the concentration of IFN- γ decreased (Kelly and Bancroft 1996). So far, the effect of IL-10 in *Listeria monocytogenes* infection during pregnancy has not yet been studied. IL-10 has been shown to stimulate HIV-1 infection of monocyte-derived macrophages by increasing the expression of CCR5, a chemokine receptor that plays a role in HIV entry. In lymphocytes, IL-10 decreases expression of CCR5 and increases expression of CXCR4 (Sozzani et al. 1998). During pregnancy, IL-10 renders cells more susceptible to HIV infections (Kwon and Kaufmann 2010). IL-10 is a multi-faceted agent of pregnancy. IL-10 levels increase markedly in women during early pregnancy and remain elevated well into the third trimester immediately prior to onset of labour (Holmes et al. 2003). *S.Tm* infections of cTBCs lead to higher secretion of IL-10 (Fig. 25). IL-10 during pregnancy is a suppressor of active maternal immunity which allows acceptance of the fetal allograft. Moreover, secretion of IL-10 by a diverse set of maternal and fetal cells has proven to aid in the orchestration of normal processes of pregnancy (Moffett and Loke 2004). Although IL-10 is important in fetal and placental development, it renders placental cells susceptible to intracellular infections. Studies have suggested that IL-10 plays a regulatory role during pathogen infection that modulates susceptibility. IL-10 produced by trophoblast cells modulates susceptibility to *Salmonella*-infection and neutralizing IL-10 activity strikingly resulted in increased phagosome maturation and curtailment of *Salmonella* replication (Fig. 26-27).

5.16 IL-10 PRODUCTION AND INCREASE IN PHOSPHO-STAT-3 EXPRESSION

Here in, we have not demonstrated the direct effect between IL-10 and STAT-3 phosphorylation has not been shown. Future experiments to examine the direct effect include using small (or short) interfering RNA (siRNA) to STAT-3 or STAT-3 clustered regularly

interspaced short palindromic repeats (CRISPR) in cTBCs, to degrade STAT-3 mRNA or to knockout STAT-3, prior to infection with *S.Tm* and examining the phagosome maturation pathway. Another experimental idea is to activate STAT-3 using EGF or IL-6 or colivelin peptide, which are activators of STAT-3, prior to infection and observing the phagosome maturation pathway.

CHAPTER 6:
CONCLUDING REMARKS
AND FUTURE
DIRECTIONS

6.0 CONCLUSION

In this Ph.D. thesis, novel insights into the mechanisms of *Salmonella* infection in human placental cells were presented. Primary placental trophoblast cells can be productively infected with *Salmonella*, and the intracellular milieu of early phagosomes supports profound bacterial replication have demonstrated. Furthermore, the trophoblast cells fail to evoke an effective immune response against *Salmonella*, which then triggers death of the placental cells through an inflammatory cell death pathway, necroptosis.

Overall, results obtained from *S.Tm* -infection in trophoblast cells *in vitro* have contributed to a better understanding of potential interaction and impact of infection during pregnancy. Briefly the main findings include (Figure 30):

[1] *S.Tm* entry in trophoblast cells not only utilizes their SPI-1 T3SS but also active phagocytosis through scavenger receptor A-mediated endocytosis.

[2] *S.Tm* infects and proliferates profoundly within placental trophoblast cell lines and primary human cTBCs. Trophoblast cells may thus provide a reservoir for systemic dissemination of *S.Tm* in maternal hosts.

[3] Once within placental trophoblast JEG-3 or primary human cTBCs, the bacteria are trapped within Rab5 positive early phagosomes and phagosomal maturation through fusion with lysosomes to form phagolysosomes is arrested.

[4] In contrast, unlike macrophages, trophoblast cells did not evoke an inflammatory cytokine response, failing to increase production of IL-12p70, TNF- α , and IL-6 upon *S.Tm*-infection.

[5] The inhibition of phagolysosomal maturation was correlated in trophoblast cell lines and primary human cTBCs to the production and signaling of anti-inflammatory cytokine IL-10 upon *S.Tm* infection.

[6] Human cTBCs undergo cell death through the inflammatory necroptosis pathway upon *S.Tm* infection:

- Lack of expression of apoptosis markers such as cleaved caspase-8, -3, or cleaved-PARP was noted.
- Lack of expression of pyroptosis markers such as cleaved caspase-1 or mature p10 subunit of caspase-1 was noted, and inhibition of pyroptosis did not rescue *S.Tm*-infected human primary cTBCs.
- Expression of necroptosis markers such as phosphorylated-RIP1 and phosphorylated-MLKL was observed and inhibition of necroptosis rescued *S.Tm*-infected human primary cTBCs.

Overall, in this thesis, I have elucidated the mechanism of pathogenesis of *Salmonella* infection at the feto-maternal interface. This knowledge can lead to novel approaches for preventing and/or managing placenta infection. It can also constitute the foundation for addressing the interaction of other placental-tropic intracellular bacterial pathogens with trophoblast cells. Understanding the trophoblast-pathogen interactions which may be the primary site of infection for pathogens such as *Salmonella* in the pregnant host, can help unravel the mechanism of systemic dissemination. My study also demonstrates the mechanisms of pathogen-induced placental inflammation which is considered a major cause of pre-term birth.

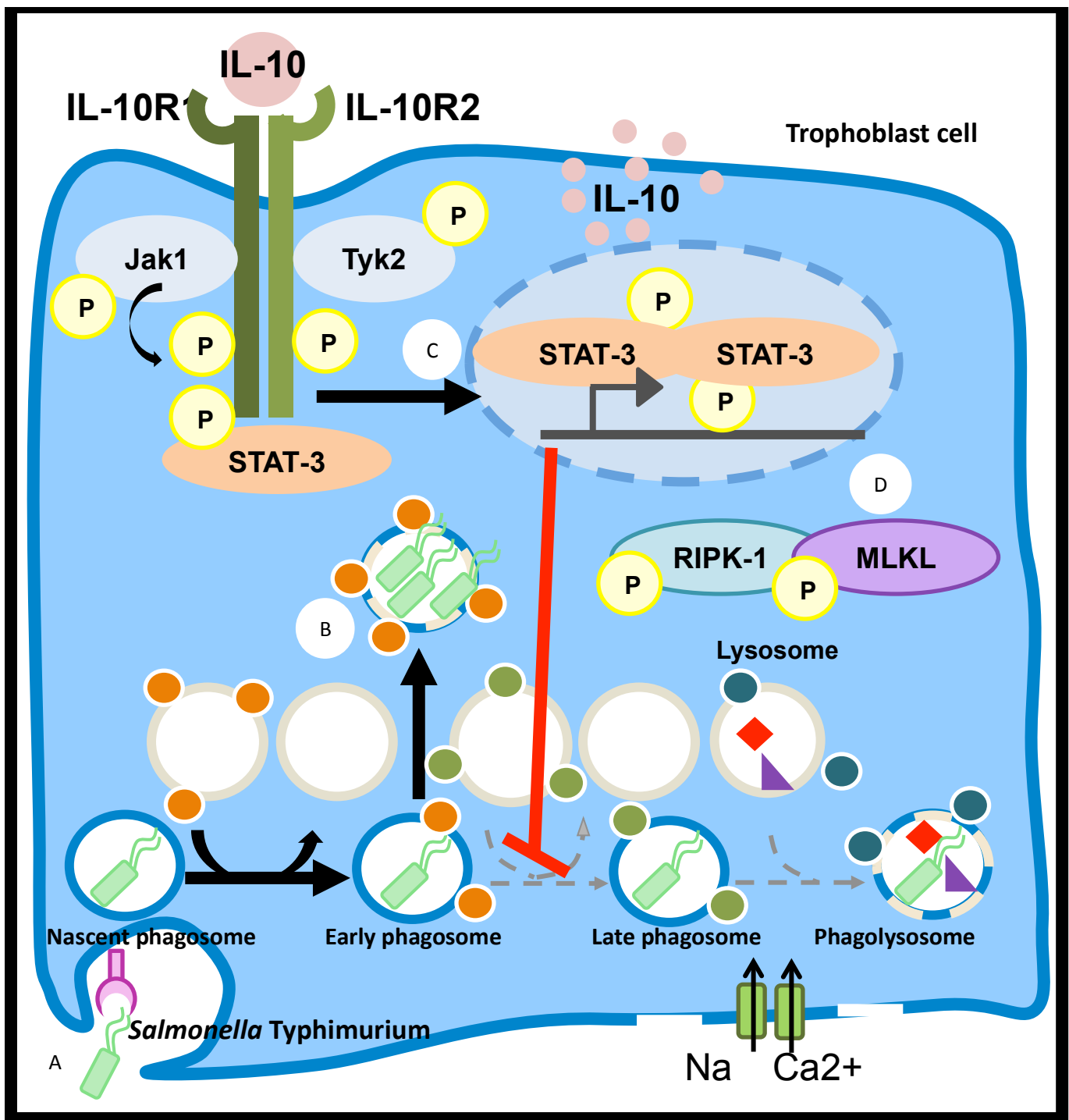


Figure 30. Schematic representation of important findings. The results obtained from the *in vitro* *S.Tm*-infection in trophoblast cells have contributed to a better understanding of infection during pregnancy. The main findings include: **A** *S.Tm*-LPS binds to scavenger receptor A which mediates phagocytosis. **B** Trophoblast cell phagosomes arrest at the early Rab5 positive phagosomal maturation state and do not fuse with lysosomes to form phagolysosomes. **C** Trophoblast cells produce IL-10 upon *S.Tm*-infected and IL-10 secreted by infected trophoblast cells exerts its effects on Jak1 and Tyk2, which phosphorylates STAT-3. **D** *S.Tm*-infected primary human cTBCs express necroptosis markers such as phosphorylated-RIPK-1 and phosphorylated-MLKL suggesting that *S.Tm*-infected trophoblasts succumb to necroptosis.

6.1 FUTURE DIRECTIONS

In this study, I have utilized *in vitro* human placental cell cultures as a convenient model to address the interaction of pathogens such as *Salmonella*. I utilized cell lines and/or defined primary placenta-derived trophoblast populations. Undertaking the study of interaction of *Salmonella* infection on placental explant cultures *in vitro* may be the next step to mimic *in utero* environments, in particular the implications of placental cellular architecture. Explant models involving placental tissue of first, second, and third trimester can provide complementary information by demonstrating how the placenta behaves during development. Additionally, studying infections of explant cultures in a perfusion model may further allow deciphering the role of placental physiology in the pathogenesis of infection. An *in vitro* placental perfusion with *S.Tm* followed by histology/confocal microscopy of placental sections can reveal the physiological entry interphase for the bacteria in an intact organ, and elucidate the specific cell types that are susceptible to *S.Tm* over a time course. I have revealed that *Salmonella*-infected placental trophoblast cells undergo an inflammatory cell death: necroptosis. This suggests that loss of integrity of the placenta due to inflammation may occur via a programmed cell death pathway. It needs to be explored if other kinds of inflammatory conditions of the placenta, such as pre-eclampsia and pre-mature birth are also associated with similar necroptotic inflammatory placental cell death. This knowledge can then further support development of novel interventions and/or management strategies to reduce inflammation-induced placental damage leading to pregnancy complications. Finally, I have demonstrated yet another role of placenta-derived IL-10 in modulating immunity to *Salmonella* infection. Understanding further the mechanism by which cytokine balance at the fetomaternal interface is maintained may have broader implications for managing maternal immunity during pregnancy.

Overall, food-borne infections are on the rise even in the developing world and are a serious concern for immunocompromised individuals. This thesis has added an important dimension in this context to reveal that specific pathogen interactions directly with trophoblast cells can be a key driver in the pathogenesis of infection in the pregnant host.

CHAPTER 7: **REFERENCES**

7.0 REFERENCES

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CHAPTER 8: **CURRICULUM VITAE**

8.0 CURRICULUM VITAE

TINA NGUYEN

EDUCATION

2010/1- In progress, (Degree expected date: 2017)

Doctorate – University of Ottawa and National Research Council of Canada - Faculty of Medicine - Department of Biochemistry, Microbiology and Immunology

Thesis title: “Mode of entry and survival of *Salmonella enterica Serovar* Typhimurium in placental trophoblast cells”

Supervisor: Dr. Lakshmi Krishnan

2005/9 – 2009/4

Bachelor’s Honours –University of Ottawa – Faculty of Science – Department of Biochemistry, Microbiology and Immunology - Honours with Specialization in Biochemistry

Thesis title: “Role of *Salmonella* virulence in modulating cytokine production by antigen-presenting cells”

Supervisor: Dr. Lakshmi Krishnan

WORK EXPERIENCE

2009/5 – 2009/9

National Research Council of Canada, Ottawa, Ontario, Canada

Summer Research Project title: “Factor(s) secreted upon *Salmonella* Typhimurium-macrophage interaction limiting intracellular growth of *Listeria monocytogenes*”

Supervisor: Dr. Nirmal Robinson and Dr. Lakshmi Krishnan

2007/5 – 2008/9 (Spring-Summer months only)

Thermo Fisher Scientific, Ottawa, Ontario, Canada.

Summer Student Customer Service Representative: Completed customer orders, quotes, and inquiries by telephone, email and/or fax for existing and prospective customers. Analyzed and resolved customer concerns using established procedures. Utilized supplier catalogues, online databases, and other appropriate resources to cross reference products to meet the customer order requirements. Made suggestions for alternative products when items are not readily available.

SPECIALIZED COURSES

Workplace Hazardous Materials Information System 2015 (WHMIS 2015)

Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans Course on Research Ethics (TCPS 2: CORE)

Canadian Council on Animal Care/National Institutional Animal User Training (CCAC/NAIUT) Program

AWARDS

2015/6 - Top 3 Clinical Research Poster Award (200 USD)

Recipient of the Top 3 Clinical Research Poster Award at the American Society for Reproductive Immunology, held at the Renaissance Inn, Kingston on June 02, 2015. Selected from a diverse pool of presentations, covering different aspects of medicine, science as well as technology.

2014/9 - Y.W. Loke New Investigator Travel Award (500 USD)

One of the 40 recipient of the Y.W. Loke Travel Award at the Federation of Placenta Association with applicants from all around the world. Held at the Paris City Hall, Paris on September 12, 2014. The purpose of the Y.W. Loke Travel Award is to recruit top-tier Canadian and international researchers and support them to attend conferences.

2013/9 - Placenta Journal Best Manuscript for 2013 (500 USD)

Recipient of the Placenta Journal Best Manuscript for 2013 Prize awarded by Elsevier. The criteria for the award include the novelty and significance of the research, the quality and quantity of the data, and the clarity of the presentation. Selected from over 70 candidates.

2013/2 – 3rd prize in the University of Ottawa Biochemistry, Microbiology and Immunology Student Research Seminar Day (50 CAD)

Recognized for my research. Selected from over 100 candidates.

SCHOLARSHIPS

2013/9 – 2016/12

Ontario Graduate Scholarship (15 000\$/year)

Ontario wide competition – A limited number of competitive studentships are offered by the Government of Ontario each year to highly qualified graduate students who are registered for a higher degree, and who are undertaking full-time research training in areas of sciences. Selection is based on academic performance, publication activity, research/academic or professional leadership and development, characteristics and abilities.

2012/9 – 2015/12 (11 900\$/year)

CIHR Training Program in Reproduction, Early Development and the Impact on Health

Canada wide competition - A limited number of competitive studentships are offered by the

Canadian Institutes of Health Research each year to highly qualified graduate students who are registered for a higher degree, and who are undertaking full-time research training in areas of sciences. Selection is based on academic performance, publication activity, research/academic or professional leadership and development, characteristics and abilities.

2013/9 – Current (3 000\$/year)

University of Ottawa Excellence Scholarship – Awarded to University of Ottawa students who hold an external scholarship.

2010/1 – 2013/9 (3 333\$/year)

University of Ottawa Admissions Scholarship – Awarded to University of Ottawa students who meet the GPA minimum of 8.0/10.

INTERNATIONAL COLLABORATION ACTIVITY

2013/8 – 2013/9

University of Rochester Medical Center, New York, United States.

Activity Description: Learned new techniques such as cytotrophoblast isolation from term placenta and placental explant whole mount in a National Institutes of Health grant collaborating laboratory, Dr. Shawn P. Murphy, at the University of Rochester, New York, United States. In addition to learning, I was able to contribute my knowledge by teaching Dr. Murphy's Ph.D Candidate *in vitro* *Salmonella*-infection.

VOLUNTEER EXPERIENCE

2012/2 – 2012/4

Director, Faculty Wellness Program Selection Committee, University of Ottawa, Ontario, Canada

Faculty of Medicine Graduate Student Representative: Representing graduate students in the Faculty of Medicine in the hiring process of a Director for the Faculty of Wellness Program at the University of Ottawa.

2012/3 – Current

Ottawa Regional Science Fair, Carleton University, Ottawa, Ontario, Canada

Junior Life Sciences Judge: Evaluating and selecting the winner in the junior life sciences category at the regional science fair.

CONTRIBUTIONS

PRESENTATIONS:

2016/4/2

Canadian Society of Immunology– Ottawa, Ontario, Canada

Title: “*Salmonella enterica* serovar Typhimurium productively infects human primary trophoblast cells and induces inflammatory cell death”

Conference was refereed

2015/8/20

National Research Council of Canada – Ottawa, Ontario, Canada

Title: “*Salmonella enterica* serovar Typhimurium productively infects human primary trophoblast cells and induces inflammatory cell death”

2015/6/2

American Society for Reproductive Immunology – Kingston, Ontario, Canada

Title: “*Salmonella enterica* serovar Typhimurium productively infects human primary trophoblast cells and induces inflammatory cell death”

Conference was refereed and awarded the Top 3 Clinical Research Poster Award

2014/9/9

International Federation of Placenta – Paris, France, Europe

Title: “*Salmonella enterica* serovar Typhimurium productively infects human primary trophoblast cells and induces inflammatory cell death”

Conference was refereed

2014/6/3

Reproduction, Early Development and Impact on Health - Montreal, Quebec, Canada

Title: “*Salmonella enterica* serovar Typhimurium productively infects human primary trophoblast cells and induces inflammatory cell death”

2013/6/12

Reproduction, Early Development and Impact on Health - London, Ontario, Canada

Title: “IL-10 Produced by Trophoblast Cells Inhibits Phagosome Maturation Leading to Profound Intracellular Proliferation of *Salmonella enterica* Typhimurium”

2013/3/11

Biochemistry, Microbiology and Immunology Student Symposium Day – University of Ottawa – Ottawa, Ontario, Canada

Title: “Mode of entry and survival of *Salmonella enterica* Serovar Typhimurium in placental trophoblast cells”

2012/5/31

Joint International Congress of the American Society for Reproductive Immunology & the European Society for Reproductive Immunology – Hamburg, Germany, Europe

Title: “Inhibition of phagosome-maturation leads to profound proliferation of *Salmonella* Typhimurium in trophoblasts”

Conference was refereed

2012/5/17

Biochemistry, Microbiology and Immunology Student Poster Day – University of Ottawa – Ottawa, Ontario, Canada

Title: “Mode of entry and survival of *Salmonella enterica Serovar* Typhimurium in placental trophoblast cells”

2011/6/12

6th Annual Human Placenta Workshop – Kingston, Ontario, Canada

Title: “Inhibition of phagosome-maturation leads to profound proliferation of *Salmonella* Typhimurium in trophoblasts”

PUBLICATIONS

JOURNAL ARTICLES:

2016/1 – Manuscript in Progress

Nguyen, T., Perry, I., Murphy, S., Krishnan, L.

“IL-10 production in human primary trophoblast cells infected of *Salmonella enterica Serovar* Typhimurium leads to profound bacterial proliferation,

2014/10 – Manuscript in Progress

Krishnan, L., Nguyen, T., Wachholz, K., Agbayani, G.

“*Salmonella* Infections in pregnancy: Pathogenesis and host immunity”

2016/1 – Manuscript in Progress

Nguyen, T., Perry, I., Wachholz, K., Gruslin, A., Murphy, S., Krishnan, L.

“*Salmonella enterica serovar* Typhimurium productively infects human primary trophoblast cells and induces inflammatory cell death”

2013/9 – Published First Listed Author

Nguyen, T., Robinson, N., Allison, S.E., Coombes, B.K., Sad, S. and Krishnan, L.

“IL-10 Produced by Trophoblast Cells Inhibits Phagosome Maturation Leading to Profound Intracellular Proliferation of *Salmonella enterica* Typhimurium”, *Journal of Placenta*, 34(9), 765-775

2013/3 – Published Co-Author

Krishnan, L., Nguyen, T., McComb, S.,

“From mice to women: the conundrum of immunity to infection during pregnancy”, *Journal of Reproductive Immunology*, 97(1), 62-73

CONFERENCE PUBLICATIONS

ABSTRACTS:

2016/4 – Published First Listed Author

Nguyen, T., Perry, I., Gruslin, A., Murphy, S., Krishnan, L.

Canadian Society of Immunology

“*Salmonella enterica serovar* Typhimurium productively infects human primary trophoblast cells and induces inflammatory cell death”

2014/9 – Published Third Listed Author

Wachholz, K., Agbayani, G., Nguyen, T., Gurnani, K., Krishnan, L.

International Federation of Placenta Associations

“Placental infection by *Salmonella enterica* Typhimurium in a murine model: mechanisms of pathogenesis and role of inflammatory cell death”

2014/9 – Published First Listed Author

Nguyen, T., Perry, I., Gruslin, A., Murphy, S., Krishnan, L.

International Federation of Placenta Associations

“*Salmonella enterica serovar* Typhimurium productively infects human primary trophoblast cells and induces inflammatory cell death”

2012/5 – Published First Listed Author

Nguyen, T., Robinson, N., Krishnan, L.

Join International Congress of the American Society for Reproductive Immunology & the European Society for Reproductive Immunology

“Inhibition of phagosome-maturation leads to profound proliferation of *Salmonella* Typhimurium in trophoblasts”

2011/8 – Published First Listed Author

Nguyen, T., Chattopadhyay, A., Krishnan, L.,

XI International Congress of Reproductive Immunology

“Host Immune and Pathogen virulence factors that contribute to placental colonization by *Salmonella* Typhimurium”