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Anindita Chattopadyay
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Microbiology and Immunology)
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Department of Biochemistry, Microbiology and Immunology
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Role of *Salmonella typhimurium* virulence in differentially modulating immune response and host susceptibility during pregnancy

TITRE DE LA THÈSE / TITLE OF THESIS

Dr. Lakshmi Krishnan
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

Dr. Subash Sad
CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

Dr. Ashok Kumar

Dr. Karen Copeland

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

Role of *Salmonella typhimurium* virulence in differentially modulating immune response and host susceptibility during pregnancy

By: Anindita Chattopadhyay

Supervisor: Lakshmi Krishnan

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Abstract

Pregnancy poses a high risk to many infections but it is unclear how maternal immunity is modulated by pathogen virulence. Typhoid fever and gastroenteritis caused by *Salmonella enterica* species are increasing globally and pregnancy poses a high risk. In 129X1Sv/J mice, *Salmonella enterica* serovar *typhimurium* (ST) infection normally leads to a systemic chronic disease. However, pregnant 129X1Sv/J mice succumb rapidly to ST infection. We aimed to decipher the host and/or pathogen-associated immune mechanisms that contribute to the loss of resistance to ST in pregnancy. Infection of pregnant mice with virulent wild-type ST promoted profound pathogen proliferation in the placenta, leading to loss of placental integrity by 72 hours. Severe systemic disease in pregnant hosts was associated with an acute increase in serum inflammatory cytokines/chemokines (ex. G-CSF, IL6, I-309). This correlated to increased placental expression of inflammatory cytokines, (G-CSF, IL-6, TNFα) and massive infiltration of polymorphonuclear cells (neutrophils) to the infected placental tissue. Contrastingly, even a high dose of the avirulent ST *aroA* mutant did not induce fetal loss or maternal illness, despite massive placental infection. This correlated to an anti-inflammatory cytokine signature (increased splenic IL10 and reduced systemic and placental inflammation) evoked by the *aroA* mutant. Thus, pathogen virulence rather than absolute bacterial burden critically influences the quality and/or quantity of the cytokine/cellular response to infection. This study provides a mechanistic insight as to why pregnant hosts are highly vulnerable to food and water borne pathogens, and may facilitate the development of potential therapeutics. ST infection in pregnancy also provides a convenient model to address the role of inflammation in placental pathology.

List of Abbreviations

Ab	Antibody
Ag	Antigen
APC	Antigen-presenting cell
BHI	Brain heart infusion
BLC	B lymphocyte chemoattractant
CCR	chemokine receptor
cDNA	Complementary deoxyribonucleic acid
CFU	Colony-forming units
DAB	Diaminobenzene
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
G-CSF	Granulocyte colony differentiation factor
H&E	Hematoxylin and Eosin
HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL1Ra	Interleukin 1 receptor antagonist
i.p.	Intra peritoneal
IPAF	ICE protease activating factor
IP-10	Interferon-gamma-induced protein
i.v.	Intra venous
LCMV	Lymphocytic choriomengitidis virus
LM	Listeria monocytogenes
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein -1
MCP-5	Monocyte chemotactic protein -5
MHC	Major Histocompatibility Complex
MIG	Monokine induced by gamma interferon
MLN	Mesenteric lymph nodes
MPO	Myeloperoxidase
Nacl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NAIP5	Neural apoptosis inhibitory protein 5
NALP	NACHT, LRR and PYRIN domain containing proteins
NaOH	Sodium hydroxide

NK	Natural killer
NOD	Nucleotide binding oligomerization domain
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PP	Peyer's patches
PRR	Pattern recognition receptor
S.C.	Sub cutaneous
SCV	*Salmonella*-containing vacuole
SPI	*Salmonella* pathogenicity islands
ST	*Salmonella typhimurium*
TBS	Tris buffered saline
TCR	T-cell receptor
TH	T helper
TLR	Toll like receptor
TNF	Tumor necrosis factor
TTSS	Type III secretion system
WHO	World Health Organisation
WT	Wild type

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1. Introduction

Salmonella enterica serovars are rod shaped gram negative, facultative anaerobes. They belong to the *Enterobacteriaceae* family and are facultative intracellular pathogens affecting around 21 million people annually, worldwide (1). More than 2500 serovars of *Salmonella* are known, which are highly related at genetic levels and are distinguished on the basis of their carbohydrate, flagellar and lipopolysaccharide structures (2). Mostly they are species specific, though some serovars infect both humans and animals, and cause discrete disorders in different hosts. *S. typhi*, *S. paratyphi and S. sendai* infect humans exclusively and cause typhoid or paratyphoid fevers which are severe systemic infections (1). In humans *S. typhimurium* (ST) causes food-poisoning or gastroenteritis, usually self-limiting in the gut, whereas in mice ST causes a fatal systemic typhoid-like disease in susceptible strains (3). Serovars such as *Choleraeuis* and *Dublin* mostly cause bacteraemia in humans, but Choleraesuis results in septicemia in pigs while *Dublin* causes bacteraemia and abortion in cows (4, 5). ST infection in mice is very similar to the pathogenesis of *S. typhi* in humans and hence is an excellent model to study systemic host immune responses to *Salmonella.*

Typhoid fever is caused by the intake of contaminated food and water and is more wide spread in the developing nations of Asia, Africa and South America with poor hygienic and sanitary conditions. Non-typhoidal intestinal disease is a global concern. With increased automation in food processing, *Salmonella* outbreaks are on the rise even in the developed world. Although, vaccines against typhoid fever such as the live attenuated strain Ty21a have been developed nearly two decades ago, they offer variable protection

against different serovars and have had limited use (6, 7). *Salmonella* species are also gradually becoming more resistant to most antibiotics (8). The young, elderly, pregnant, HIV-infected individuals form the high risk groups for *Salmonella* infections (9, 10). Much of our knowledge regarding the virulence mechanisms of *Salmonella* has been obtained through studies done on *in vitro* cultured epithelial cells and using the mouse model of *S. typhimurium* infection (11). However, relatively, less is known about the underlying mechanisms of virulence and immune evasion *in vivo* particularly in immunocompromised hosts.

Mammalian pregnancy is believed to be a state of selective immunological tolerance against the fetus and any defect in maintaining allo-tolerance may lead to pregnancy complications (12). Since maternal allo-reactive lymphocytes are not systemically depleted (13, 14) several local mechanisms at the feto-maternal interface appear to come into play to evade immunological attack on the fetus. For example, cytolytic activity of NK cells are inactivated in the uterine environment (15). Fetal antigen specific maternal T cell activity is suppressed by depleting tryptophan (16). Additionally Th1/Th2 cytokine balance is considered to be critical in maintaining fetal survival (17). Pregnancy is viewed as a Th2 phenomenon and there is a bias away from cell mediated immune response during a successful pregnancy (18). Suppression of Th1 immunity during pregnancy can weaken maternal responses to intracellular pathogens (19). Despite evidence of increased host susceptibility to intracellular pathogens during pregnancy (20, 21) the exact mechanisms in case of each infection is less clearly understood. *Salmonella* is known to cause several pregnancy complications in both humans and farm animals such as chorioamnionitis, trans-placental fetal infection, abortions and even life

threatening septicemia in the mother (22-24). ST infection in pregnant mice compromises both maternal and fetal survival (21). Thus the aim of this study is to unearth the specifics of host-pathogen interactions in the context of immune mediated alterations during pregnancy.

1.2 Salmonella pathogenesis

1.2 a) Invasion: Salmonellosis is normally caused by ingestion of contaminated food and water. The natural route of entry for *Salmonella* is the gastrointestinal tract. Pathogen entry into the systemic compartment occurs through the intestinal epithelial cells or enterocytes and specialized 'M' cells located in the small intestine (25). M cells are highly endocytic cells with microvilli at their apical end. They serve as a port of entry for the pathogen into the Peyer's patches (PP) which is its favored site of invasion (26). Dendritic cells (DC's) can also serve as vehicles to transport the bacteria from the lumen into the intestinal epithelium (27). An important feature of S*almonella* invasion is its ability to induce phagocytosis by normally non phagocytic cells (28). The bacterium induces host cell cytoskeletal changes and formation of membrane ruffles on epithelial cells. The bacteria are then rapidly internalized by these membrane ruffles on epithelial cells and once inside they lead to the formation of spacious vacuoles in both epithelial cells and phagocytes (29). On reaching the PP the bacterium encounters numerous resident macrophages and DC's. *Salmonella* infects the resident macrophages and induces Caspase 1 mediated cell death early on in the infection. This leads to the production of pro-inflammatory cytokines such as IL18 and IL1-β which in turn recruit additional phagocytes resulting in further dissemination of the bacteria to deeper tissues (30, 31). In the murine typhoid model, it has been seen that from the PP *Salmonella*

makes its way to the mesenteric lymph nodes, gains entry into the blood and then disseminates to spleen and liver (32).

1.2 b) **Salmonella *virulence genes:*** *Salmonella* has evolved a sophisticated array of virulence genes to support invasion and intracellular survival in the host. These virulence genes are clustered in two *Salmonella* Pathogenicity Islands (SPI), SPI 1 and SPI 2 located in distinct regions in the chromosome (33, 34). These islands code for two Type III secretion systems (TTTS) which are needle complexes which the bacteria uses to inject effector proteins into the host cell to interfere with the host's cellular processes. SPI 1 encoded proteins are used to gain entry into the gut epithelium and are significant in inducing early intestinal inflammation (35). SPI-2 encoded TTSS and effector proteins are involved in promoting intracellular survival such as evading killing by NADPH oxidase by macrophages and systemic proliferation (36, 37).

1.2 c) **Salmonella typhimurium *mutants:*** Much of our knowledge regarding *S. typhimurium* pathogenesis comes from invasion assays wherein cultured intestinal epithelial cells are infected with various *Salmonella* mutants The SPI 1 and 2 encoded TTTS is a central component of the *Salmonella* virulence machinery. A group of genes *invA, B, C* have been identified which confer the ability to invade epithelial cells. Null mutations in *invA* and *invC* result in a total absence of the needle structure of the TTTS (38). *S. typhimurium* strains with *invA* mutation are unable to invade intestinal epithelial cells in tissue culture models, though they can successfully attach on them and are significantly less virulent in murine oral infection models (26). However, in intraperitoneal infection models *invA* mutants are completely virulent, as they can bypass

invasion through intestinal epithelium to reach the systemic compartment. *Salmonella* strains auxotrophic for aromatic amino acids show high degree of attenuation as well. *Aro* mutants such as *aroA* and *aroD* are unable to produce aromatic metabolites like typtophan and tyrosine, which are not freely available inside the host and hence they are unable to replicate efficiently intracellularly (39, 40). The availability of *Salmonella* mutants has significantly enhanced our understanding of how virulence factors influence pathogenesis in the host.

1.3. Innate immune responses to Salmonella:

Upon oral ingestion of *Salmonella*, the intestinal epithelium forms the interface for triggering of innate immune responses. The Toll like receptors (TLR's) and the intracellular nucleotide binding and oligimerization domain (NOD) receptors form the two main families of Pattern Recognition Receptors (PRR's) in the intestinal epithelium which identify Pathogen Associated Molecular Patterns (PAMPS) on *Salmonella* (41). Prominently, Flagellin (monomeric subunit of flagellar machinery) and lipopolysaccharide (LPS) are recognized by TLR5 and TLR4 on the host cells respectively (42, 43). TLR4 interaction triggers both the innate cascade and directs adaptive immunity against *Salmonella*. For example, TLR4 deficient mice show increased suseptibility to infection in murine typhoid model (44). Nod 1 and Nod 2 detect *Salmonella* expressed peptidogylcan and muramyl peptides (45, 46). TLR-5 on the basolateral surface of intestinal epithelial cells interacts with *Salmonella* flagellin and is considered as a potent pro-inflammatory response mediator during intestinal inflammation in humans but does not appear to be critical in the murine typhoid model (47, 48).

In mice, many pro-inflammatory cytokines and chemokines are released in response to *Salmonella* by the intestinal epithelial cells (49), macrophages (50) and dendritic cells (51). Bacterial cell envelope components such as LPS evoke massive inflammatory cytokines like IFNγ, IL6, IL12, IL18, TNFα and MCP-1 which recruit immune cells to the intestinal tissue (52-54). Activation of the NFκβ signal transduction pathway and IL8 secretion has been shown to be critical in initiating pro-inflammatory responses to *Salmonella* (55, 56). Neutrophils are the first innate immune cell type to be recruited at the site of infection within a few hours of infection and are considered to be vital in murine defense responses to *Salmonella* infection (57). Neutrophil infiltration is followed by the influx of macrophages and immature DC's and these cell type numbers rapidly expand in the next 4-5 days in the spleen (58). Macrophage activation by cytokines such as IFNγ and TNFα is important for destruction of *Salmonella* (54). Natural killer cell numbers also go up in the gut during *Salmonella* infection and are an important source of IFNγ which is significant in triggering intestinal inflammation to the pathogen invasion (59).

However, *Salmonella* infection is challenging to the host immune system. Despite the inflammatory response, the bacteria are not completely eliminated from the system. Intracellular pathogens such as *Salmonella* have developed several different mechanisms to favor their survival in the host. *Salmonella* have the unique ability to induce phagocytosis even by non phagocytic cells (28). They survive inside modified vacuoles called *Salmonella* containing vacuoles (SCV) for extended periods in both macrophages and non phagocytic cells (60, 61). *In vivo* studies suggest that macrophages are the most

permissive cell types for *Salmonella* growth (62) and survival within the macrophages is crucial for systemic infection.

1.4. Evasion of T cell immunity by Salmonella

In contrast to the rapid influx of cells of the myeloid lineage following *Salmonella* infection, the number of B and T cells in the infected tissues does not alter considerably. Virulent *Salmonella* infection stimulates DC maturation but antigen (Ag) presentation by both MHC I and II is greatly diminished (63-66). *In vitro* studies show that *Salmonella* survives inside the macrophages and DC's for extended periods of times in spacious SCV's and it interferes with the phgosome-lysosome fusion in those cells (64, 65, 67). Escaping the toxicity of the lysosomes favors intracellular bacterial survival. Phagosomal lifestyle of ST has been attributed to decreased amount of bacterial antigens available to be presented by the DC's. Also, it is known to alter the expression of more than 40 genes and thus avoid T cell recognition (68, 69). This phenomenon has been reported as 'genetic phase variation'.

It has been shown that $CD4^+$T cells play an important role in clearing the bacteria during primary infection. Mice depleted of $CD4^+$T cells rapidly succumb to infection (70). In vaccinated mice depletion of $CD4^+$T cells results in decreased resistance to rechallenge (71). $CD4^+$T cells induce mainly a Th1 response to ST. $CD4^+$T cells have been shown to display an activated phenotype with ~ 20% of $CD4^+$T cells producing IFNγ upon *in vitro* restimulation following 2-3 weeks of ST infection. However, despite displaying an activated phenotype, the $CD4^+$T cells do not show substantial proliferation in response to ST infection (72). In a SM1 TCR transgenic mouse model which allows visualization of

Salmonella flagellin specific T cells, it has been shown that low doses of *Salmonella* infection does not lead to the activation of flagellin specific $CD4^+$T cells. It appears that a low challenge dose enables *Salmonella* to escape detection by flagellin specific $CD4^+$T cells (63). Thus, *Salmonella* appear to have devised intelligent ways of escaping $CD4^+$ T cell immunity.

Potent $CD8^+$T cell responses which are vital for the elimination of intracellular pathogens is substantially delayed in case of *Salmonella* infection. In the murine ST infection model, the bacteria proliferate rapidly (doubling time 26 min) but fail to generate a rapid $CD8^+$T cell response (73). The $CD8^+$T cells peak only around day 21. In addition, Sad et al have recently reported that $CD8^+$T cells generated in response to ST infection produce very little IL2 (important for the responsiveness and proliferation of $CD8^+$T cells) and are defective in their cytolytic function *in vitro* (74). Thus the delay in T cell immunity probably facilitates the establishment of chronic infection by *Salmonella* which lasts around 60-90 days beyond which the bacterial burden falls below detection level.

15. Mammalian Pregnancy

1.5a Immunoregulation during pregnancy: Pregnancy is an immunological paradox as the mammalian fetus that bears 50% of paternal (foreign) genes and has similar chances of rejection by the mother's immune system, as a transplanted graft, escapes rejection and thrives. The mother's immune system is not oblivious to the fetus but accepts the foreign entity by undergoing numerous anatomical, physiological and immunomodulatory processes to protect the fetus from destructive allo-immune responses (13, 75, 76). Since a mother's allo-immune responses are not systemically depleted, numerous local

mechanisms come into effect to prevent fetal rejection. These include mechanisms such as: expression of non classical MHC molecules on the trophoblast which inhibits NK cell attack (77); expression of indoleamine 2,3-dioxygenase (IDO) by the trophoblast which degrades tryptophan, thereby depriving maternal T cells of tryptophan and preventing their overt proliferation (78); significant increase in the population of $CD4^+$ $CD25^+$ T regulatory cells during pregnancy which facilitates tolerance (79); expression of Fas-Ligand by the trophoblast which causes apoptosis of any maternal lymphocyte expressing Fas if it recognizes paternal antigens (80). Despite several immunoregulatory mechanisms, anti-paternal lymphocytes do survive during gestation, although they remain hyporesponsive.

In the early nineties, Wegmann *et al* proposed that successful pregnancy favors a bias towards Th2 type cytokines and a shift away from Th1 responses (81). In accordance, it was shown that the placenta secretes an abundance of Th2 cytokines such as IL-10 and TGFβ that limit the secretion of Th1 cytokines such as IFN-γ and TNF. Furthermore, cytokines such as TNFα, IFNγ, IL2 and IL12 when administered to pregnant mice induced fetal resorptions, and IL10 could rescue resorptions rates (82) reiterating the benefit of a biased Th2 environment in pregnancy.

Moreover, the local Type2/humoral shift also appeared to influence the systemic maternal immune response as cell mediated autoimmune disorders such as rheumatoid arthritis ameliorate during pregnancy. In contrast, systemic lupus erythematosus (which involves excessive autoantibody production) is often exacerbated during pregnancy (83). In the same vein, women experience less exacerbation of multiple sclerosis during pregnancy, but the relapse rate often enhances during the post partum period (84). Nevertheless,

Th1/inflammatory cytokines appear to play a beneficial role in early implantation and may also trigger uterine changes conducive to labor (85). Furthermore, cytokines such as IL6, IL18, and most recently IL16 can play differential roles during different phases of pregnancy. Moreover, the Th1/Th2 cytokines bias in pregnancy may be modulated by non-immune/non-T cells such as trophoblast cells, dendritic cells and NK cells.

NK cell functions are tightly regulated as they play a key role in angiogenesis and spiral artery remodeling during the implantation and peri-implantation phases of pregnancy (86). A unique population of uterine NK cells which constitute the most abundant lymphocyte population in the uterus, but are compromised in their cytolytic ability, aid in this role (85, 87, 88). In contrast to the many mechanisms that act to suppress allo-responses in pregnancy, there are clinical and experimental reports indicating that certain components of maternal innate immunity may be activated even during normal pregnancy (89). An increase in the number of circulating monocytes with an activated phenotype (increased CD11b, CD14, elevated intracellular reactive oxygen species) in normal third trimester pregnancy has been demonstrated (90, 91). Neutrophil counts increase during gestation with their numbers almost doubling at term (92, 93). Monocytes from healthy pregnant women have been shown to produce high amounts of IL12 which is involved in differentiation of Th1 cells, upon *in vitro* stimulation (94).

Overall, a more encompassing current view of immunoregulation in pregnancy suggests a kinetic shift from implantation/peri-implantation Type I/inflammatory response to Type 2/anti-inflammatory bias during mid-gestation and finally a reversion to an inflammatory scenario triggering labor (95).

1.5b Infections and pregnancy: Clinically, although infections during pregnancy are common, pregnant women are not overwhelmingly susceptible to all infections. However, pregnancy may make the mother more vulnerable or more severely affected by infection with intracellular pathogens. Miscarriages and/or premature births are often caused by *Chlamydia trachomatis* and *Trichomonas vaginalis* (96, 97). These infections can breach the intermediate trophoblast layers which express TLR 2 and 4 and trigger inflammatory responses which can lead to placental apoptosis. Compromising the fetus may be a survival mechanism for the pregnant host in case of life endangering infections. Diseases such as leishmaniasis, toxoplasmosis and listeriosis can also be exacerbated during pregnancy (19, 20). In the U.S, up to one–third of listeriosis cases reported occur in pregnant women. In the malaria endemic regions, pregnant women are at a much higher risk of contracting malaria which is detrimental to pregnancy (98). Relapse of tuberculosis during pregnancy is also commonly seen in developing countries (99). Pregnancy complications due to *Salmonella* include endomyometritis, transplacental infection, chorioamnionitis, neonatal septicemia and abortions etc (22, 100). Krishnan et al have shown that down-regulation of Th1 responses during pregnancy worsens systemic *Leishmania major* infection, and the Th1 response to the pathogen is debilitating to pregnancy (19, 101). Importantly, most of the above infections mentioned are caused by intracellular pathogens which require vigorous cell-mediated T cell responses to eliminate them. Nevertheless, the majority of these infections are generally damaging to the fetus and only slightly alter maternal immunity. In most cases infections during pregnancy are not a threat to maternal survival.

1.5c Inflammation and pregnancy loss/placental pathology: While the above sections clearly outline the complexity of immune regulation during the different phases of mammalian pregnancy, it is widely accepted that overt placental inflammation leads to pathology and adverse outcomes. Regulated inflammation is important for reproductive success. Localized regulated inflammatory changes are required at several stages of pregnancy from implantation of the blastocyst into the uterine wall to parturition (102, 103). However a systemic exaggeration in inflammation during pregnancy results in a common pregnancy disorder called preeclampsia. It is a major cause of maternal and fetal mortality worldwide (104), caused by defective remodeling of uterine spiral arteries resulting in placental ischemia and production of inflammatory cytokines by the placenta (105). Plasma of preeclamptic women has been shown to contain elevated levels of pro-inflammatory cytokines such as IL12, TNFα, IL8, IL1β and IL6 (106-108). Predominance of these cytokines has been proposed to promote systemic vascular damage and pathogenesis of preeclampsia. Marked increase in the number of decidual macrophages and absolute neutrophil count along with highly activated monocyte and neutrophil populations have been reported in preeclamptic patients (109-111). Monocyte infiltration into the placenta has also been shown to be responsible for preterm delivery in infections such as malaria (112). Increased Th1 cell counts and more IFNγ production by PBMC's of preeclamptic women than normal pregnant women has been observed (113). Aberrant NK cell activation in the decidua is also an implicated mechanism in preeclampsia (114-116). NK cells may also become overtly activated through recognition of PAMPs leading to pregnancy loss (117). In case of intrauterine infection chorioamnionitis which is a leading cause of preterm labor, high levels of inflammatory

cytokines have been found in the amniotic fluid of the pregnant women (118). Thus overt inflammation, either endogenous dysregulation or extraneously triggered by infection, is a major factor contributing to poor pregnancy outcomes.

***1.5d Structure of the mouse placenta*:** The word placenta has been derived from the latin word 'flat cake'. The mammalian placenta is a remarkable organ which is indispensable for the growth and viability of the fetus. It allows delivery of nutrients from the mother to the fetus as well as protects the fetus from maternal immunological attack and ensuring its survival (119). Human and murine placentae have some very basic similarities; both consist of maternal and zygote derived cells and is thus considered hemochorial in nature, meaning that there is direct physical contact between maternal and fetal blood in the placenta (120). Since the focus of this thesis is the mouse model of pregnancy, the murine placentae structure is described in detail. Mouse placenta can be distinguished into three physiologically and anatomically discrete regions. The placental layer bordering the fetus is called the labyrinth. This region is highly vascular containing a network of maternal and fetal blood vessels. The labyrinth is crucial for the exchange of nutrients, gases and waste between the maternal and fetal blood. The ultra structure of labyrinth consists of three layers; the cytotrophoblast and two synctiotrophoblast layers. The labyrinth is lined by a layer of trophoblast cells along with extracellular matrix towards the fetal side, which is known as the chorionic plate. The adjoining layer to the labyrinth towards the maternal side is the junctional zone or the spongiotrophoblast zone. This zone is devoid of any fetal blood vessels and consists of giant spongiotrophoblast cells and trophoblast glycogen cells. Next to the trophoblast giant cell zone is the maternal uterine tissue known as the deciduas basalis. The glycogen trophoblast giant cells invade the decidua

intermittently forming feto-maternal interfaces. Normally, the deciduas basalis consists of a central maternal artery and numerous peripheral maternal veins which allow maternal blood flow in and out of the placenta (120, 121) (Illustration 1).

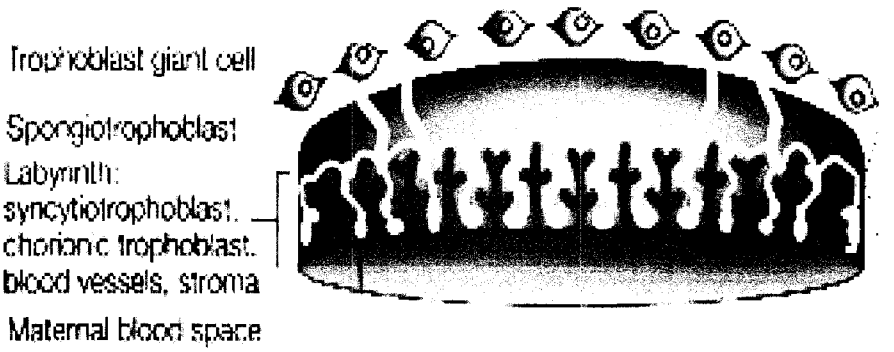

Illustration 1: Structure of the mouse placenta

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2. Rationale, hypothesis and objectives

2.1 What is missing? Many immune alterations occur during pregnancy to shield the fetus from maternal immunological attack. But how these immune alterations influence a mother's capability to fight infections is not clearly known. As discussed earlier inflammation is detrimental to pregnancy and hence is tightly regulated at the feto-maternal interface. Nevertheless, does the mother make the appropriate inflammatory responses during pregnancy, at least in the systemic compartment to retain her ability to fight infection?

Infections during pregnancy are common, especially in endemic areas. But usually, it is the fetus which is compromised. The mother by and large does not succumb to infections and only select pathogens or infections are a threat to a mother's survival. Infections caused by pathogens such as *Plasmodium falciparum*, *Mycobacterium tuberculosis*, *Salmonella enterica* sp. can be detrimental to the mother during pregnancy (9, 21, 122, 123). Are these highly virulent and evasive pathogens utilizing the 'altered immune status' of the pregnant host to proliferate unabatedly? **What pregnancy factors or immune components are responsible for weakened and/or altered immune responsiveness of the maternal host to certain pathogens or infections? The specifics of immune interactions between a pregnant host and an intracellular pathogen are not well elucidated.** Addressing these issues is critical for designing vaccines and devising interventions to safeguard the mother and the fetus against infections and better management of disease outbreaks.

***2.2 Rationale*:** Pregnant hosts show increased vulnerability to *Salmonella* infections. Studies done previously in our lab demonstrated that resistant mice which normally develop a non lethal chronic ST infection, upon getting pregnant and infected with ST, exhibit ~100% fetal loss and die within 7 days. This correlated to significantly lowered serum IL12 levels and elevated serum IL6 levels as compared to non pregnant infected controls. Besides, pregnant ST infected mice exhibited lack of recruitment/activation of innate immune cell types in the peripheral lymphoid organs. Thus, the systemic inflammatory/innate immune response to ST in maternal hosts was reduced (21).

Thus the primary question was: Does ST interact differentially with the feto-maternal interface, leading to rapid fetal death and adverse maternal outcome? Evolutionarily, a mother's survival is given preference over the fetus so that the mother survives for future pregnancies. Then why is ST infection lethal not only to the fetus, but a threat to maternal survival as well?

Several factors could be responsible for the above observations. ST is a highly virulent pathogen which devotes ~4% of its genome towards virulence mechanisms (124). Even in an immuno-competent host, adaptive immunity to *Salmonella* is significantly delayed (73). Although, immunotolerance during normal pregnancy is an area of active research, not many studies have addressed the mechanisms of immunity to intracellular infections such as ST in the pregnant host.

The observation that normally resistant mice become fatally susceptible to ST infection during pregnancy within 7 days implies that the first line of defense, innate immunity, against the pathogen is defective. Experiments outlined in this study were designed to

gain a rational understanding of how the specific interaction of ST with placental tissues and pregnancy-related immune components modulates pathogenesis of infection.

As discussed previously, ST has evolved a sophisticated array of virulence genes that cause fatal infection even in certain immunocompetent mice strains such as C57BL6/J. These elusive tactics used by ST may be even more drastic during pregnancy, due to the altered/weakened immune system. ST mutants differing in key virulence factors, also exbihit differential colonization habits, replication rates and nature of inflammation generated. Thus each mutant may interact with the host in a unique manner. Infecting the pregnant hosts with various ST mutants may aid in deciphering what ST effector mechanisms such as SPI 1 or 2 proteins or colonization properties facilitate immune evasion and/or modulate the function of innate immune cell types of the pregnant host.

2.3 Hypothesis:

Virulence, but not pathogen burden, triggers a unique innate immune signature and inflammatory response at the feto-maternal interface, accelerating pathogenesis in the maternal host.

2.4 Objectives:

1) **HOW DOES PREGNANCY ALTER HOST INNATE RESPONSES TO ST?**
    - 1.1 Characterization of ST wild type (WT) infection in pregnant and non pregnant 129.B6F1 mice.
    - 1.2 Assessment of the role of pregnancy hormones and T regulatory cells in influencing ST infection resolution.

2) **DOES BACTERIAL VIRULENCE INCREASE SUSCEPTIBILITY TO ST?**

2.1 Characterization of ST mutants (*invA* and *aroA*) infection in pregnant and non pregnant 129.B6F1 mice.

2.2 Examine the cytokines and cellular mechanisms that are triggered at the feto-maternal interface.

3. Materials and methods

3.1 Mice: 129.B6F1 mice were bred in the animal facility at the Institute for Biological Sciences of the National Research council (NRC-IBS, Ottawa, ON, Canada), by crossing 129X1Sv/J female x C57BL6/J male; both were acquired from The Jackson Laboratory (Bar Harbor, Maine). 129X1Sv/J or 129.B6 F1 mice were used for all studies since they do not succumb to ST, and develop a chronic infection in the non-pregnant state. 129X1Sv/J mice, ovariectomised 129X1Sv/J mice, 6–8 wk of age, were purchased from The Jackson Laboratory. Mice were maintained in the animal facility at NRC-IBS in accordance with the recommendations of the Canadian Council on Animal Care. For mating, 1 male mouse and 2 female mice were caged overnight and females were observed for vaginal plugs the following morning. The day of vaginal plug detection was considered day 0 of pregnancy.

3.2 Bacteria and Infection: *In vitro* growth of bacteria: ST (WT, *invA* SL1344, *aroA* SL3261) were grown in liquid culture in brain-heart infusion (BHI) medium (Difco Laboratories). At mid-log phase (OD_{600} = 0.8), bacteria were harvested and frozen at –80°C (in 20% glycerol). CFU were determined by performing serial dilutions in 0.9% NaCl, which were spread on BHI agar plates. Frozen stocks of bacteria were thawed and mice were inoculated with 1×10^3 (WT and *aroA*) or 1×10^6 (*aroA*) organisms suspended in 200 µl of 0.9% NaCl, via the lateral tail vein (i.v.). Infection was initiated at middle pregnancy (days 12-13) and in non pregnant age-matched mice.

3.3 CD25 depletion: For depletion of $CD25^+$ T cells *in vivo*, mice were treated with 100 μg/100 μl (i.p.) of mouse anti-CD25 antibody. Antibody was purified from hybridoma clone PC61 5.3 (ATCC, Manassas, USA) in the laboratory and administered (4 days pre infection or 1 day post infection).

3.4 Estrogen administration: Ovariectomised, non pregnant mice were treated with 4 or 40 μg β-estradiol (Sigma, Oakville, Canada) suspended in water (day 0, 1 and 2 of infection) (i.p.) or sesame oil (Sigma) (day -1 of infection) (s.c.). For estrogen administered in water a special carrier formulation of cyclodextrin encapsulated 17 β-estradiol was used.

3.5 Assessment of bacterial burden in organs: Mice were euthanized by CO_2 asphyxiation and spleen, liver, placentas/uteroplacental tissue, and fetal liver were aseptically removed. For the spleen, single-cell suspensions were obtained by squishing the organ between frosted ends of a glass slide. Liver, placentas (pooled per mouse), and fetal liver (pooled per mouse) were homogenized using a motorized homogenizer. In some cases, wherein it was difficult to discern the placentas of resorbed fetuses, the entire uteroplacental unit was homogenized. An aliquot of the cell suspension or homogenate was lysed with water for 30 s, and then ten-fold serial dilutions of the tissue homogenates, in 100-μl volume of 0.9% NaCl were plated on BHI agar. For assessing bacterial burdens at early time points after infection, the entire volume of tissue homogenate was spread onto several plates. Colonies were counted after 24 h of incubation at 37°C.

3.6 Assessment of pregnancy outcome: Fetal resorptions were identified by the notably smaller size and necrotic or hemorrhagic appearance of the fetus and/or placenta when

compared with normal viable fetuses and/or as resorbing necrotic scars in the uterus (Illustration 2). The percentage resorption rate was calculated using the formula $R/(R + V) \times 100$, where R is the number of resorbing fetuses and V is the number of viable fetuses per animal.

3.7 ***Cytokine ELISAs*****:** Blood was obtained by cardiac puncture under anesthesia, before euthanasia of mice, and collected in microtainer serum separator tubes (BD Biosciences, Mississauga, Canada). After the blood was allowed to clot at 4°C, the serum was separated by quick high-speed centrifugation, and stored at –70°C. Levels of serum IL6 and IL12p40/p70 were assayed by sandwich ELISA. IL6 (clone MP5-20F3) and IL-12p40/70 (clone 551219) antibody pairs were purchased from BD Biosciences, Mississauga, Canada. Cytokine standards were purchased from ID Labs (London, Canada). Duplicate standard curves encompassing several doubling dilutions of the standard were included on each plate. All serum samples were assayed at the same time to minimize day-to-day error in cytokine detection. The detection limits for cytokines in the ELISAs were 200 pg/ml for IL6, 100 pg/ml for IL12p40/70.

3.8 Mouse proteome array*:* Blood was obtained by cardiac puncture under anesthesia, before euthanasia of mice and collected in microtainer serum separator tubes (BD biosciences). After the blood was allowed to clot at 4°C, the serum was separated by quick high speed centrifugation, and stored at -70°C. Relative levels of 40 different mouse cytokines/chemokines were detected using a proteome profiler array from R&D systems (Minneapolis, USA). Each array kit consists of 4 nitrocellulose membranes each containing 40 different anti-cytokine antibodies printed in duplicate, detection antibody

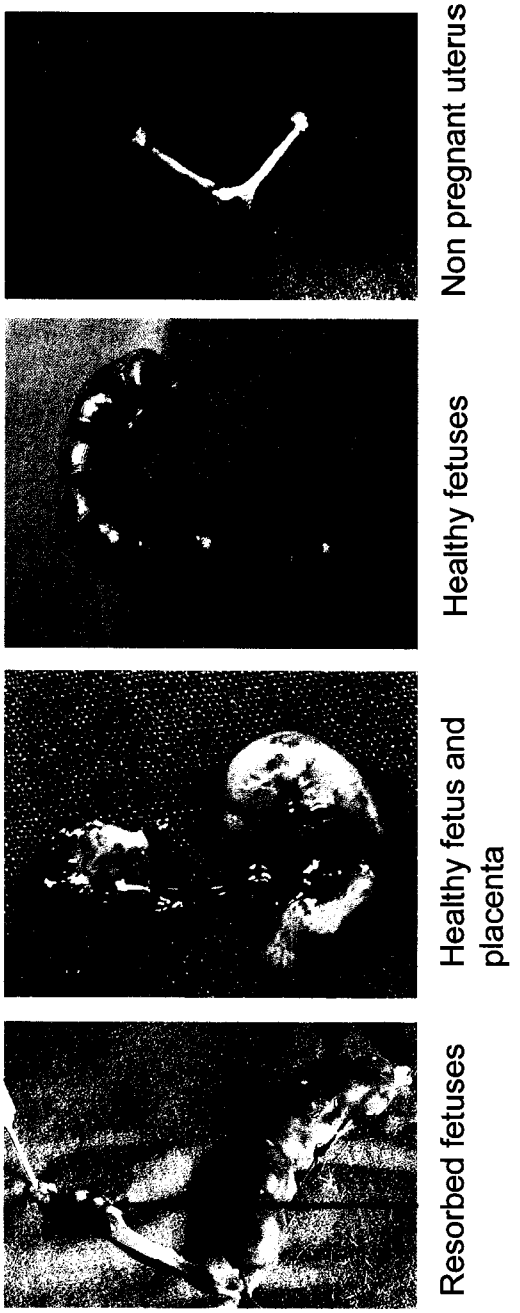


Illustration 2: Images of murine pregnant and non pregnant uterus

cocktail, array buffers (buffer number 4, 5 and 6) and wash buffer. Membranes were processed using the manufacturer's protocol and treated with Super signal West Femto chemiluminiscent detection reagent for 2-3 minutes. The photons emitted by the membranes were then captured by a Flourochem 8900 (Alpha Innotech) imager.

3.9 Assessment of spleen and placental cytokine expression by quantitative RT-PCR:

Spleen and placentas from individual mice were dissected out (placentas were pooled), and snap-frozen in a dry ice/100% ethanol bath. In the case of ST-infected pregnant mice, where it was not always possible to discern individual placenta, the whole uteroplacental unit was snap-frozen. Total RNA was extracted using the Qiagen RNeasy Mini kit according to the instructions of the manufacturer along with rapid mechanical lysis. Briefly, the spleens, placentas or uteroplacental units were cut into pieces and lysed in 1 ml of lysis buffer in a MiniBeadbeater 3110BX (BioSpec Products) with glass beads (ø = 0.5 mm and ø = 0.1 mm; BioSpec Products). Total RNA from homogenates was extracted and treated with RNase-free DNase I (Roche Applied Science) for 30 min at 37°C. DNase was then removed according to the instructions of the manufacturer. A total of 2–5 µg of total RNA was taken for cDNA synthesis. cDNA was synthesized using AncT primers (Sigma-Aldrich). RNA was made linear at 65°C for 5 min and cDNA was synthesized in a 40-µl reaction volume containing: 1.5 µl of AncT primers (100 pM/µl), 8 µl 5 x first-strand buffer, 4 µl of DTT (100 mM), 5 µl of dNTP (5 mM), 1 µl of RNase OUT (40 U/µl), 2 µl of Superscript II (200 U/µl) (Invitrogen Life Technologies), and 15 µl of RNA template. Reverse transcription was performed in a Thermo Cycler 9700 (Applied Biosystems) at 42°C for 15 min and 45°C for 2 h. Identical samples not treated with Superscript II were also prepared as controls to measure DNA contamination. The

remaining RNA template was hydrolyzed with 1 M NaOH at 65°C for 5 min and neutralized with 1 M HCl. cDNA was purified using Microcon YM-30 centrifugal filter units (Millipore). The number of amplicons was measured by quantitative real-time PCR using gene-specific primers and quantitative PCR SYBR green supermix (ABgene). Primers were designed using Primer Express 2.0. βactin was used as an internal reference control. Ten-fold dilutions of cDNA were used as template to generate the standard curve for each primer-template set (1x, 1/10x, 1/100x, 1/1000x). This standard curve was run together with triplicate reactions of the uncharacterized samples. PCR was performed in sealed tubes in a 96-well microtiter plate in an ABI Prism 7000 thermocycler (Applied Biosystems). The 25-µl reaction consisted of 12.5 µl of quantitative PCR SYBR green supermix, 2.5 µl of primer mix (1.5 pM/µl each), and 10 µl of template. Thermal conditions were as follows: activation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Fluorescence was measured during the annealing step and plotted against the amplification cycle. Relative quantitative analysis of the data was extrapolated from the standard curve. Primer efficiencies were 98–100%.

3.10 Immunohistological studies: 12-13 days pregnant mice were infected with ST WT or *aroA*. 24 h, 48 h and 72 h post infection placentas/uteroplacental unit were retrieved from each mouse and placed separately into identified tissue cassettes. These cassettes were placed in jars containing 4% para formaldehyde and allowed to fix for 24 h on a moving platform. After 24 h the fixative was discarded using institutional waste protocol and replaced with 70% ethyl alcohol. The fixed tissues were sent to University of Ottawa histology services (Dept., of Biochem., Microbiol., Immunol.,) for paraffin embedding

and tissue sectioning. 40 micron tissue sections were obtained and stained with Hematoxylin and Eosin (H&E) by University of Ottawa histology services. Briefly, the protocol followed was: slides containing paraffin sections were deparaffinized and rehydrated for 3 x 3 min toluene, 3 x 1 min 100% ethanol and 1 x 5 min deionized H_2O. Hematoxylin was filtered though coffee filters to remove oxidized particles. Hematoxylin staining: 1 x 7 min Hematoxylin (Fisher Scientific), rinsed with deionized water, 1 x 5 min tap water (to allow stain to develop) dipped 30 fast dips (total 15 sec) in Acid ethanol, rinsed 2 x 1 min with tap water and finally rinsed 1x 2 min with deionized water. Excess water was blotted from the slides. Eosin staining: 1 x 30 sec Eosin (Alcoholic Eosin/Potassium dichromate/Picric Acid Solution (AFIP 2nd Edition), 3 x 3 min 100% ethanol, 3 x 5 min with xylene or left overnight in xylene to get good clearing of alcohol. Slides were coverslipped using permount (xylene based) (Fisher Scientific) and slides were dried overnight in the hood.

Myeloperoxidase staining: The paraffin embedded placental sections were stained with Myeloperoxidase (MPO) antibody to stain for neutrophils at our own laboratory. The tissue sections were deparaffinized with xylene (3 x 5 minutes) and dehydrated in 95% ethanol (2 x 10 dips). The sections were rehydrated in running water for 5 minutes and transferred to Tris-Buffered Saline (TBS) for 5 minutes. Antigen retrieval was performed on the sections. 250 ml of 10 mM sodium citrate buffer, pH 6.0 was taken in a Coplin jar and warmed at 37°C for 5 minutes. The sections were placed in pre-warmed antigen retrieval solution such that they are completely covered with the sodium citrate buffer. The Coplin jar containing the sections and another jar containing water were microwaved on high for 5 minutes. Cooling was allowed for 5 minutes and again microwaved on high

for 5 minutes. Following the microwaving, the coplin jar was removed and cooled at room temperature for 60 minutes. After that the sections were transferred to running water for 5 minutes and then into 50 mM TBS. Next, to block endogenous peroxidase activity, sections were incubated in 3% H_2O_2 in TBS for 15 minutes in dark. Sections were then rinsed in TBS (2x for 5 minutes). Tissues sections were then covered with universal blocking solution (DAKO diagnostics Canada Inc., Mississauga) and incubated in a humid chamber at room temperature for 30 minutes. Excess blocking agent was removed and the sections were covered with MPO primary antibody (Thermo Fisher Scientific, USA) diluted 1:100 in antibody diluent (DAKO diagnostics Canada Inc., Mississauga) and incubated for 4°C overnight in a humid chamber. Next morning, excess TBS was shaken off and sections were washed in TBS (3x for 5 minutes). Next the sections were incubated with R.T.U biotinylated universal horse anti-mouse/anti-rabbit antibody (R.T.U Vectastain universal ABC kit) for 30 minutes at room temperature in a humid chamber. Sections were rinsed in TBS (3x for 5 minutes). Next sections were incubated with R.T.U ABC reagent (R.T.U Vectastain universal ABC kit) for 30 minutes at room temperature in a humid temperature followed by rinsing the sections in TBS (3x for 5 minutes). Sections were next incubated with diaminobenzene (DAB, 0.02%, Sigma) and H_2O_2 (0.0006%) for 10 minutes at room temperature with constant stirring in the dark. Sections were rinsed with TBS (2x for 5 minutes) and transferred to running water. Sections were counterstained with hematoxylin (filtered prior to use) for ~1 minute and then transferred to running water for 2 minutes. Sections were dipped in 0.2% acid alcohol 10x. Sections were transferred to running water for 2 minutes. Subsequently, sections were dipped in LiCO3 for ~30-40 seconds and transferred to running water for 2

minutes. Finally, the sections were dehydrated 2x for 5 minutes in 95% ethanol, 2x for 5 minutes in xylene and mounted in Dako cytomation permanent mounting media.

4. Results

Salmonella typhimurium infection in mice leads to contrasting host outcomes in different strains of mice. While susceptible strains of mice such as C57BL6/J succumb to even low doses of ST infection (10^2) i.v., within 7 days, resistant 129x1Sv/J mice develop a chronic infection lasting ~60-90 days with even higher dose of (10^3) bacteria. Previous studies in our laboratory demonstrated however that pregnant 129x1Sv/J mice upon infection with 10^3 ST, succumb to infection within 7 days with ~100% fetal loss. *Salmonella* infection initiated during mid-pregnancy resulted in ~1000 fold higher bacterial burden in the spleens in comparison to non pregnant mice rapidly resulting in loss of host resistance to infection (21). I first wanted to substantiate the above findings in an F1 hybrid (between susceptible and resistant mice) 129.B6F1 mice. It has been shown previously that 129.B6F1 mice are also as resistant to ST as 129x1Sv/J mice (73). Additionally, as 129.B6F1 strain of mice are better breeders than the 129x1Sv/J mice, I chose this strain to facilitate my studies on unraveling the mechanisms of increased susceptibility to *Salmonella* infection during pregnancy.

4.1 Exacerbation of virulent ST infection in pregnant 129.B6F1 hosts

Infection with 10^3 (i.v.) ST WT (SL1344) in middle pregnant 129.B6F1 mice resulted in rapid increase in the bacterial burden in the systemic organs. Within 3 days post infection, there was a massive increase in the bacterial burden in the spleen (Fig.1A) and liver (Fig. 1B) of pregnant mice relative to infected non pregnant controls. The aggravated infection in pregnant mice correlated to massive bacterial colonization of the placenta, with ~10^8 bacteria being recovered from uteroplacental tissue by 3 days of

infection (Fig.1C). This correlated ~100% fetal loss (Fig. 1D). Thus, the 129.B6F1 mice were also highly susceptible to *Salmonella* infection during pregnancy.

Infection by an oral route with 10^5 dose in C57BL6/J mice also showed increased susceptibility of pregnant mice to ST infection. While none of the non-pregnant mice exhibited detectable bacteria in their spleen on day 3 post-infection, all of the pregnant mice had signs of systemic infection as early as day 3, with CFU's ranging from 500 to 6000 bacteria. Furthermore, placentas of some mice showed CFU's as high as ~10^8, although there was variability among individual animals with some placentas remaining non-infected at this early stage after infection. Nevertheless, these results suggested that pregnancy poses an increased risk of systemic ST infection, even when initiated through the natural oral route.

Figure 1: Effect of ST WT infection in pregnant hosts. A) Splenic bacterial burden on day 3 after ST WT (10^3 i.v.) infection in non pregnant or pregnant 129.B6F1 mice. B) Liver bacterial burden on day 3 after ST WT infection in non pregnant or pregnant 129.B6F1 mice. C) Uteroplacental unit of 129.B6F1 mice at day 3 of infection with ST WT. Pregnant mice were infected in mid pregnancy (12-13 days). D) % resorptions on day 3 of infection with 10^3 ST WT (i.v). Each data point represents individual mice values. The median bacterial burden and resorption rate in various groups is indicated by a horizontal line. The bacterial burden in the spleen and liver of pregnant mice was significantly higher from non pregnant mice ($p<0.005$), and resorptions induced by infection was significantly higher than in non infected controls ($p<0.0005$), based on the two tailed, Mann-Whitney *U* test.

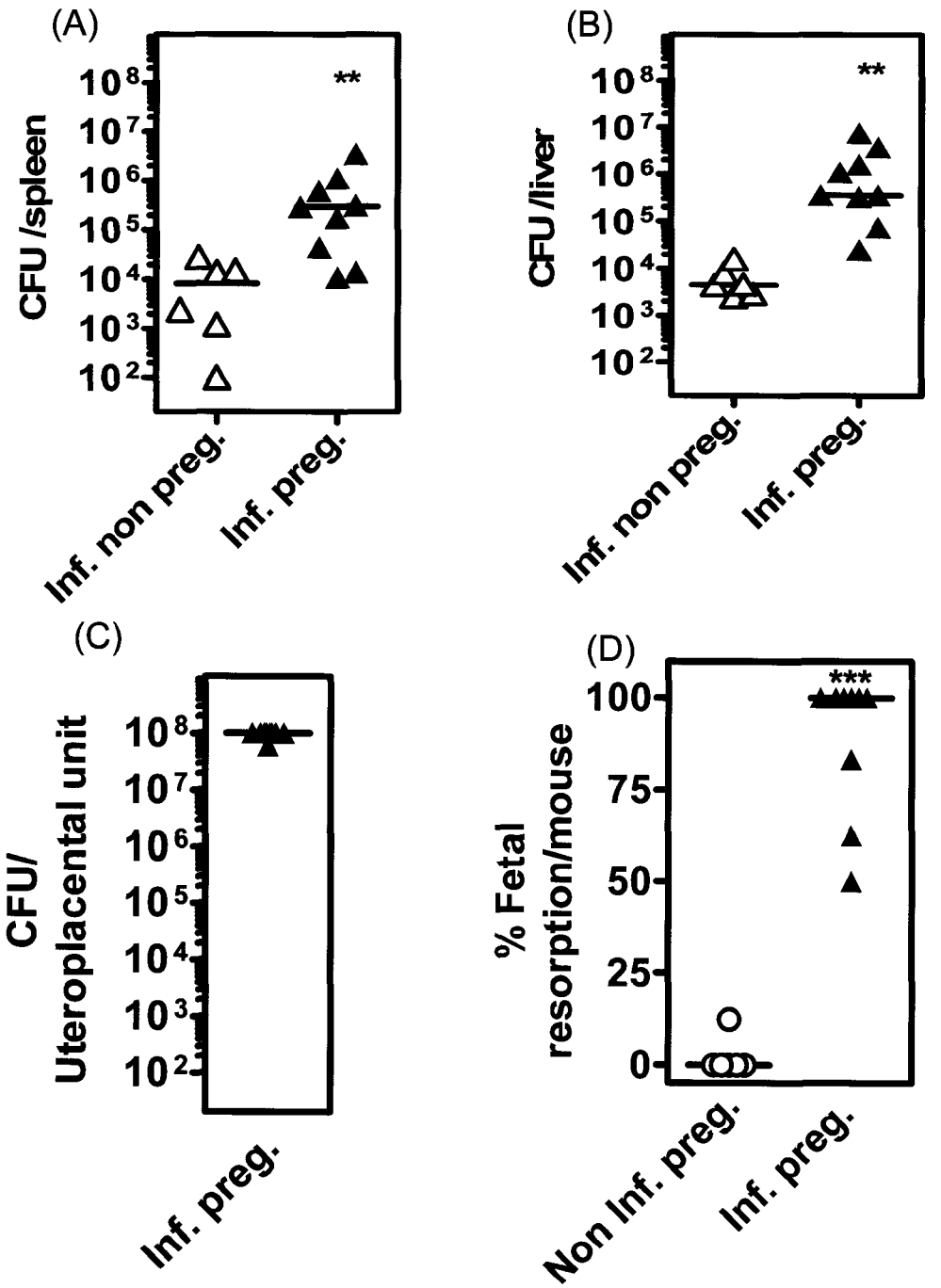


Figure 1

4.2 Pregnancy hormones: The susceptibility of hosts to ST-infection is not attributable to direct effects of pregnancy hormones.

Pregnancy hormones such as estrogen and progesterone have been known to bear immunomodulatory and immunosuppressive roles during pregnancy (125-127). Since, ST infection gets exacerbated during pregnancy, we examined if pregnancy hormones had any role in influencing host susceptibility to ST during pregnancy. Ovariectomised mice were treated with 4 or 40 μg of estrogen dispensed in oil 1 day prior to ST infection (Fig. 2A) or repeated daily injections of 1, 4 or 20 μg of estrogen in water (Fig. 2B),. Estrogen treatment did not alter normal host resistance of 129x1Sv/J mice to ST infection. Similar observations were obtained on treating the ovariectomised mice with progesterone (data not shown).

4.3 The susceptibility of pregnant hosts to ST infection is not attributable to direct effects of T regulatory cells.

$CD4^+$ $CD25^+$ regulatory T cells (T_{regs}) are reported to increase during pregnancy and are considered crucial in maintaining healthy pregnancy, particularly in the context of an allogeneic fetus (79, 128). To address whether T_{regs} may be responsible for suppressing pregnant host's innate immunity, we depleted T_{regs} *in vivo* by anti-CD25 antibody treatment. Depletions were carried out pre-infection (Fig. 3A) to deplete the natural T_{reg} populations and (Fig. 3B) post-infection to potentially deplete T_{reg} populations that may have induced/expanded post-infection in pregnant hosts. Fig. 3A indicates that depletion of T_{regs} prior to infection, results in a reduced trend in the day 3 splenic bacterial burden in the pregnant animals as compared to non-pregnant susceptible hosts. However, the observation lacked any statistical significance. Furthermore, depletion of T_{regs} in the

Figure 2: Effect of estrogen on ST WT infection. A) Non pregnant ovariectomised 129X1Sv/J mice were injected with estrogen (4 or 40 μg) (i.p), suspended in sesame oil, oil alone or no treatment followed by ST WT infection, 10^3 dose (i.v) the following day. The bacterial burden in spleen was determined 3 days post infection. B) Non pregnant ovariectomised mice were injected with water soluble carrier-associated estrogen (i.p) (1, 4 and 20 μg) followed by ST WT infection (10^3 i.v.). 2^{nd} and 3^{rd} doses of estrogen were given on days 1 and 2 post infection. The bacterial burden in spleen was determined on day 3 post infection. Data points represent bacterial burden in organs of individual mice, the median bacterial load is indicated by a horizontal line.

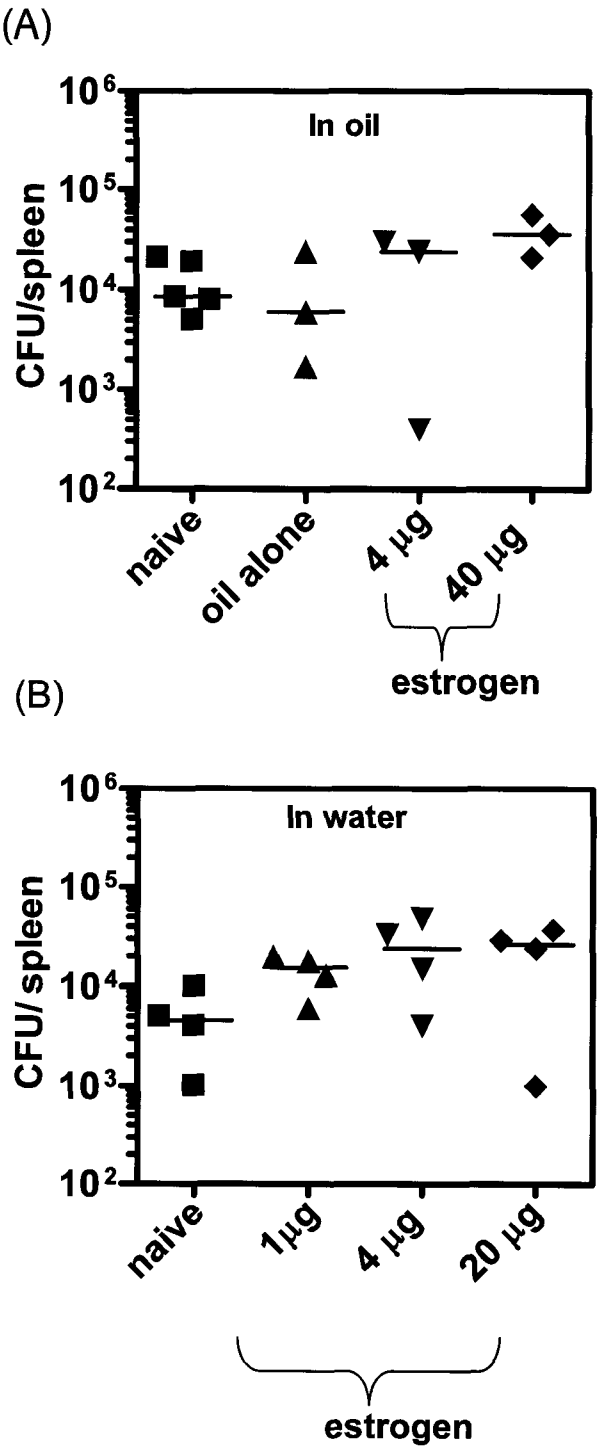


Figure 2

Figure 3: Effect of T regulatory cells on ST infection in pregnant hosts. A) T_{reg} depletion preceding ST WT infection. 150 μg of anti-CD25 antibody was administered (i.p.) into non pregnant or pregnant 129X1Sv/J mice. After 4 days, mice were infected with 10^3 ST WT (i.v.) B) T_{reg} depletion post-ST WT infection. 150 μg of anti-CD25 antibody was administered (i.p.) into non pregnant or pregnant 129X1Sv/J mice one day after infection with 10^3 ST WT. Bacterial burden in the spleen of individual mice was determined 3 days post infection. The median bacterial load is indicated by a horizontal line.

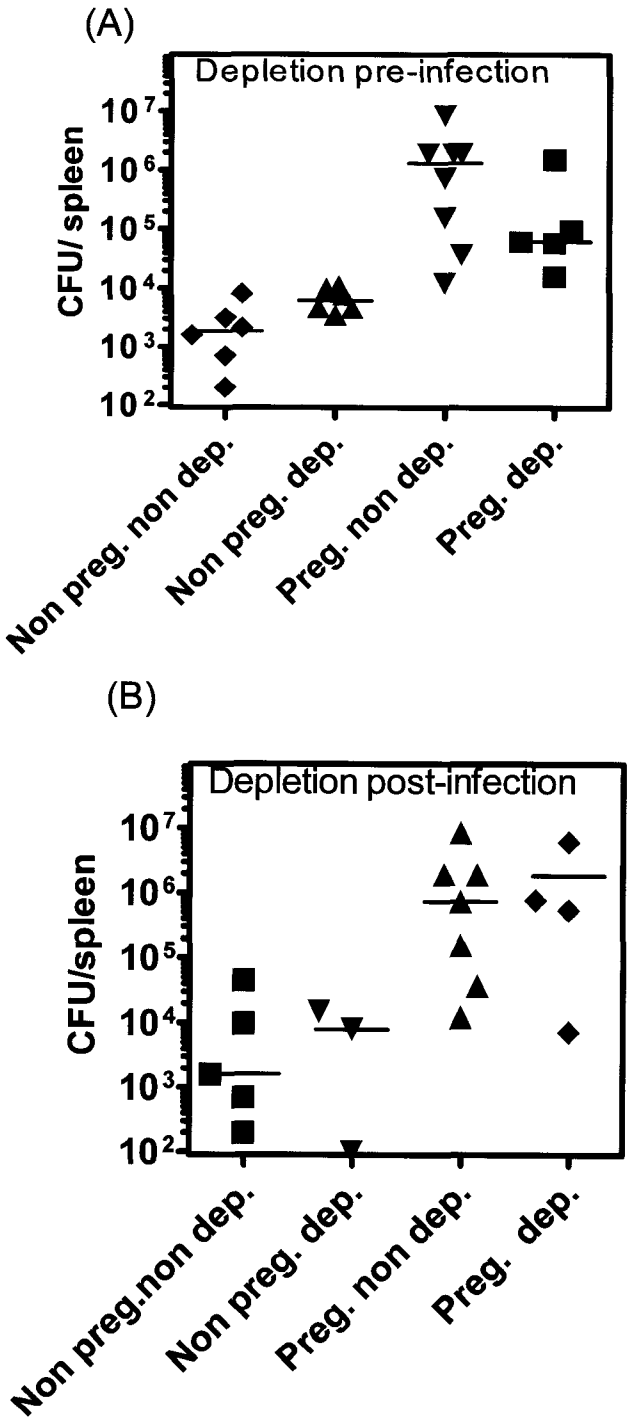


Figure 3

post-infection period did not confer resistance to the pregnant hosts. Thus, it is not clear if T_{regs} play a role in altering host resistance to ST during pregnancy.

4.4 Infection with mutant ST- *invA* is also exacerbated in pregnant hosts.

To further delineate host–pathogen interactions, we characterized infection with ST *invA* mutant in pregnant and non pregnant hosts. *InvA* strain has a mutation in the SPI 2 genes that alters invasive ability, and exhibit an attenuated infection profile after oral challenge (129). However, we noted that after intravenous infection, similar to ST WT infection, *invA* mutant resulted in increased bacterial burden in the organs of pregnant mice within 3 days of infection. ST *invA* infected pregnant mice showed increased bacterial burden in spleen (Fig. 4A) and liver (Fig. 4B) as compared to non pregnant mice. Furthermore, bacterial proliferation in the placenta was profound with $>10^8$ *invA* bacteria being recovered by day 3 (Fig. 4C). Nevertheless, the fetal resorption rates were highly variable among individual mice, and also between strains. For example, while 129x1Sv/J mice showed resorption rate of ~50%, 129.B6F1 mice had highly variable resorption rates, and the median resorption rate not being significantly different from healthy controls (Fig. 4D). Overall, systemic infection with *invA* mutant was no less severe than WT infection in pregnant hosts. Furthermore, placental invasion appeared to be unaffected by the *invA* mutation although its effects on fetal loss needs further investigation.

4.5 Pregnant hosts are resistant to infection with ST *aroA* strain

We next studied the effects of avirulent ST *aroA* mutant infection on pregnant hosts. ST *aroA* is an auxotrophic mutant and is a highly attenuated strain. 10^3 ST *aroA* results in a

Figure 4: Effect of ST *invA* mutant infection on pregnant hosts. A) Splenic bacterial burden on day 3 of ST *invA* infection in non pregnant or pregnant 129.B6F1 or 129X1Sv/J mice. B) Liver bacterial burden on day 3 of ST *invA* infection in non pregnant or pregnant 129.B6F1 or 129X1Sv/J mice on day 3. C) Uteroplacental bacterial burden on day 3 of infection in pregnant 129.B6F1 and 129X1Sv/J mice. Pregnant mice were infected in mid pregnancy (12-13 days). D) % resorptions per mouse on day 3 of infection. Mice were injected with 10^3 ST *invA* (i.v). Data points are indicative of values for individual mice and the median bacterial burden and resorption rate in various groups is indicated by a horizontal line. The bacterial burden in the spleen and liver of pregnant mice in both the strains was significantly different from non pregnant mice ($p<0.001$); based on the two tailed, Mann-Whitney *U* test.

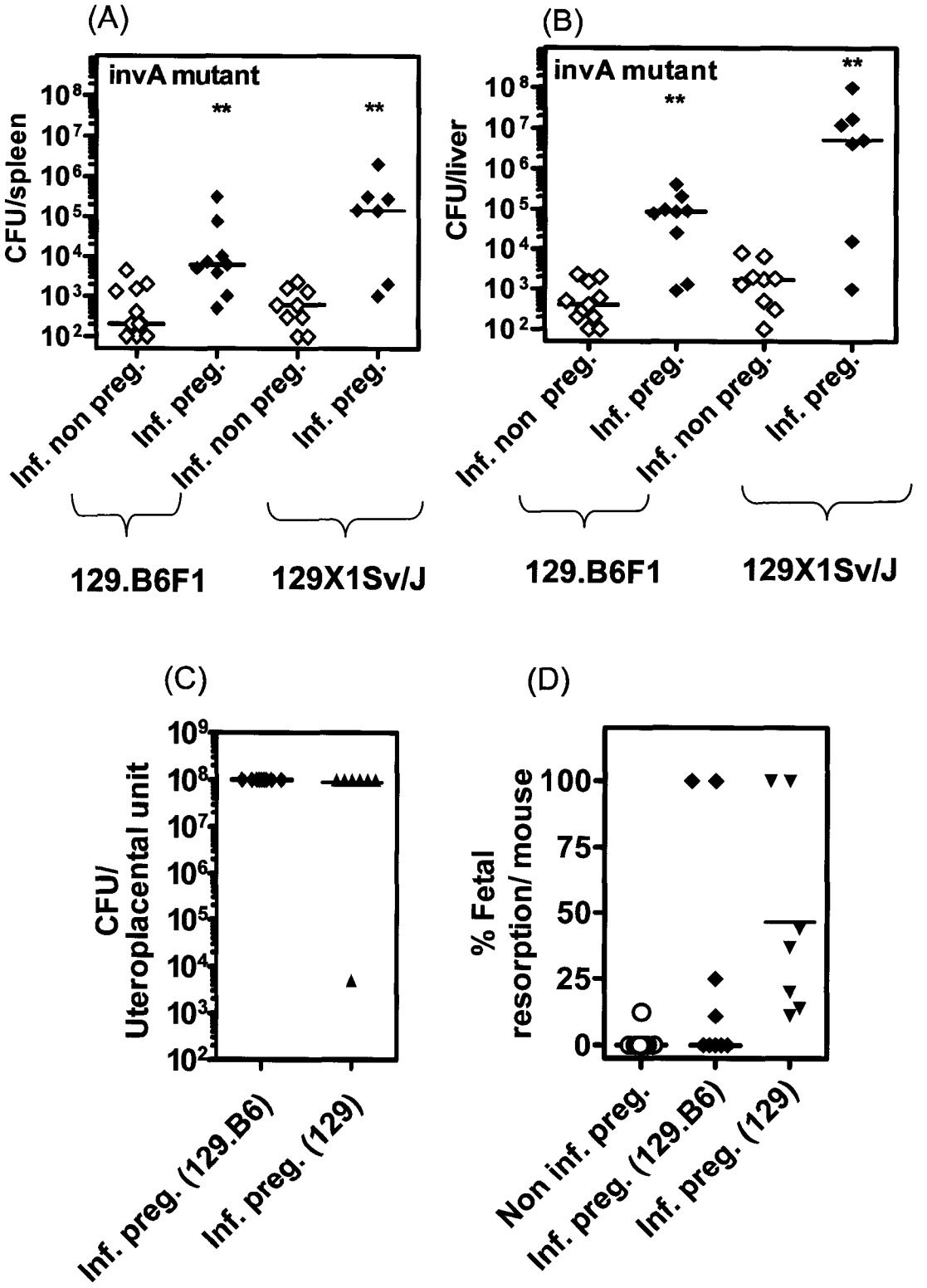


Figure 4

very low bacterial burden in the peripheral organs of infected mice (Fig. 5A). Furthermore, there was no increased susceptibility observed with pregnant hosts. Thus, a higher infection dose (10^6) was chosen to achieve a bacterial burden comparable to that of ST WT in the peripheral organs of non pregnant infected mice. ST *aroA* (10^6) infection exhibited similar bacterial burden in the organs of pregnant and non pregnant mice (Fig. 5B, 5C). Even with a higher dose infection, the pregnant mice did not show significantly increased bacterial burden in the peripheral organs relative to non pregnant controls. Interestingly, the placentas showed increased bacterial colonization similar to that of ST WT infection (Fig. 5D). However, despite high bacterial colonization of the placenta, in contrast to ST WT infection, the fetal resorption rate in case of ST *aroA* infection was significantly low with both 10^3 and 10^6 dose (Fig. 5E). Indeed, the resorption rates in the ST *aroA* infected animals were comparable to background resorption rates of non-infected healthy pregnant mice.

4.6 Kinetics of ST WT versus *aroA* infection

Next we examined the kinetics of ST infection in order to compare the rate of replication of WT and *aroA* mutant strains in the systemic tissues of non pregnant and pregnant hosts and in the placentas of the pregnant mice.

After infection with 10^3 ST WT (Fig. 6A), within 24 h a bacterial burden of $>10^5$ was observed in the spleens of pregnant mice. In contrast, the non pregnant mice did not show a rapid increase in the bacterial burden and a CFU of $\sim10^3$ was recovered after 24 h. Over the next 48 h the CFU increased to $>10^6$ in case of pregnant mice infected with WT ST, whereas the non pregnant mice showed a CFU of $<10^4$. Therefore, pregnant mice

exhibited a rapid rate of bacterial replication in the peripheral organs and a significant difference in the bacterial burden in the pregnant and non pregnant mice was evident as early as the first 24 h.

In case of *aroA* infection (Fig. 6B), even after an infection with 10^6 bacteria, both pregnant and non pregnant mice exhibited muted bacterial replication and exhibited a CFU of ~10^5 in the first 24 h. In the next 48 h the splenic bacterial burden did not change considerably in the non pregnant and pregnant mice, though the pregnant mice showed a marginal increase in the splenic burden as compared to non pregnant mice.

In the placenta, 10^3 WT and 10^6 *aroA* showed very similar trend. Within the first 24 h both the infections resulted in a burden of ~10^8 in the placentas. The burden in both cases showed minor increase over the next 48 h (Fig. 6C)

Thus, with a 10^6 *aroA* infection, an absolute bacterial burden in the systemic organs and at the feto-maternal interface similar to infection with 10^3 ST WT could be achieved. Nevertheless, it was clear that the *aroA* mutant does not replicate substantially relative to the initial intake infection.

As described in Fig. 1C and Fig. 5D, both ST WT and ST *aroA* result in massive placental infection. Nevertheless, ST *aroA* does not cause fetal loss. Thus, we wanted to ascertain if both strains of ST lead to invasive fetal infection. Fetal livers were obtained 24 h after maternal infection with either ST WT or ST *aroA*. As shown in Fig. 7, the fetal livers were infected in all cases showing a variable bacterial burden between 100 and 10^7 bacteria. Thus, placental infection with both virulent and attenuated ST resulted in

vertical transmission of infection. Nevertheless, only the virulent bacterium triggered fetal loss. Indeed all ST *aroA* infected pregnant mothers went on to have full term pregnancies and delivered live pups. These pups survived into adulthood even though many were infected (data not shown) and were euthanized ~40 days post-partum.

Comparing the survival rate of ST WT and ST *aroA* infected 129.B6F1 pregnant and non pregnant mice, we observed that ST WT infected non pregnant mice survive for more than 100 days. However ~80% of the pregnant ST WT infected mice succumbed to infection within 3 days (Fig 8A). In contrast, both non pregnant and pregnant ST *aroA* infected mice go on to survive for more than 100 days, even when infected with a high dose (10^6).

Overall, ST *aroA* (10^6) infection provides a convenient model for further study of mechanisms of host resistance as despite bacterial colonization of both systemic and placental tissue (that is comparable to WT virulent strain) there is no adverse consequence for the host and fetus.

4.7 Serum cytokine profiling

The severity and/or end result of *Salmonella* infection is dependent on many host and pathogen factors. Cytokines are often crucial regulators of host immune response to infection by intracellular pathogens. Previous results from the lab have shown that ST WT infection in 129X1Sv/J mice, results in increased IL6 and reduced IL12 levels in the serum of pregnant infected mice as compared to non pregnant infected controls. Therefore, we examined, if a similar trend was observed in 129.B6F1 strain as well.

Figure 5: Effect of ST *aroA* mutant infection on pregnant hosts. A) Splenic bacterial burden on day 3 after ST *aroA* 10^3 (i.v) infection in non pregnant or pregnant 129.B6F1 mice. B) Splenic bacterial burden on day 3 after infection with 10^6 ST *aroA* in non pregnant and pregnant 129.B6F1 mice. C) Liver bacterial burden on day 3 after ST *aroA* 10^6 infection in non pregnant or pregnant 129.B6F1 mice. D) Bacterial burden in the placentas of pregnant 129.B6F1 mice on day 3 of infection with ST *aroA*. Pregnant mice were infected in mid pregnancy (12-13 days). E) % resorptions on day 3 of infection with 10^3 and 10^6 ST *aroA* infection. The median bacterial burden and resorption rate in various groups is indicated by a horizontal line.

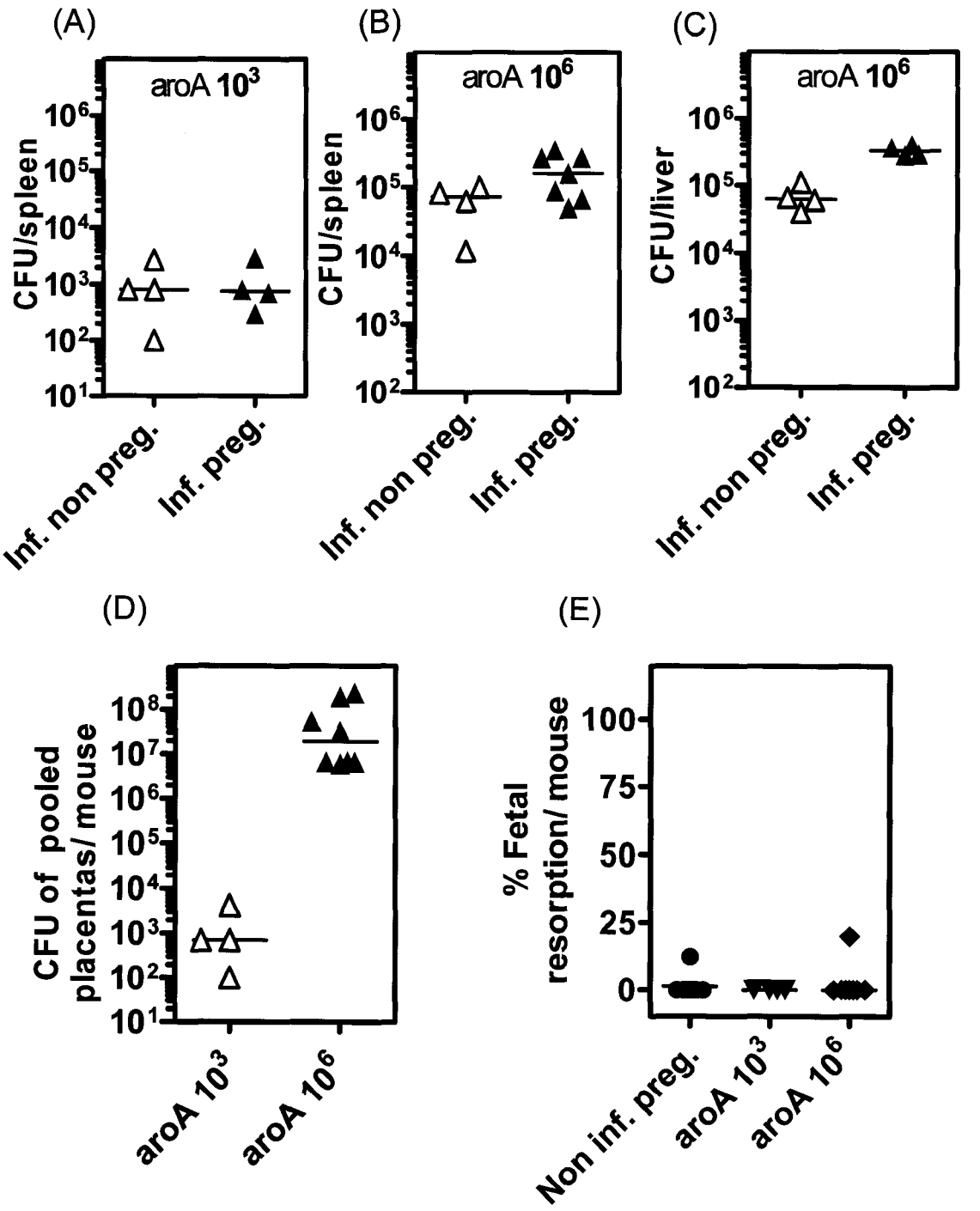


Figure 5

Figure 6: Kinetics of ST WT versus *aroA* infection in spleen and placenta. Non pregnant or pregnant 129.B6F1 mice were infected with 10^3 ST WT or 10^6 ST *aroA* (i.v) Bacterial burden in the Spleens (A & B) and uteroplacental units in case of ST WT and placentas in case of ST *aroA* infection (C) (n=3-6/time point) were determined at 24 and 72 hours post infection. Mean ± SEM of CFU over time in the spleen and placentas is indicated.

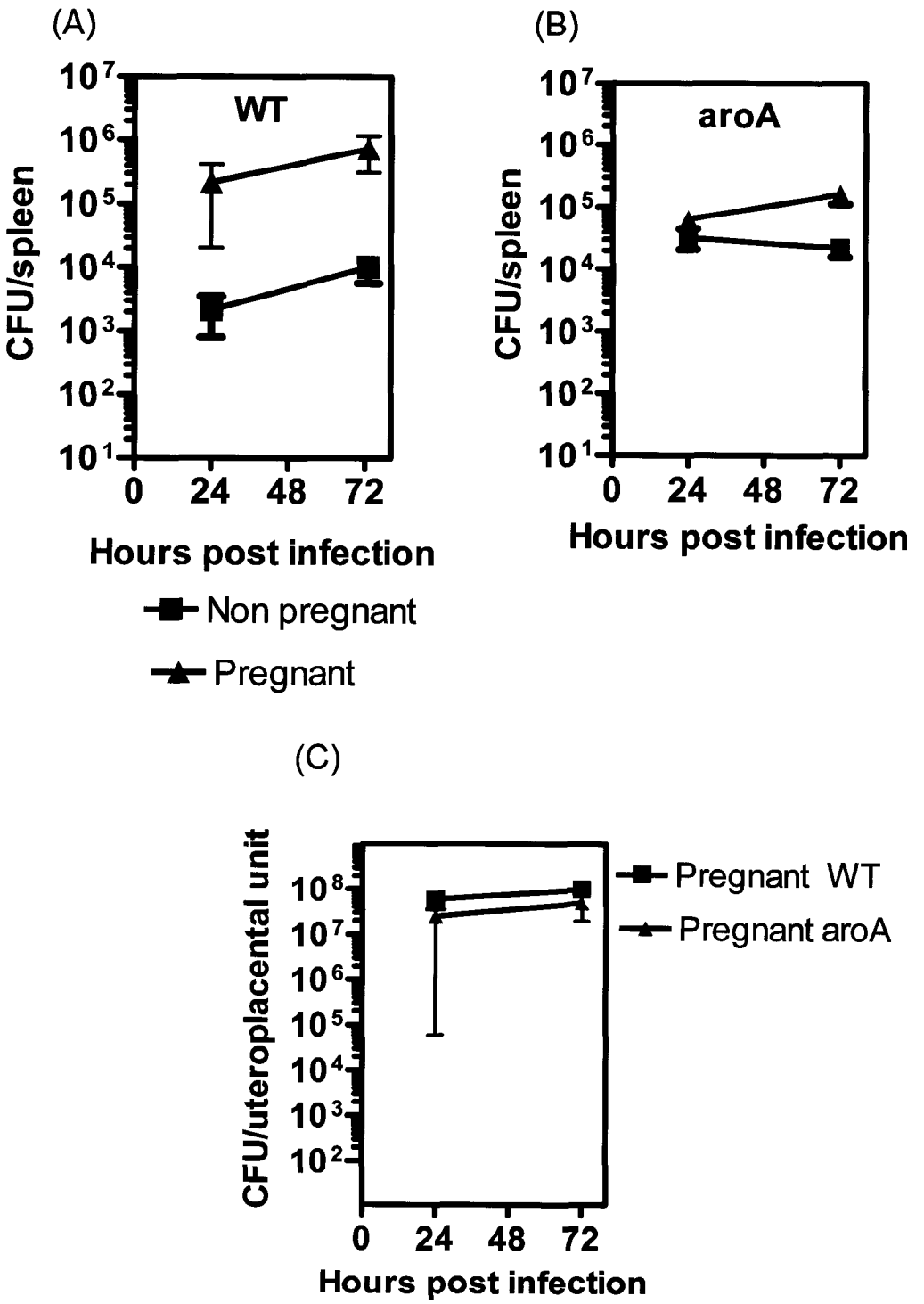


Figure 6

Figure 7: Invasive fetal infection with ST WT and ST *aroA*. 129.B6F1 mice were infected with 10^3 ST WT or 10^6 ST *aroA* in middle pregnancy. 24 h after infection, fetuses from each mouse was recovered, the livers pooled and bacterial burden assessed. Median fetal liver bacterial burden is indicated with a horizontal line.

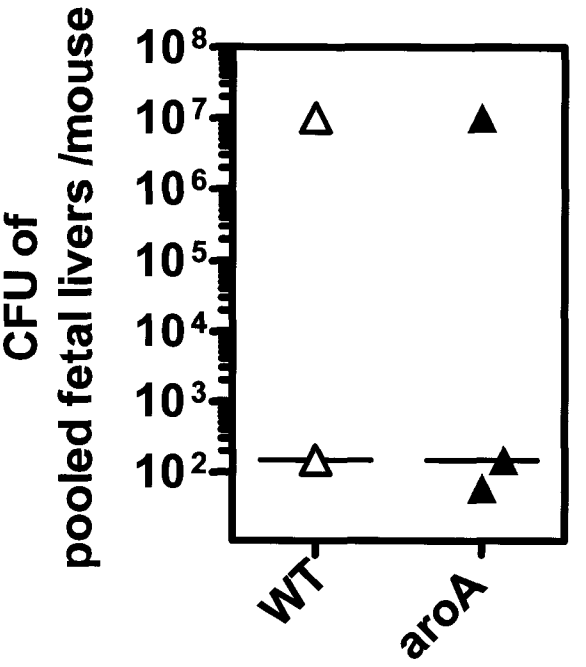


Figure 7

Figure 8: Course of ST WT and ST *aroA* infection in 129.B6F1 hosts Non pregnant and pregnant B6.129F1 mice were infected with A) 10^3 ST WT B) 10^6 ST *aroA* (i.v). Pregnant mice were infected in middle pregnancy. Survival curves are based on (6 mice/ group). In the ST WT infected group mice were euthanized once they showed 2-3 visible signs of morbidity; pilorection, slow/retarded movement, >20% weight loss.

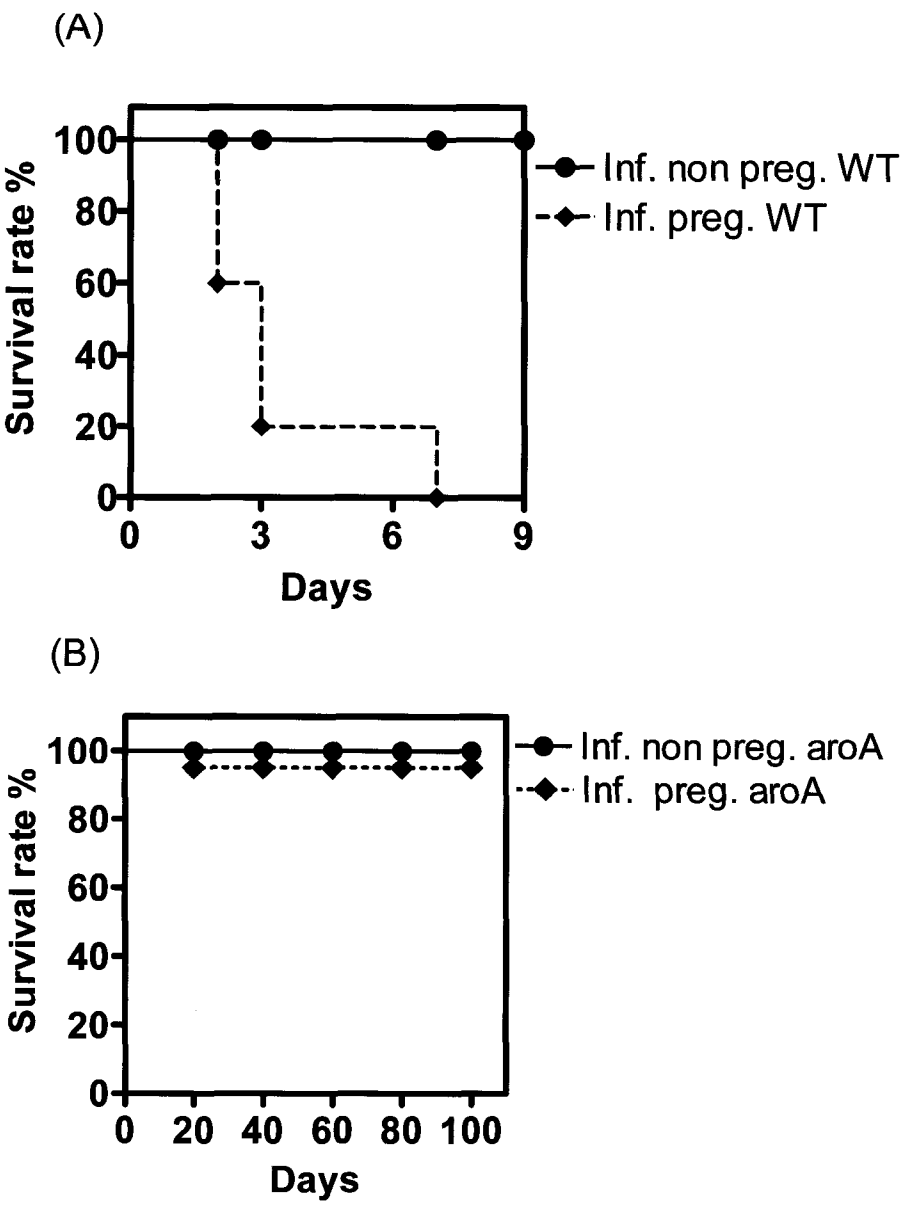


Figure 8

4.7a. ST WT infected 129.B6F1 mice produce high levels of IL6

IL6 is a pro-inflammatory cytokine and is considered to play an important role in anti-*Salmonella* defense (130). IL12 is also an early cytokine triggered following pathogen recognition and results in activation of innate immune cell types such as DC's, NK cells, and subsequently modulates T cell differentiation. Thus, IL12 is a key cytokine in the pathogen signaling pathway that contributes to host resistance in many infection models. Serum cytokine levels of IL6 and IL12 were measured in individual mice by cytokine-specific ELISA. IL6 level was found to be elevated in the serum of pregnant ST WT infected mice on day 3 of infection compared to non pregnant infected controls. However, the level of IL12 was found to be similar in both the groups. Thus, while 129.B6F1 pregnant mice showed an increase in IL6, there appeared little difference in the level of serum IL12 relative to non-pregnant infected controls (Fig. 9).

4.7b. Proteome profiling indicates specific increase in inflammatory cytokines in pregnant infected hosts. Determining cytokine levels from serum one at a time requires a large sample volume, and limits the number of cytokines that can be assayed. In order to ascertain a holistic picture of cytokine/chemokine expression following ST infection, we used a mouse cytokine array system which allows simultaneous determination of 40 cytokines/chemokines at once from a single sample. Fig. 10 shows a representative chemiluminscent blot obtained from the analysis of individual serum of 6 different groups of mice. The six different groups of mice that were analyzed were: non pregnant ST WT infected, pregnant ST WT infected, non pregnant *aroA* infected and pregnant *aroA* infected, non pregnant non infected.

Figure 9: Serum IL6 and IL12 levels in response to ST WT infection. Individual serum samples were analyzed for A) IL6 and B) IL12 levels by ELISA. Serum samples were collected on day 3 from ST WT 10^3 infected 129.B6F1 mice. Median serum cytokine level in each group is indicated by a horizontal line. The level of IL6 in pregnant infected mice was found to be significantly higher in comparison to non pregnant mice ($p<0.05$) on the basis of two tailed, Mann-Whitney *U* test.

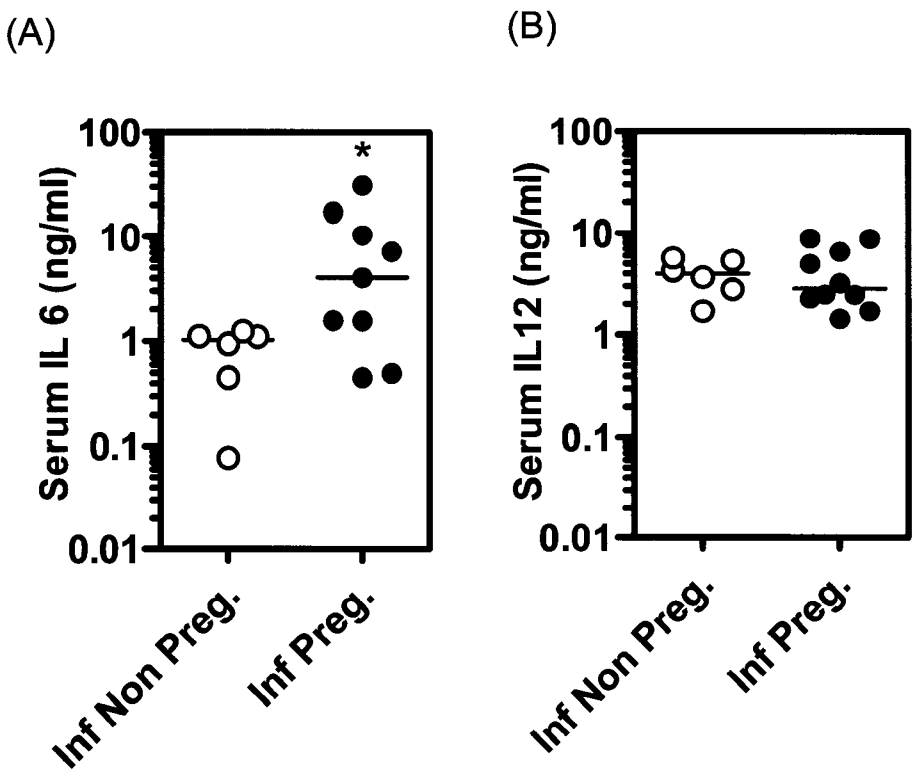


Figure 9

infected (naïve), pregnant non infected (healthy pregnant). Each cytokine is represented in duplicate on the blot, and the intensity of each spot was quantitated using densitometric software to derive the level of expression of the particular cytokine relative to the positive control spots on each blot (Fig. 10). Using this strategy, I further compared the expression level of the cytokines in serum of infected mice with the level of expression in non infected controls to derive fold-change expression for the various cytokines.

Firstly, we found that in response to both ST WT and ST *aroA* infection, there was an early increase in the expression of certain chemokines, and this remained similarly modulated in both pregnant and non pregnant hosts. For example, all the four infected groups of mice showed an increase of 5-10 folds in the levels of IP10, CCl2 and CXCL9 relative to their respective non infected controls (Fig. 11).

Next the levels of several inflammatory cytokines/chemokines were found to be elevated particularly in the pregnant ST WT infected group. IL6 and BLC/CXCL13 levels were increased ~30 fold relative to pregnant non infected mice. G-CSF, IL1Ra and I-309/CCL1 showed an increase of 10-20 folds relative to pregnant non infected group (Fig. 12). Most importantly, these 5 inflammatory cytokines/chemokines were not increased in non pregnant ST WT infection, suggesting that these inflammatory mediators may play a role in the aberrant response to virulent ST WT infection in pregnant hosts. In lieu with this thinking, these cytokines were also relatively unchanged from non-infected control mice, in the serum of non pregnant or pregnant *aroA* infected

mice. Cytokines/chemokines such as G-CSF and I-309 are known to be major chemoattractants for monocytes and neutrophils suggesting that these may be the innate cell types that are dysregulated in pregnant ST WT infected mice.

Several other inflammatory mediators were also increased 5-10 fold especially in the pregnant ST WT infected group (Table1). Furthermore, such increases were not seen in the pregnant ST *aroA* infected mice, again reiterating that defective and/or overt inflammation triggered in response to ST WT infection during pregnancy may have contributed to the adverse host outcome. The changes in the expression level of the inflammatory cytokines appeared to be specific responses to infection in pregnant hosts, as many other cytokines/chemokines remained unchanged among the four groups of infected mice analyzed (Table 2).

Figure 10: Proteome profiling blot

Representative proteome blots carried out on serum of individual mice, in the 6 different groups are shown. Membrane was coated with primary capture antibody, followed by a mixture of sample and biotinylated detection antibody. This was followed by treatment with streptavidin- HRP and finally chemiluminescent detection. The spot intensity is proportional to the amount of cytokine bound. Each cytokine is represented as duplicate spots, and there are 3 sets of positive control spots on each blot. Using a software program, the intensity of spots was quantitated relative to the positive control spot.

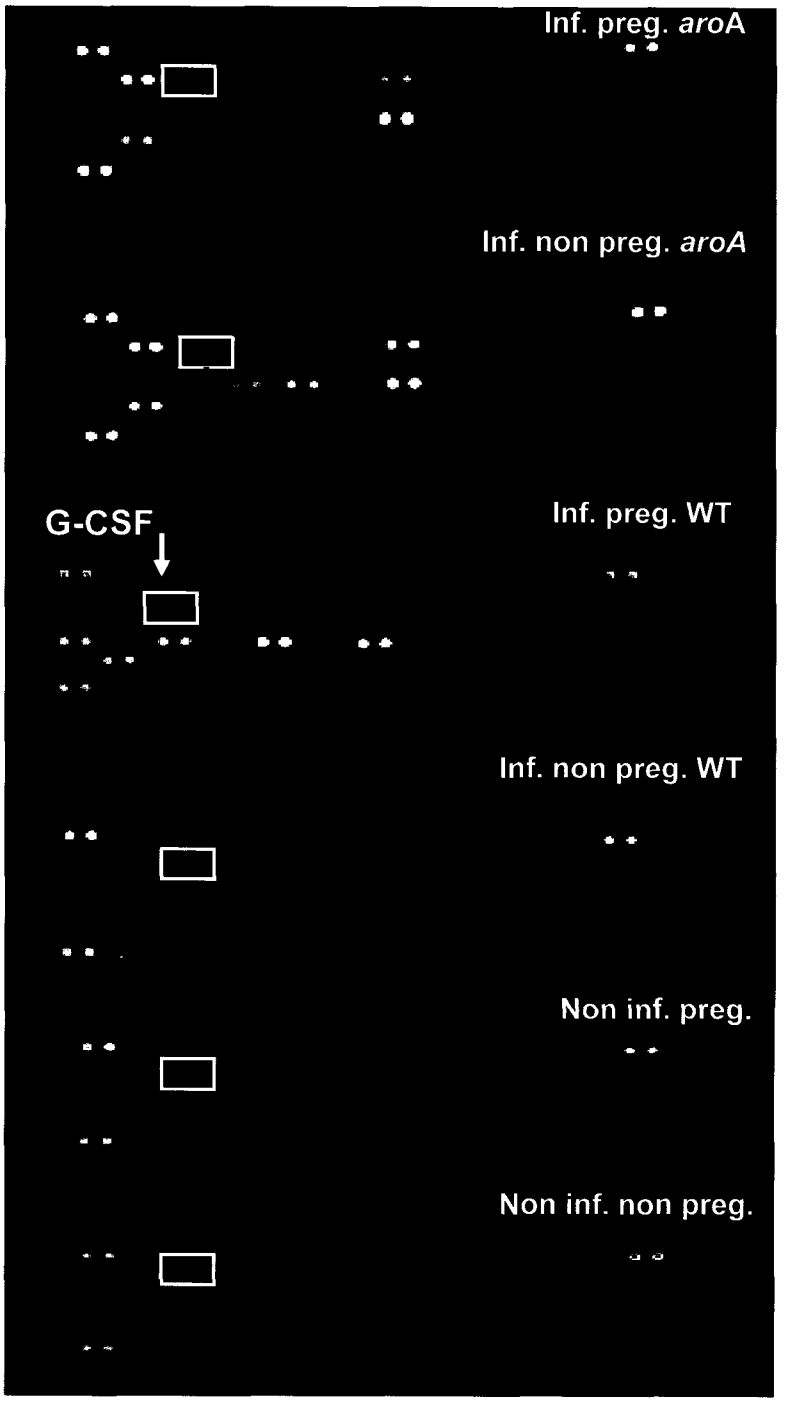


Figure 10

Figure 11: Increase in select chemokines/cytokines in early response to infection. Cytokine levels were determined in the serum samples from individual mice, using proteome profiling. 129.B6F1 mice were infected with either WT (10^3) or *aro A* (10^6) in the non-pregnant state or at days 12-13 of gestation. Serum cytokine levels on day 3 of infection in four groups of mice are shown; Non pregnant infected with ST WT , non pregnant infected with ST *aroA*, Pregnant infected with ST WT, and pregnant infected with ST *aroA* (n=3-5/group). Data is represented as Mean fold change ± SEM relative to the cytokine level in the respective non infected control group.

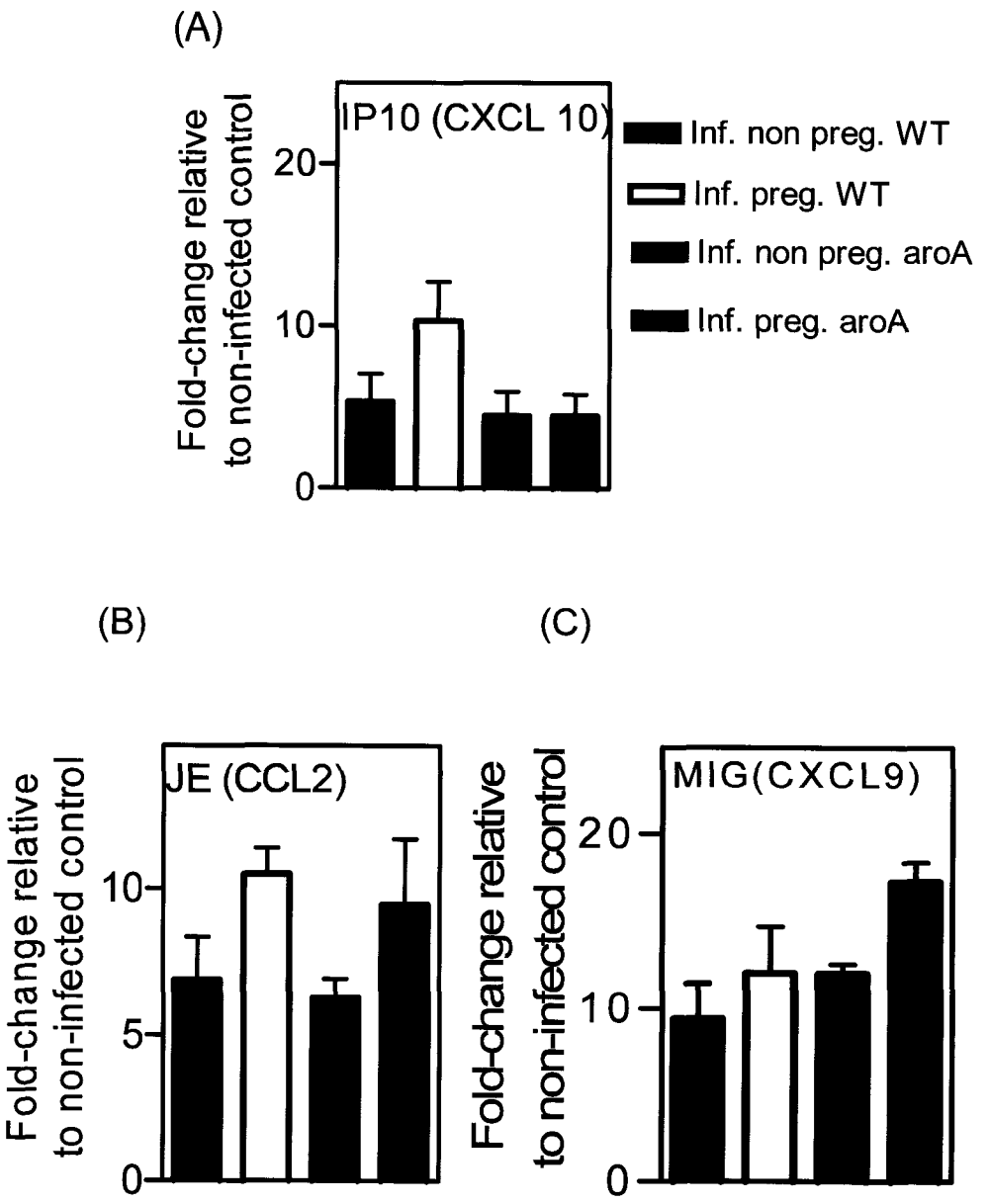


Figure 11

Figure 12: Specific increase (>10 fold) in inflammatory cytokines/chemokines in pregnant hosts infected with ST WT. 129. B6F1 mice were infected with either WT (10^3) or *aro A* (10^6) in the non-pregnant state or at days 12-13 of gestation. Analyte levels on day 3 of infection relative to the respective non-infected control group are indicated. Data represent Mean fold change ± SEM of values obtained for individual mice serum (n-3-5/group). * indicates $p < 0.05$ in comparison with non pregnant WT infected group by Mann Whitney U test.

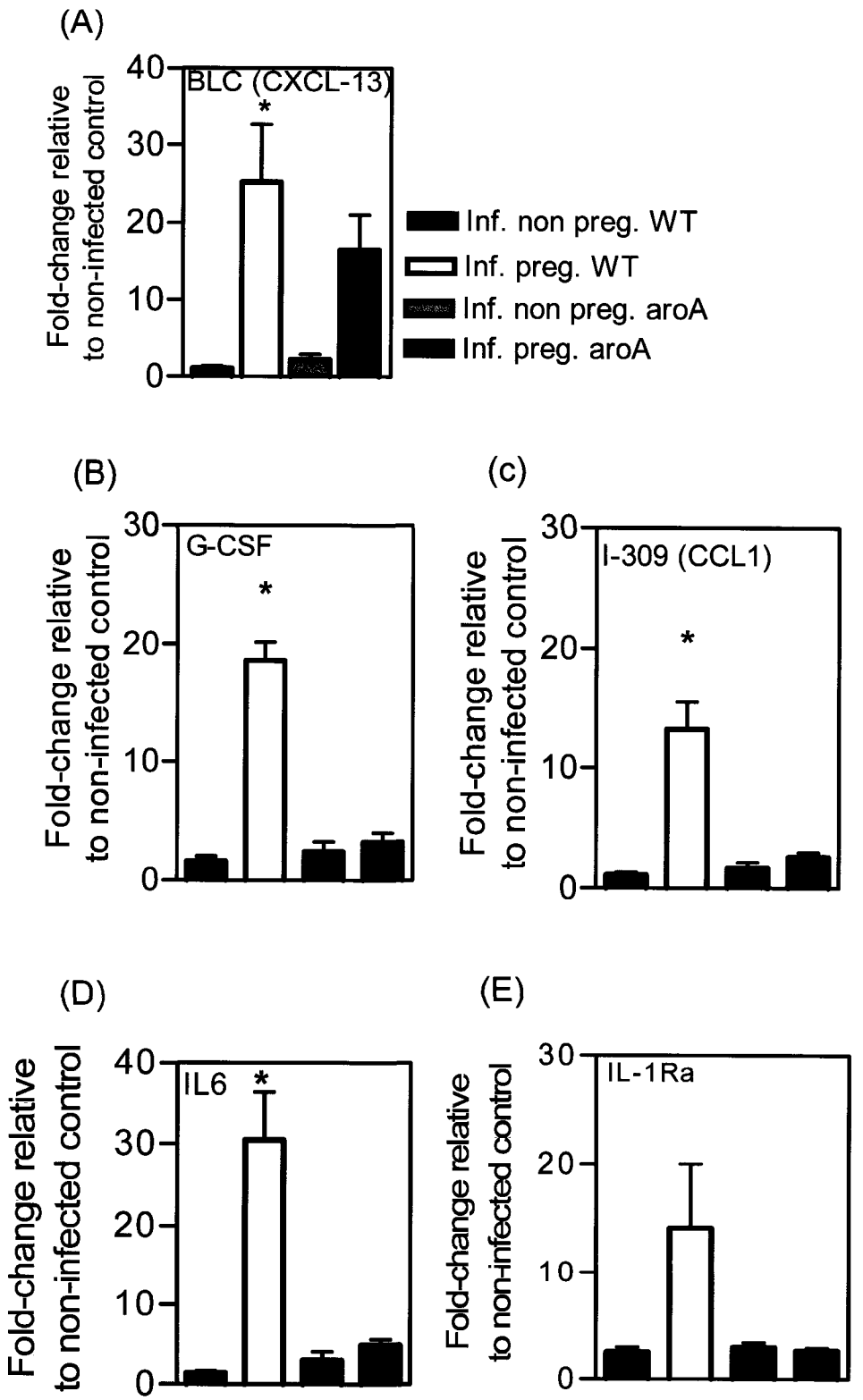


Figure 12

Table 1: Cytokines/ chemokines showing a change of 5-10 folds in the serum relative to respective non infected controls. 129. B6F1 mice were infected with either WT (10^3) or *aro A* (10^6) in the non-pregnant state or at days 12-13 of gestation. Analyte levels on day 3 of infection relative to the respective non-infected control group are indicated. Data represents fold-increase for each infected group relative to the mean serum values for non infected- non pregnant or pregnant groups respectively. n=4 mice per group. * Values are significantly increased in the pregnant infected (ST WT) animals relative to non pregnant infected group, by Mann Whitney U test.

Table 1
Serum cytokines/chemokines that are increased 5-10-fold in Pregnant ST WT infected hosts.

Cytokine/ Chemokine	Group Mean ± SEM			
	Non Preg Inf. ST WT	Preg. Inf. ST WT	Non Preg. Inf. ST *aroA*	Preg. Inf. ST *aroA*
KC	1.96 ± 1.04	***8.20 ± 2.67**	1.83 ± 1.25	2.15 ± 1.03
MCP-5 (CCL12)	1.63 ± 0.65	***5.23 ± 0.72**	1.62 ± 0.57	1.71 ± 0.48
MIP-2	1.32 ± 0.59	***5.02 ± 2.98**	2.17 ± 1.66	1.94 ± 0.44
IL-10	1.24 ± 0.58	***6.33 ± 0.94**	2.28 ± 1.20	***4.34 ± 1.73**

Table2: Serum Cytokines/Chemokines that remain unchanged in pregnant ST WT infected mice relative to non pregnant and pregnant ST *aroA* infected hosts

Table 2

Cytokine/Chemokine	Group (Mean ± SEM)			
	Non Preg. Inf. ST WT	Preg. Inf. ST WT	Non Preg. Inf. ST *aroA*	Preg. Inf. ST *aroA*
C5a (Complement Component 5a)	0.98 ± 0.57	1.13 ± 0.45	0.77 ± 0.48	1 ± 0.73
GM-CSF	1.16 ± 0.49	3.95 ± 0.98	2.59 ± 1.43	3.12 ± 1.28
Eotaxin	1.18 ± 0.48	4.2 ± 1.62	2.4 ± 1.31	3.55 ± 1.71
sICAM-1 (CD54)	1.02 ± 0.17	1.26 ± 0.24	0.78 ± 0.48	0.92 ± 0.62
IFNγ	1.2 ± 0.6	1.58 ± 0.36	1.53 ± 0.92	1.56 ± 0.63
IL1α	0.92 ± 0.41	2.35 ± 0.64	1.92 ± 1.34	2.05 ± 0.75
IL1β	1.07 ± 0.41	2.29 ± 0.58	2.25 ± 1.67	1.97 ± 0.75
IL2	1.26 ± 0.59	2.25 ± 0.58	1.75 ± 0.72	1.70 ± 0.68
IL3	1.07 ± 0.49	1.40 ± 0.47	1.62 ± 0.89	1.10 ± 0.38
IL4	0.98 ± 0.31	1.53 ± 0.22	1.49 ± 0.67	1.56 ± 0.60
IL5	1.06 ± 0.67	3.98 ± 1.75	2.49 ± 1.43	3.07 ± 1.55
IL7	1.18 ± 0.37	2.26 ± 0.39	1.69 ± 0.79	2.20 ± 0.89
IL13	0.97 ± 0.37	1.46 ± 0.30	1.30 ± 0.66	1.69 ± 0.93
IL12p70	0.95 ± 0.51	3.77 ± 2.51	2.46 ± 1.40	4.42 ± 2.48
IL16	1.01 ± 0.31	1.88 ± 0.36	1.68 ± 0.77	1.76 ± 0.57
IL17	1.06 ± 0.31	2.61 ± 1.08	1.85 ± 1.30	1.84 ± 0.82
IL23	1.04 ± 0.49	2.86 ± 0.78	2.22 ± 1.31	2.52 ± 1.18
IL27	1.45 ± 0.53	2.08 ± 1.07	1.80 ± 0.94	1.65 ± 0.79
I-TAC (CXCL11)	1.48 ± 0.69	2.59 ± 0.82	2.28 ± 1.34	2.31 ± 1.27
M-CSF	1.11 ± 0.48	1.34 ± 0.60	0.91 ± 0.55	0.89 ± 0.52
MIP-1α (CCL3)	1.10 ± 0.38	2.16 ± 0.36	2.18 ± 1.15	2.56 ± 0.99
MIP-1β (CCL4)	0.97 ± 0.46	4.36 ± 2.31	2.67 ± 1.92	3.63 ± 2.32
RANTES (CCL5)	1.44 ± 0.67	2.81 ± 0.68	2.97 ± 1.98	2.3 ± 0.75
SDF-1 (CXCL12)	0.65 ± 0.21	1.00 ± 0.44	0.57 ± 0.31	0.79 ± 0.42
TARC (CCL17)	1.10 ± 0.59	2.80 ± 0.82	2.40 ± 0.93	2.85 ± 1.30
TIMP-1	1.60 ± 0.65	3.09 ± 0.56	1.94 ± 1.26	1.54 ± 0.98
TNFα	1.22 ± 0.37	2.65 ± 0.51	1.97 ± 0.75	2.24 ± 0.45
TREM-1	1.19 ± 0.51	3.1 ± 0.60	1.97 ± 0.89	2.03 ± 0.76

Data represents fold-change for each infected group relative to the mean serum values for non infected- non pregnant or pregnant groups respectively. n=4 mice per group.

4.8 Quantitation of cytokine RNA expression: Spleen

The mouse cytokine array suggested that some of the inflammatory cytokines were elevated in the systemic compartment of pregnant WT infected mice. Thus, we wanted to ascertain the cytokine profile in the peripheral lymphoid organs. We determined the relative levels of key inflammatory and non inflammatory cytokines in the spleens of the individual mice by QRT-PCR using cytokine-specific primers and normalized the values to β-actin expression levels. Six groups of mice were included in the study: Non pregnant non infected (naïve), Pregnant non infected, Non pregnant infected WT, Pregnant infected WT, Non pregnant infected *aroA*, Pregnant infected *aroA*.

4.8 a IL10: Firstly, expression levels of IL10 were found to be significantly higher in the pregnant non infected mice relative to naïve (non pregnant non infected) mice and is consistent with the increased IL-10 levels reported to be associated with successful pregnancy (131). Interestingly, IL-10 levels were also elevated in response to ST WT and *aroA* infection in non pregnant mice, compared to naïve controls. Moreover the pregnant mice infected with WT ST showed significantly higher expression levels of IL10 as compared even to pregnant non infected mice. However, the most dramatic increase in IL-10 expression was seen in pregnant *aroA* infected mice relative to both pregnant healthy controls and pregnant WT infected mice (Fig.13A).

4.8 b IL6: IL6 expression levels in non pregnant and pregnant mice infected with WT ST showed an increased trend as compared to naïve, pregnant non infected, non pregnant and pregnant mice infected with ST *aroA*, but lacked statistical significance (Fig. 13B).

4.8c TGFβ: The naïve, pregnant non infected, non pregnant WT and pregnant WT infected mice showed similar TGFβ expression levels in the spleens. In contrast, non pregnant *aroA* and pregnant *aroA* infected mice showed significantly low TGFβ expression levels (Fig. 13C).

4.8d IFNγ: Naïve (non infected non pregnant) mice exhibited the lowest levels of splenic IFNγ expression. Splenic IFNγ expression significantly increased in response to infection as both non pregnant WT and non pregnant *aroA* infected groups exhibited elevated levels relative to naïve mice. Surprisingly, pregnant non-infected, mice showed higher IFNγ expression levels in the spleen as compared to naïve (non pregnant) mice. Thus the levels IFNγ were found to be similar in the pregnant WT infected and pregnant *aroA* infected mice spleens relative to pregnant non infected mice (Fig. 14A).

4.8e TNFα: Non pregnant mice, in response to ST WT infection, appeared to exhibit an increased trend in TNFα expression relative to naïve (non-infected) mice, although this was statistically insignificant. Nevertheless, pregnant ST WT infected mice showed a significantly decreased TNFα levels relative to non pregnant ST WT infected mice, suggesting a role for this cytokine in early effective and beneficial response to ST infection. *AroA* infection in non pregnant mice resulted in similar TNFα induction relative to non pregnant naïve controls and ST WT infected mice. Moreover, the pregnant ST *aroA* infected mice, showed no decrease in TNFα levels relative to all other groups (Fig. 14B).

4.8f IL12p40: The expression level of IL12p40 was found to be significantly higher in the spleens of pregnant non infected mice as compared to the naïve mice. The pregnant

WT infected and pregnant *aroA* infected mice also showed an increased trend in IL12p40 expression levels. IL12p40 expression did not appear to be elevated in any of the infected non pregnant group of mice. We could not detect IL12p35 subunit by qRT-PCR, thus it is unclear whether the lack of IL12p40 increase in response to infection bears any correlation to the expression of dimeric IL12p70 (Fig. 14C).

4.8g G-CSF: levels were undetectable in the spleen by QRT-PCR

Overall, the splenic cytokine expression pattern correlates with a decreased inflammatory (decreased TNF, increased IL-10) response in pregnant mice infected with ST WT. In contrast, non-pregnant hosts in response to infection appeared to evoke an increased systemic inflammatory response (increased TNF, IFNγ).

Figure 13: Splenic anti-inflammatory cytokine expression in response to ST WT or ST *aroA* infection.

Cytokine mRNA expression was determined in the spleen samples from individual mice by QRT-PCR. 129.B6F1 mice were infected with ST WT (10^3) and *aroA* (10^6) in the non pregnant state or on day 12-13 of gestation. Expression levels in six groups of mice are included; Naïve, pregnant non infected, non pregnant ST WT infected, non pregnant ST *aro A* infected, pregnant ST WT infected and pregnant ST *aroA* infected (n= 4-5/group). Spleens were obtained on day 3 of infection. Data are graphed as relative mRNA abundance normalized to β-actin expression levels of individual samples. Each data point is representative of expression level in individual mouse tissue, and the median is indicated by a horizontal line. * indicates ($p < 0.05$) in comparison with naive, * indicates significant difference in comparison with pregnant non infected and # indicates significant difference in comparison with pregnant group infected with ST WT. $P < 0.05$ by Mann Whitney U test.

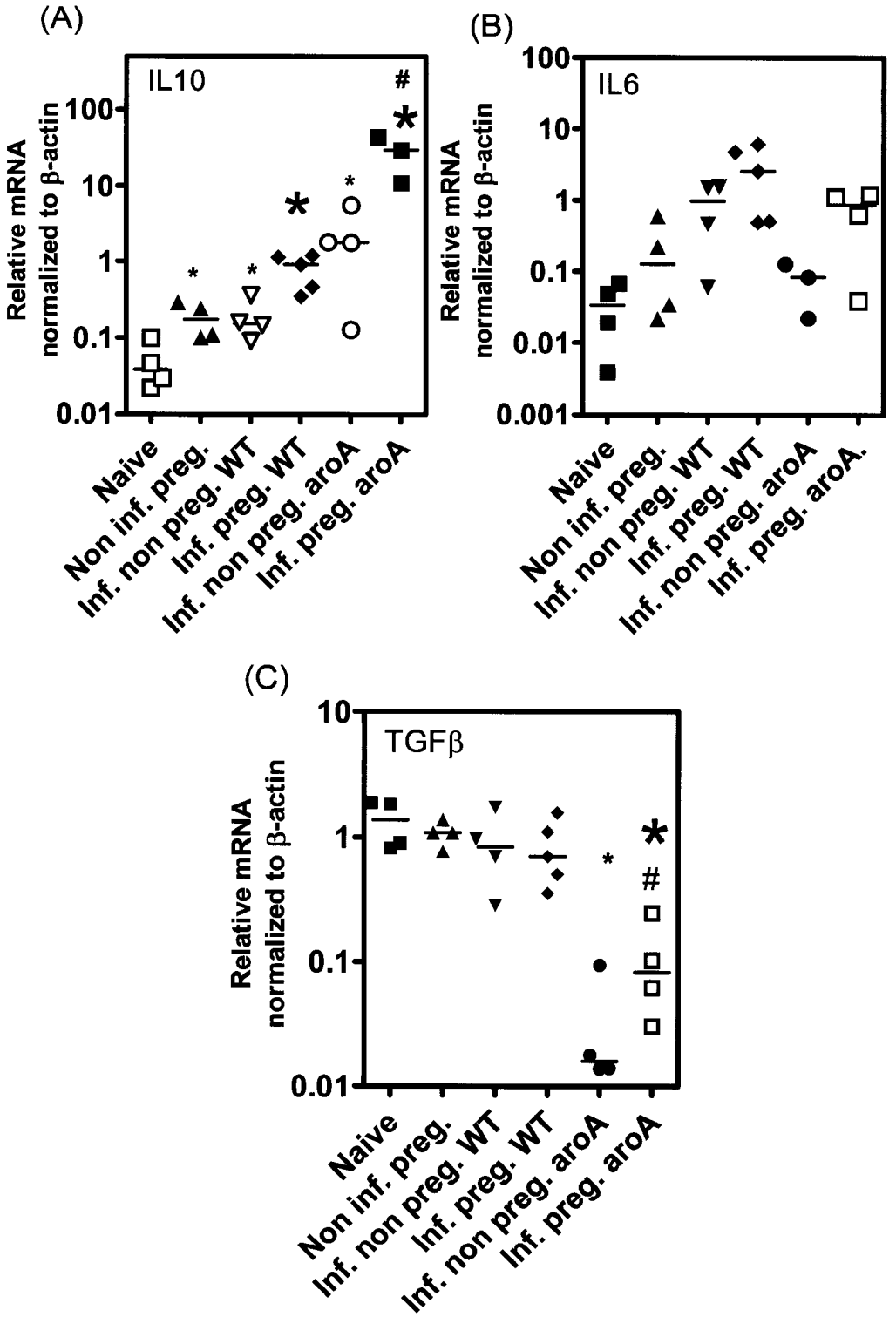


Figure 13

Figure 14: Splenic pro-inflammatory cytokine expression in response to ST WT or ST *aroA* infection.

Cytokine mRNA expression was determined in the spleen samples from individual mice by QRT-PCR. 129.B6F1 mice were infected with ST WT (10^3) and *aroA* (10^6) in the non pregnant state or on day 12-13 of gestation. Expression levels in six groups of mice are included; Naïve, pregnant non infected, non pregnant ST WT infected, non pregnant ST *aro A* infected, pregnant ST WT infected and pregnant ST *aroA* infected (n=4-5/group). Spleens were obtained on day 3 of infection. Data are graphed as relative mRNA abundance normalized to β-actin expression levels of individual samples. Each data point is representative of expression level in individual mouse tissue, and the median is indicated by a horizontal line. * indicates ($p < 0.05$) significant difference in comparison with naive and ☆ indicates significant difference in comparison to non pregnant ST WT infected mice. $P < 0.05$ by Mann Whitney U test.

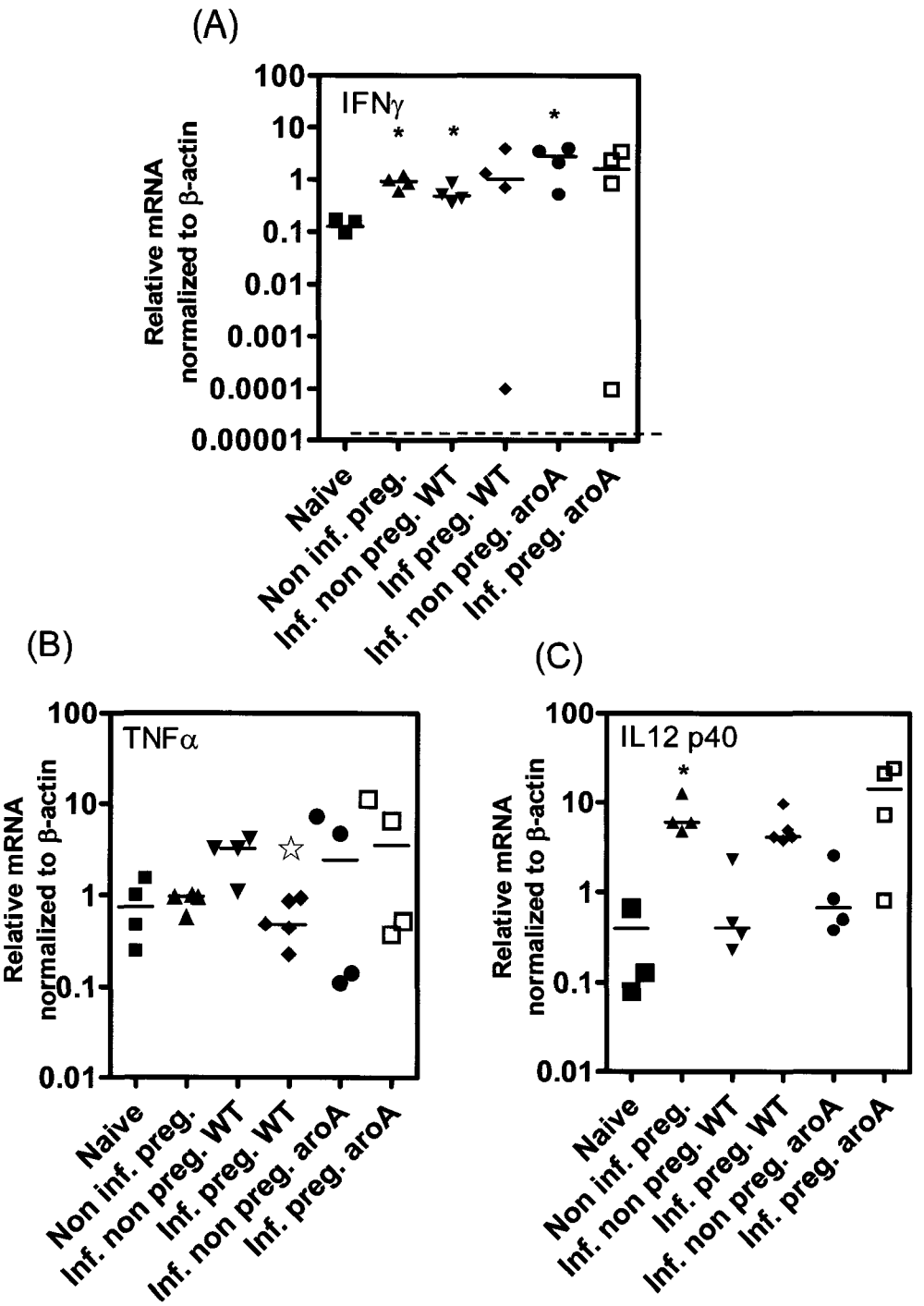


Figure 14

4.9 Quantitation of cytokine RNA expression- Placenta

ST WT infection results in a significant increase in fetal loss, whereas ST *aroA* infection does not compromise fetal survival despite high placental bacterial burden. Thus we sought to investigate the cytokine profile in the uteroplacental unit/placentas of pregnant infected mice. QRT-PCR was performed from the placental samples from individual mice at days 15-16 of gestation, 3 days post infection. Expression of cytokines was profiled in the placenta of mice infected with either ST WT or *aroA* infection and compared to expression levels in the placenta from healthy non infected mice.

4.9a G-CSF: The pregnant WT infected mice showed very high expression levels of G-CSF (~400 fold) as compared to pregnant non infected mice. In contrast, G-CSF expression in the placentas of *aroA* infected mice was similar to expression levels in healthy controls, and significantly less than pregnant WT infected mice (Fig. 15A).

4.9b TNFα: Placentas from pregnant WT infected mice also showed dramatic increase (~1000 fold) in the levels of TNFα relative to healthy placentas from non infected mice. In contrast, placentas from the *aroA* infected mice, expressed TNFα at levels similar to healthy placenta and significantly reduced relative to the pregnant WT infected mice (Fig. 15B).

4.9c IL6: The expression level of IL6 was increased ~200 fold in the placenta of ST WT infected mice relative to healthy non infected placentas. Furthermore, as above, placentas from *aroA* infected mice had significantly lower IL6 expression, similar to healthy control placentas (Fig. 15C)

4.9d IFNγ: Uteroplacental units from pregnant WT infected mice showed an increased trend of IFNγ expression levels relative to the naïve, but the differences were not statistically significant. Nevertheless, placentas from pregnant *aroA* infected mice showed significantly lower levels of IFNγ expression as compared to the pregnant WT infected mice (Fig. 15D).

4.9e In contrast to the above cytokines, expression levels of **IL10**, **TGFβ** and **IL12p40** were found to be similar in infected and non infected placentas (Fig. 16 A, B, C).

Thus, the overt expression of inflammatory cytokines in the feto-maternal milieu of pregnant ST WT infected mice may have contributed to rapid placental death and necrosis. Consistent with this, there was a clear absence of these inflammatory signals in the case of *aroA* infected placentas that supported live fetuses to full-term.

4.10 Immunohistological studies on ST infected placentas:

The mouse proteome array revealed that cytokines/chemokines which were chemo attractants for neutrophils and monocytes (G-CSF, I-309) were specifically elevated in the ST WT infected pregnant mice. This was further corroborated by QRT-PCR data that showed that inflammatory cytokines (G-CSF, TNFα, IL6) were particularly elevated in the placenta of ST WT infected pregnant mice. Therefore, we performed histological studies on the placentas retrieved from pregnant infected and non infected mice to determine the profile of the innate immune cell types in the tissue. Fig. 17 depicts H & E staining of placental sections from healthy non-infected and infected placentas at various times. These sections are representative of 8-10 fields viewed at 3 mm distances from each other as per systematic uniform random sampling protocol (132). These sections

Figure 15: Placental cytokine expression in healthy and ST WT or ST *aroA* infected tissues 129.B6F1 mice were infected with ST WT (10^3) and *aroA* (10^6) in the non pregnant state or at days 12-13 of gestation. Cytokine mRNA expression was determined in the uteroplacental/placental tissue samples from individual mice. Expression levels are indicated for pregnant non infected, pregnant ST WT infected and pregnant ST *aroA* infected (n=4/group) mice. Placentas or uteroplacental units were obtained on day 3 of infection. Placentas from healthy age matched non infected mice were obtained on day 15-16 of gestation. Data are graphed as relative mRNA abundance normalized to β-actin expression levels for each corresponding sample. Median of expression levels for cytokines in each group is indicated by a horizontal line. * indicates significant difference ($p<0.05$) in comparison with pregnant non infected and *indicates ($p<0.05$) in comparison with pregnant WT infected group by Mann Whitney U test.

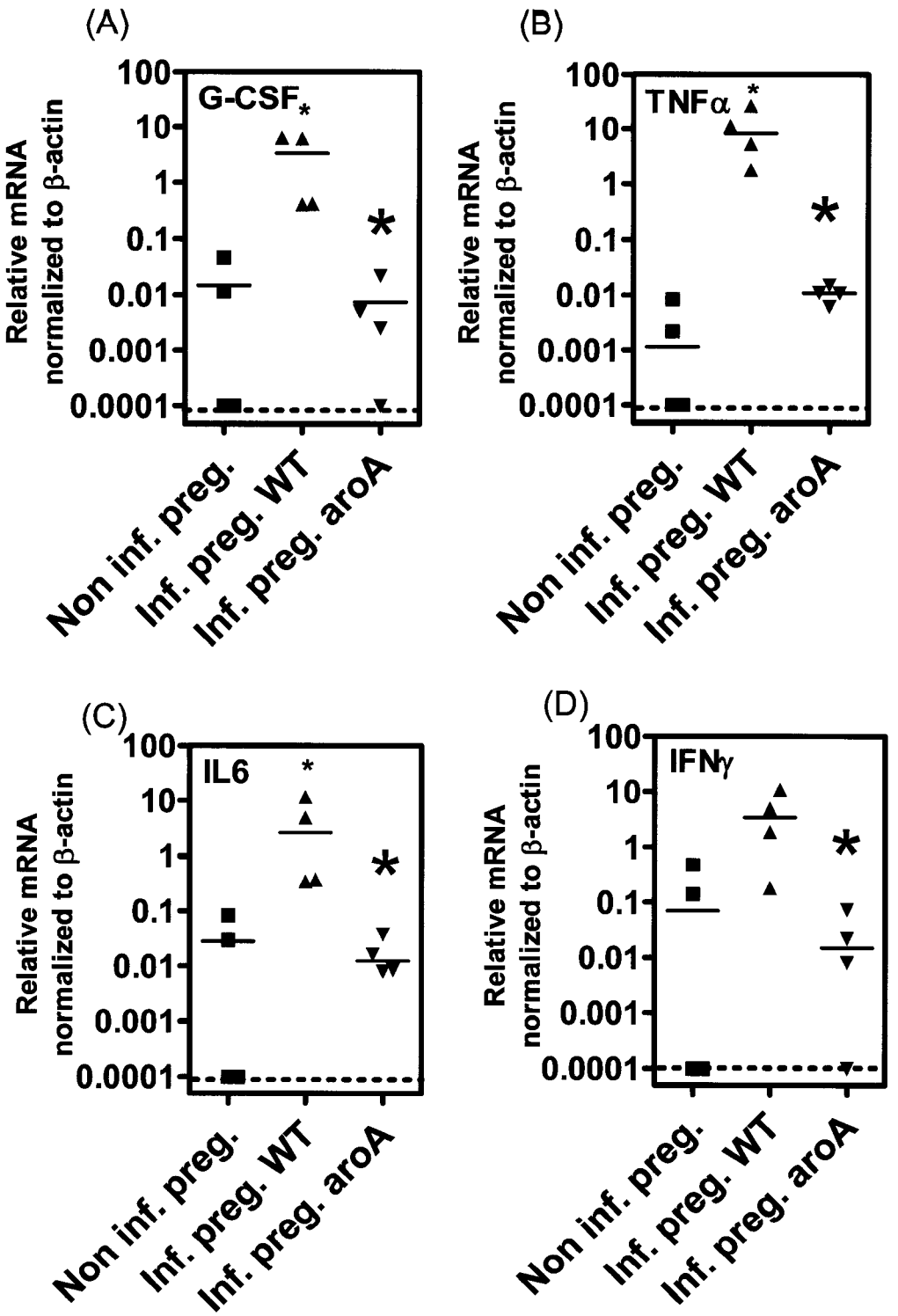


Figure 15

Figure 16: Placental cytokine expression in healthy and ST WT or ST *aroA* infected tissues 129.B6F1 mice were infected with ST WT (10^3) and *aroA* (10^6) in the non pregnant state or at days 12-13 of gestation. Cytokine mRNA expression was determined in the uteroplacental/placental tissue samples from individual mice. Expression levels are indicated for pregnant non infected, pregnant ST WT infected and pregnant ST *aroA* infected (n=4/group) mice. Placentas or uteroplacental units were obtained on day 3 of infection. Placentas from healthy age matched non infected mice were obtained on day 15-16 of gestation. Data are graphed as relative mRNA abundance normalized to β-actin expression levels for each corresponding sample. Median of expression levels for cytokines in each group is indicated by a horizontal line. * indicates significant difference ($p<0.05$) in comparison with pregnant non infected and * indicates ($p<0.05$) in comparison with pregnant WT infected group by Mann Whitney U test.

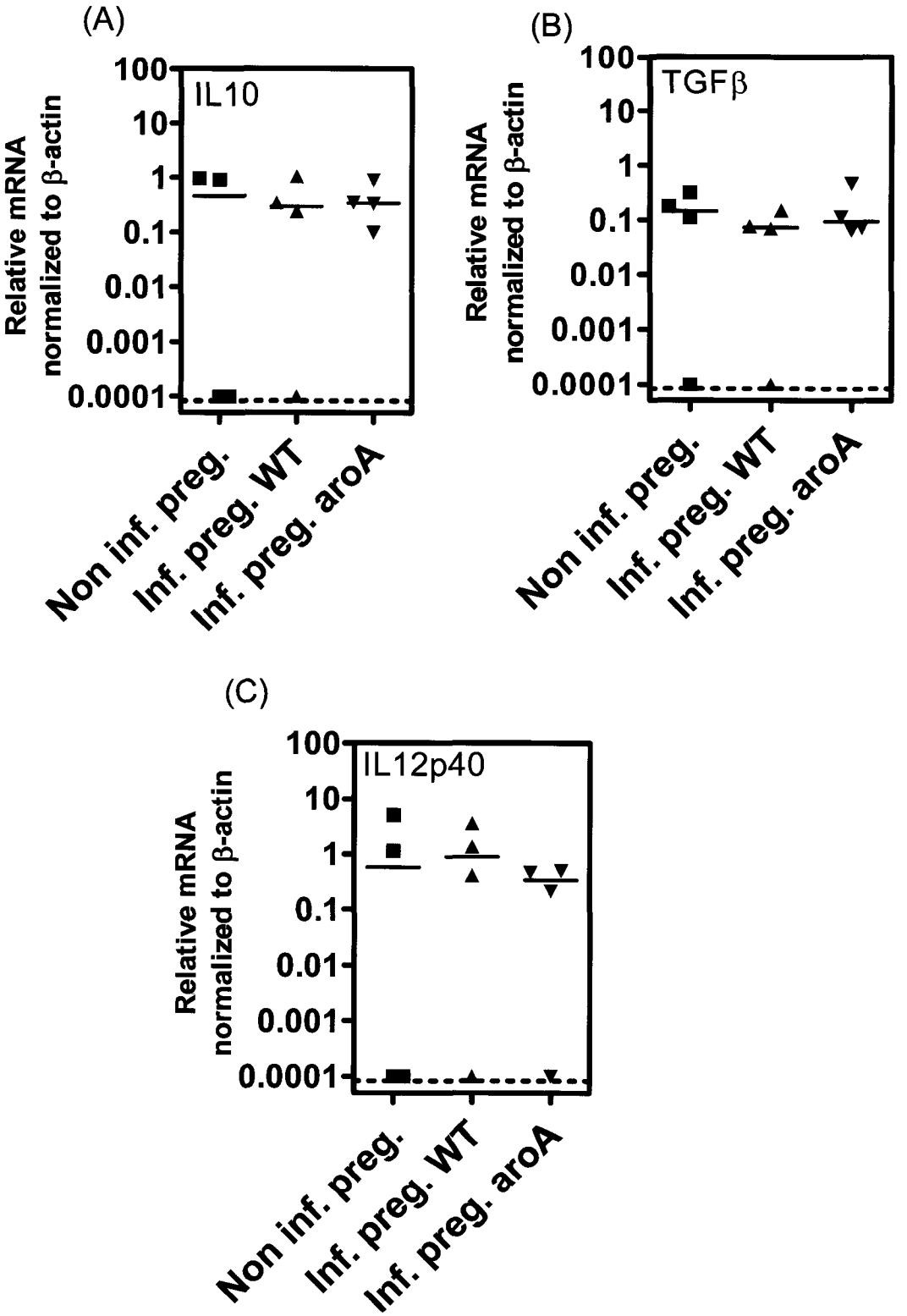


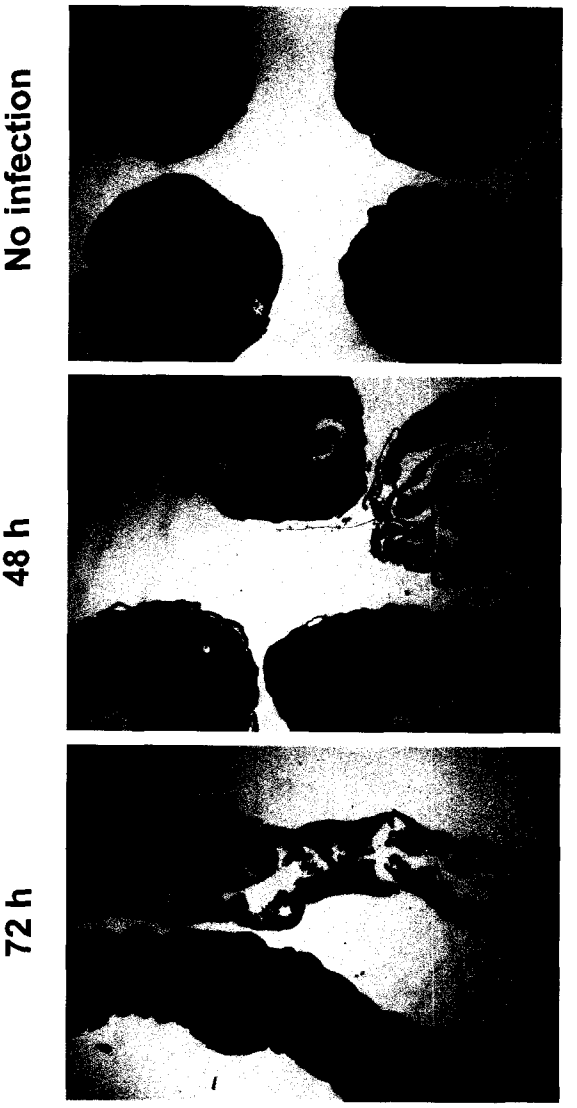
Figure 16

depict the labrynthine vascular region of the trophoblast. At 24h of infection, the placental tissue still looks healthy. Indeed at this time point, a few polymorphonuclear cells were spotted only in the decidual region (data not shown). But within 48 h of WT infection, rampant necrosis of the decidual region, with ruptured blood vessels, loss of trophoblast giant cells and connective tissue with marked increase in the polymorphonuclear cell numbers in the decidual basalis region was noted. Astoundingly, by 72 h the placenta becomes completely necrotic, totally losing its integrity and the placental tissue gets completely replaced by a bed of polymorphonucleated cells which appear to be mainly neutrophils based on their morphological presentation (Fig. 17). By this time the placental tissue layers become totally indiscernible. In contrast, the *aroA* infected placental tissues looked healthy and very similar in appearance to the non infected placentas (Fig. 18, 19) with clear maternal artery and cellular appearance in the labrynthine trophoblast region. To ascertain that the placenta is indeed infiltrated by neutrophils, granulocyte specific MPO staining was performed (133). Cell specific MPO was quite obvious in the WT infected placental samples as opposed to the pregnant non infected samples. Placentas infected with *aroA* also showed few very large cells positive for MPO staining, which appear from their size and morphological presentation to be macrophages which have engulfed dying neutrophils (Fig. 20). MPO has also been shown to be produced by human placental tissues. More specifically by the fetal membranes, basal plate and maternal and fetal blood cells (134). Mice have very similar hemochorial placentation as humans (135). This may be the reason for a diffused background MPO staining found in the non infected healthy placenta.

Overall the histology studies support the conclusion that overt placental cytokine storm results in the massive recruitment and activation of neutrophils precipitating acute pathology in ST WT infected hosts.

Figure 17: H&E staining on ST WT infected placental tissues

129.B6F1 mice were infected with ST WT 10^3 at day 12 of pregnancy. At days 1, 2 and 3 post-infection placentas/uteroplacental units were dissected out and fixed in formalin and paraffin embedded followed by transverse sectioning. Hematoxylin and Eosin staining of section at magnification 10 is shown. These section views are representative n=2-3 individual mice per group.



ST WT infection

Figure 17

Figure 18: H&E staining on ST WT and ST *aroA* infected placental tissues- low magnification

129.B6F1 mice were infected with ST WT 10^3 or ST *aroA* 10^6 at day 12 of pregnancy. At days 1, 2 or 3 post infection placentas/uteroplacental units were dissected out and fixed, paraffin embedded followed by transverse sectioning. Hematoxylin and Eosin stained images at magnification 100. Images are representative of n=3 mice per group, and screening of 8-10 randomized views per slide.

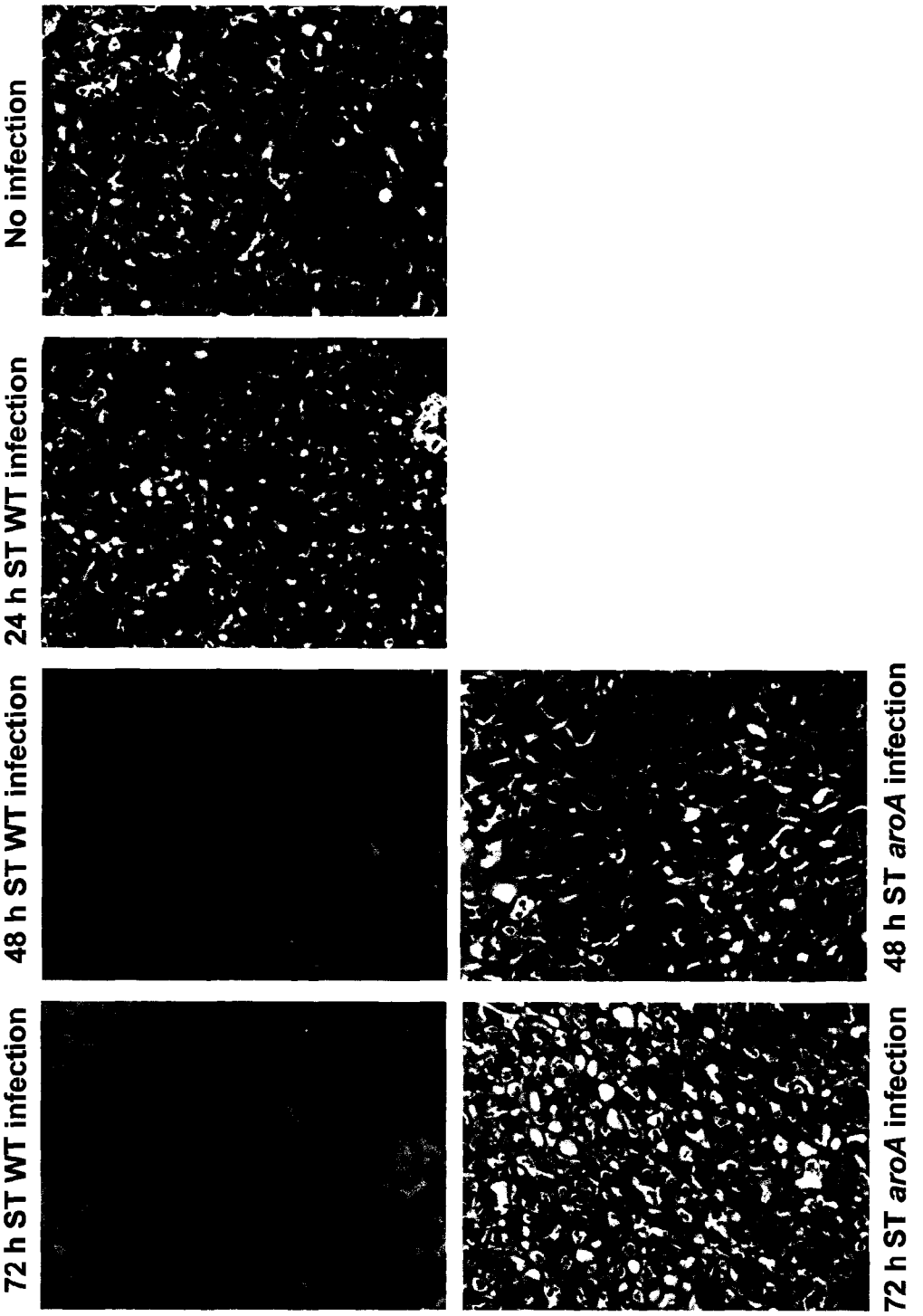


Figure 18

Figure 19: H&E staining on ST WT and ST *aroA* infected placental tissues- high magnification

129.B6F1 mice were infected with ST WT 10^3 or ST *aroA* 10^6 at day 12 of pregnancy. At days 1, 2 or 3 post infection placentas/uteroplacental units were dissected out and fixed, paraffin embedded followed by transverse sectioning. Hematoxylin and Eosin stained images at magnification 400.

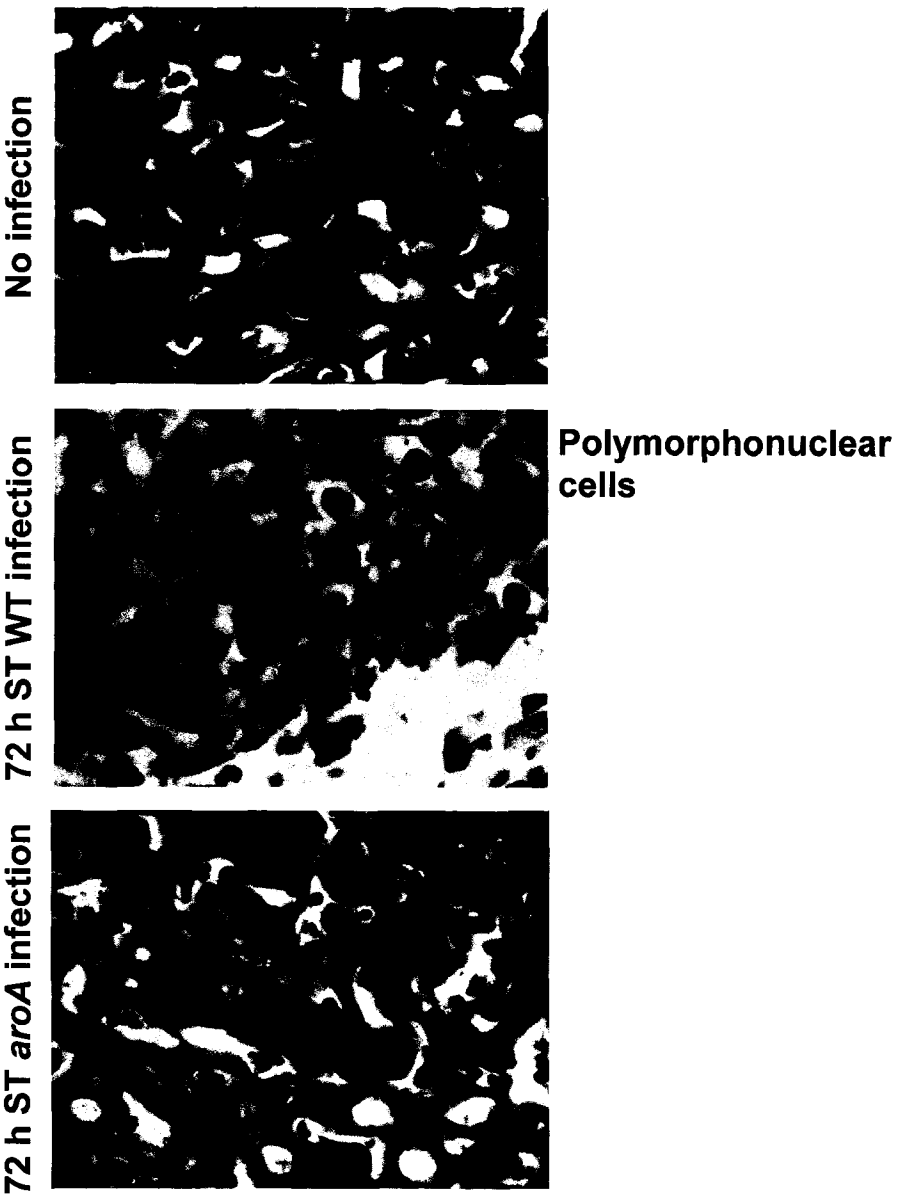


Figure 19

Figure 20: MPO staining on ST WT and ST *aroA* infected placental tissues

129.B6F1 mice were infected with ST WT 10^3 or ST *aroA* 10^6 at day 12 of pregnancy. At day 3 post infection placentas/uteroplacental units were dissected out and fixed, paraffin embedded and transverse sectioned. The paraffinized sections were stained with myeloperoxidase primary antibody and revealed with HRPO. Magnification 400, indicating myeloperoxidase positive polymorphonuclear cells. Slide is representative of staining done for 2-3 mice in each group.

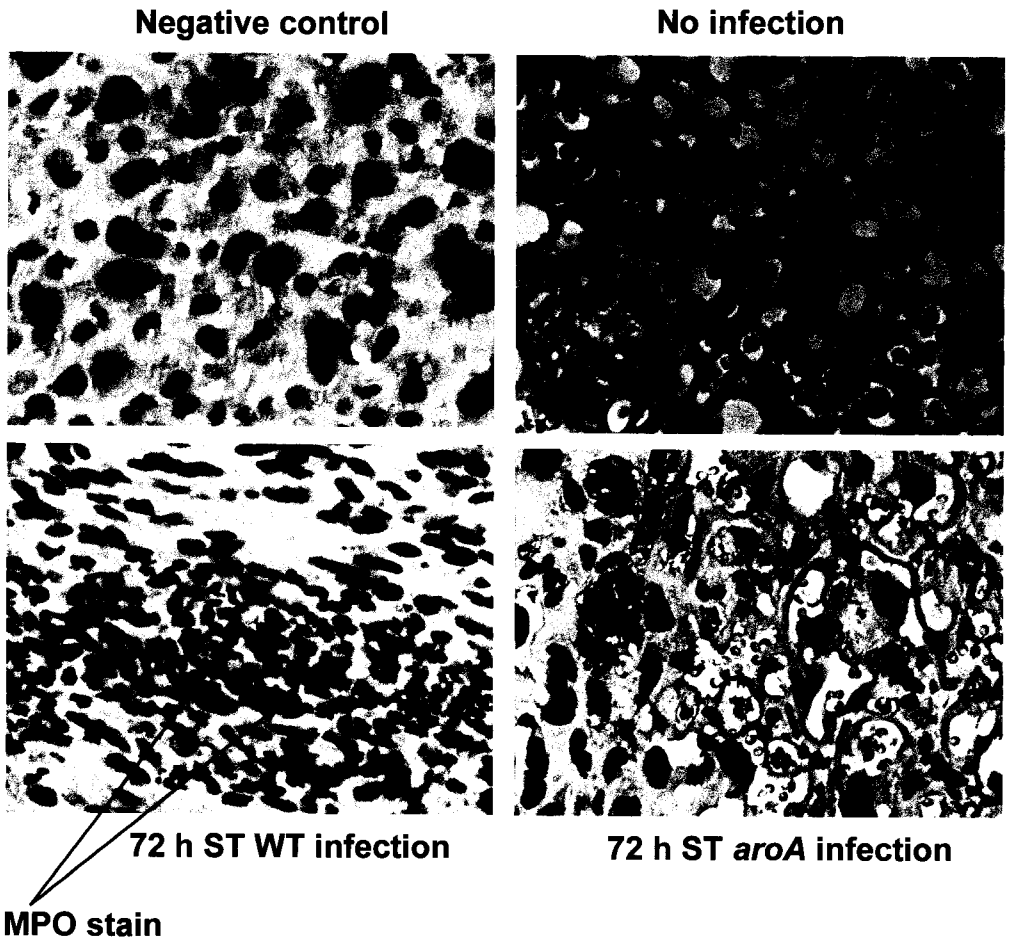


Figure 20

5. Discussion

Food borne disease outbreaks are one of the most challenging health concerns affecting public health world wide. Mass food production, packaged foods and the ease with which food can be shipped worldwide have increased the incidence of food borne diseases. *Salmonella* species are one of the most widespread food borne pathogens affecting public health (136). Disease incidences caused by *Salmonella* have been estimated to be ~1.4 million cases in the United States alone (137). Typhoid fever in humans is a major health issue in the developing nations, whereas non-typhoidal salmonellosis incidence is on the rise even in the developed world. The young, old, HIV infected individuals, pregnant or immunocompromised individuals are at a higher risk of contracting *Salmonella* infection, leading to bacteraemia or systemic infection (138, 139). In pregnant individuals *Salmonella* can result in several complications such as chorioamnionitis, endomyometritis, neonatal septicemia, meningitis, hepatic dysfunction and even lethal septicemia in the mother (9, 22, 140). Non typhoidal *Salmonella* infection in the mother can also result in fatal transplacental infection (23, 141). *Salmonella* is also considered a serious pathogen for livestock, causing huge economical impact due to maternal and fetal loss (24). In this study we have addressed the immune mechanisms leading to loss of host resistance to infection during pregnancy using a murine model of *Salmonella* pathogenesis.

5.1 Mouse pregnancy-ST infection model

Previous work in the laboratory demonstrated that ST infection adversely effects both maternal and fetal survival. ST grew unabatedly in the placenta leading to invasive fatal

infection and ~100% fetal loss. The infected pregnant hosts succumbed within 7 days of infection, displaying a much higher bacterial burden in the peripheral organs as compared to non pregnant infected mice (21). Thus, this study was undertaken to decipher what is lacking or differentially modulated during pregnancy precipitating the rapid adverse outcome. Although *Salmonella* infections are normally contracted through the oral route, it is hard to imitate the natural epidemiology of infection in a murine model. When we initiated ST infection via the oral route, as expected a higher infective dose was required to establish systemic infection. While placental infection was observed even after oral infection, there was a lot of variability among individual mice, as may be expected for such an infection route even in the non pregnant state. Nevertheless, as *Salmonella* infection in susceptible strains of mice ultimately leads to systemic pathogenesis and consequences, we utilized the intravenous route of infection initiation to provide a controlled population study for systemic effects.

The previous studies in our laboratory showing susceptibility of maternal hosts to ST infection were done using the inbred 129x1Sv/J strain of mice. 129x1Sv/J mice have a mutation in the nramp1 gene which confers them resistance to *Salmonella* infections (21). In this strain, the non pregnant mice develop a chronic infection lasting 60-90 days and do not succumb to the infection like other susceptible strains such as the C57BL6/J mice. However, pregnancy converts the resistant 129x1Sv/J mouse into a highly susceptible one. We first wanted to reiterate the above observations in F1 hybrids, to substantiate that the above findings are not strain specific. Our observation that the pregnant 129.B6F1 mice also succumb to ST infection during pregnancy indicates a generalized pregnancy-specific modulation of host responses to ST, rather than a strain-dependant effect. The

placenta was the most permissive to bacterial proliferation, with a 10^3 infection dose resulting in ~10^8 bacterial burden post 72 h of infection along with ~100% fetal resorptions. The uteroplacental unit in the 129.B6F1 mice shows a much higher bacterial burden (~10^8) as compared to the peripheral organs (~10^5-10^6), suggesting that ST, similar to some pathogens such as *Listeria, Toxoplasma, plasmodium* etc., preferentially invades and proliferates in the placenta causing fetal demise (20)(142)(143) (Fig. 1)

5.2 Pregnancy-specific immunosuppression and pathogenesis of ST

Pregnancy may be viewed as a state of tolerance when several immune alterations occur to prevent rejection of the allogenic fetus (144). It is possible that some pregnancy-related immunosuppressive mechanisms resulted in diminished host resistance towards ST. Serum levels of sex hormones such as progesterone go up during pregnancy and play an important role in maintaining healthy pregnancy (145). Sex hormones are known to alter host defenses during pregnancy (125-127). Estrogen and progesterone have been shown to regulate changes in the recruitment of neutrophils and macrophages in the female reproductive tract in mice throughout the estrous cycle (146, 147). Estrogen is also known to decrease the severity of some autoimmune diseases such as experimental autoimmune encephalomyelitis in mice and multiple sclerosis in humans (148) whereas it known to increase susceptibility to certain genital tract infections by *Mycoplasma hominis* and *Ureaplasma urealyticum* in murine models (149, 150). Estrogen treatment in mice leads to enhanced FoxP3 expression, a hall mark of T_{regs}, as well as IL10 and Programmed death receptor-1 (PD-1) expression (151)(152). PD-1- PD-Ligand1 interactions have been implicated in promoting feto-maternal tolerance (153). Progesterone secreted by the placenta during pregnancy is critical in protecting the fetus

from T cell mediated rejection (154). This is attained by a shift towards Th2 cytokine environment and inhibition of lymphocyte activation (155, 156). Expression of progesterone receptors on decidual NK cells and peripheral blood lymphocytes increases during pregnancy (157). Binding of progesterone to its receptor induces secretion of a protein called *progesterone induced protein blocking factor* which has direct inhibitory effects on the cytolytic activities of NK cells and lymphocytes (158). All together it is possible that pregnancy hormones may influence host immune responses to ST in a bystander fashion. We therefore examined if administrating exogenous hormones could mimic the immune status of the pregnant host and susceptibility to ST.

Two ways of estrogen administration were included in our study; fat soluble β estradiol which we emulsified in sesame oil, and a water soluble form of estrogen bound to a carrier. Serum levels of estrogen and progesterone in pregnant mice are 14-22 ng/ml and 100-170 ng/ml respectively (127). In our case, estrogen treatment of 4 μg or 40 μg (these injection doses should result in serum levels of estrogen that are higher than those in pregnant mice) to ovariectomized mice (to avoid any endogenous estrogen) did not have any significant effect on the day 3 spleen CFU's of the treated animals. To rule out any possibility that estrogen may not have dissipated from the oil emulsion properly, we also treated the mice with a water soluble estrogen formulation that has been previously used in animal models to achieve systemic circulating levels (159) similar to those observed in pregnancy. But similar to the previous observation, water soluble estrogen did not affect the infection outcome. Progesterone treatment also did not make any difference to the CFU's (data not shown), thus suggesting that the adverse effects of ST infection on

pregnant hosts may not be attributable to direct immune suppressive effects of pregnancy hormones (Fig. 2).

Populations of $CD25^+$ $CD4^+$ T_{regs} increase during both murine and human pregnancies and have been suggested to play a crucial role in suppressing paternal antigen specific T cell responses (79, 160). Abortion prone strain combinations in mice have been shown to generate low levels of T_{regs} and transfer of T_{regs} from pregnant mice rescues these mice from spontaneous abortions (161). Although T_{regs} are important in the maintenance of allogenic pregnancies, they are present even in syngenic pregnancies (79). Therefore, it is likely that the immunosuppressive effects of T_{regs} during pregnancy leads to increased vulnerability of pregnant mice towards ST infection. Our observation that T_{reg} depletion prior to infection leads to a slightly decreased systemic ST burden in pregnant hosts is suggestive of a role for T_{regs} in modulating host susceptibility to infection. However, the data lack statistical significance, and depletion post-infection had little effect. Thus, further studies are required, in particular profiling of $FoxP3^+$ T_{reg} populations in ST-infected pregnant hosts to definitively reveal the role of this population (Fig. 3).

5.3 Bacterial virulence and pathogenesis in pregnant hosts

Next we focused on how bacterial virulence may influence ST infection in pregnant hosts. ST is a highly virulent pathogen which has developed a complex array of virulence genes clustered in pathogenicity islands (162). Several mutants of ST are available which are defective in different virulence mechanisms. We surmised that infecting pregnant hosts with these mutants will aid in deciphering what bacterial genes or key virulence factors manipulate ST pathogenesis in pregnant hosts. Previous studies carried out in our

laboratory (21) have shown that infection with ST *ssaR* mutant a highly attenuated mutant, defective in SPI-2 TTSS does not result in exacerbated infection in pregnant 129x1sv/J mice. However, *ssaR* infection showed high placental colonization which correlated to ~50% fetal resorption. In this study we have ascertained the effect of the ST *invA* mutant on pregnant hosts. *InvA* mutants have a defect in SPI-1 and are defective in invasion after oral challenge. However, we observed that intravenous infection with *invA* results in severe infection in pregnant hosts similar to ST WT. Furthermore, our observation of very high bacterial load in placental tissues suggests that the *invA* mutation may not affect placental invasive ability. Nevertheless, the variable resorption rate observed with *invA* mutation, may suggest a differential interaction with placental cells, relative to ST WT (Fig. 4).

Next, we decided to characterize the infection during pregnancy using a nonvirulent ST *aroA* mutant. ST *aroA* is a highly attenuated strain and 10^3 *aroA* infection in 129.B6F1 mice resulted in very low bacterial load in the peripheral organs and the placenta along with no discernable effects on the fetus. However, resistance of pregnant hosts to 10^3 *aroA* infection may be due to a very low rate of bacterial burden. Therefore, we wanted to compare ST WT with *aroA* infection wherein similar bacterial burden could be achieved in the various organs. To this end, we used a higher dose (10^6) *aroA* such that the bacterial burden in peripheral organs was comparable to that achieved with 10^3 WT infection. Interestingly, the pregnant and the non pregnant mice exhibited a similar bacterial load in the systemic compartments even with the higher dose. Nevertheless, the placenta showed very high bacterial colonization similar to ST WT and *invA* (~10^8). But

in contrast to WT and *invA* infections, despite the high placental bacterial colonization, infection with *aroA* resulted in very low fetal resorption rate (Fig. 5).

These results suggest that it is not the mere absolute bacterial numbers in the placenta which lead to fetal loss but the virulence of the bacteria that triggers unfavorable responses leading to fetal demise.

Our results also indicate that virulent WT, *invA* mutant as well as the non virulent *aroA* mutant all invade the placenta. Previous studies (21) in our laboratory have shown that for WT infection (with 10^3 CFU) in 129x1Sv/J mice, ~50 bacteria reach the placenta in the first two hours. However, this number grows stupendously and becomes ~10^7 within 14-30 h. In contrast, after infection with 10^3 ST WT, ~200 bacteria go into the spleen initially but their number only marginally increases in the first 24 h. In this study we show that the kinetics of bacterial replication with ST WT is similar for the 129.B6F1 strain (Fig. 6). Moreover, based on the calculation that ~10% of the infective load after an intravenous injection probably circulates to different organs, we can envisage that a 10^6 CFU infection of the *aroA* mutant, may result in ~10^5 bacteria tracking to the various organs. We may then surmise that number does not remarkably increase in the spleens of pregnant-*aroA* infected mice, consistent with the muted replication ability of this strain. In case of the placenta, both WT and *aroA* infection show a colossal burden of ~10^8 by the first 24 h highlighting the vulnerability of the placental tissue to ST invasion. However it is quite evident that the replication rate of WT is very high as compared to *aroA* as a 3-log higher infection dose with the latter is required to achieve bacterial burdens similar to 10^3 WT infection. Clearly the placental tissue seems to facilitate ST

replication, providing a protective niche for bacterial survival and/or the bacteria have some unique invasive mechanism which allows exquisite colonization of the placenta. In the case of pregnancy associated malaria, *Plasmodium* infected erythrocytes sequester in the placenta of the mother and the protein chondroitin sulphate A serves as an adhesion receptor for the parasite in the placenta (163). *Listeria monocytogenes* exploits act A to gain accesses and favorably invade the placenta (164). Thus, the exact mechanism behind ST's increased tropism towards the placenta needs to be further delineated.

Although *aroA* does not replicate as actively as the WT bacteria, given the high placental bacterial burden achieved we surmised that at least some bacterial surface interactions with placental cells may be similar as that achieved with WT infection. Thus it is interesting, that while virulent ST leads to ~100% fetal death, non virulent *aroA* infected mothers go on to have full term pregnancies and give birth to live pups. Moreover while WT infection adversely affects maternal survival, *aroA* infection has no such effect (Fig. 8). CFU's of WT and *aroA* infected fetal livers revealed that both WT and *aroA* lead to vertical transmission of the infection (Fig. 7). Thus, even in case of attenuated *aroA* infection, the pathogen invades the placenta infecting the fetus, but the infection is not detrimental to the pregnancy. These results clearly indicate that it is qualitative nature of the interaction of the bacteria with the host that differentially modulates fetal and maternal survival.

Infections during pregnancy are not uncommon. However many infections caused by organisms such as *Listeria*, *Plasmodium*, *GroupB streptococcus*, *Human papilloma virus*, mostly have a profound impact on the fetus but are often not life threatening to the

mother (143, 163, 165, 167). Then why is ST WT infection detrimental not only to the fetus but also fatal to the mother within 7 days? To address this issue we decided to look into the cytokine and cellular mechanisms that may influence host susceptibility to ST. As an infection dose of 10^6 *aroA* resulted in an absolute bacterial burden comparable to 10^3 WT but contrasting fetal and maternal outcomes, we used these two infection models to further delineate host-pathogen interactions and the role of virulence.

5.4 Cytokines and resistance to ST infection

Cytokines/chemokines are the soluble mediators of the immune system and they often regulate the course of the host's immune response to any infection. Previous data on the 129X1sv/J mice showed that IL6 was upregulated while IL12 was downregulated in the serum of pregnant ST WT infected mice as compared to non pregnant infected mice (21). However, in this study we observed that in the 129.B6F1 mice, while IL6 was elevated in the serum of pregnant ST WT infected mice, no major difference was observed in serum IL12 levels (Fig. 9). Therefore, the decrease in serum IL12 levels in the 129X1Sv/J mice may be a strain-specific observation. However, when the serum levels of 40 different cytokines/chemokines were detected using the proteome assay, key differences in the overall cytokine response were noted between non-pregnant and pregnant, ST WT and ST *aroA* infected groups.

Firstly, select cytokines were elevated in response to infection, and this remained similar in ST WT and ST *aroA* infections in both pregnant and non-pregnant hosts. For example, IP10, CCL2 and CXCL9 showed a similar 5-10 fold increase in all infected hosts relative to their naïve non-infected counterparts (Fig. 11). IP10/CXCL10 is released by a variety

of cells such as monocytes and endothelial cells in response to IFNγ and LPS (168), CXCL9, a T cell chemoattractant that is also induced by IFNγ (169), and CCL2/Monocyte chemotactic protein1 is involved in recruiting monocytes and T cells to the site of infection (170). The elevation of these cytokines/chemokines demonstrated that both non pregnant and pregnant hosts rapidly initiate the recruitment of innate immune cells such as monocytes to the sites of infection. However, the levels of certain other inflammatory cytokines and chemokines were found to be highly elevated specifically in the pregnant ST WT infected group. These included BLC, IL6, G-CSF, IL1Ra and I309 (Fig. 12). The increase in the levels of BLC which is a major chemoattractant for B cells (171) may be due to the effect of pregnancy, which is believed to be more biased towards humoral responses (83). IL6 is a multifunctional cytokine with both pro- and anti-inflammatory properties (172, 173). It may be secreted by monocytes, macrophages, lymphocytes and epithelial cells under different conditions. A high level of IL6 has been implicated in contributing to pathogenesis of many diseases such as rheumatoid arthritis and inflammatory bowel disease. It has been shown to be involved in B cell differentiation, monocyte proliferation, regulating neutrophil recruitment to the site of injury or infection (174- 176). Thus overt production of IL6 can influence many cell types and result in an overt and/or faulty inflammatory response. G-CSF and I-309/CCL1 are major chemoattractants for neutrophils and monocytes (177, 178). G-CSF is important for the proliferation of granulocyte precursors, their differentiation, survival and function (179). Previously it has been shown that in case of *Pseudomonas aeruginosa* infection, G-CSF plays a very critical role in balancing bacterial clearance and inflammation (180). High G-CSF levels have been associated

with pregnancy complications such as preterm labor and chorioamnionitis (96, 181). Elevated amounts of G-CSF may also result in excessive inflammation and in turn lead to tissue injury. Some additional inflammatory mediators such as KC (mouse homologue for human IL8), MCP-5 and MIP-2 which are known to activate neutrophils and monocytes (182-184)) were also found to be elevated specifically in the pregnant WT infected group. Since these cytokine responses are not elevated in the pregnant *aroA* infected mice, it reaffirms that defective and/or overt inflammation in response to WT infection during pregnancy may be the root cause for the maternal pathology. Moreover, as many other cytokines/chemokines were found to remain unchanged among the infected non pregnant and pregnant groups (Table 2), a specific role is implicated for those cytokines profoundly increased in pregnant ST WT infected mice in contributing to adverse pathogenesis.

While the proteome array provided a comprehensive picture of circulating cytokine/chemokine levels in pregnant hosts adversely responding to ST, immune responses are initially triggered locally at sites of infection. Thus, we wanted to determine the cytokine profile in the organs of the infected mice. The QRT-PCR analysis of key inflammatory and anti-inflammatory cytokines in the spleens of individual mice revealed a differential picture of cytokine responses in the non pregnant and pregnant infected animals.

First, we considered the anti-inflammatory cytokines. IL10 expression was found to be elevated in the spleens of pregnant non infected mice as compared to naïve mice consistent with the notion that high IL10 environment is favored for successful pregnancy

(185). Indeed as the healthy placenta has been reported to overproduce IL10 (185), it can be envisaged that a similar systemic environment may be favored in the non infected pregnant state. On the other hand, the increase in systemic IL10 levels in non pregnant mice in response to WT and *aroA* infection may be a normal regulatory response, triggered in the host to keep in check the inflammation caused by infection. Similarly, the significant increase in the IL10 levels in pregnant WT infected mice may be a host attempt to down modulate the overt inflammation triggered by ST WT infection. In line with this possibility that IL10 may regulate ST-induced overt inflammation, *aroA* infected pregnant mice showed the most remarkable increase in the levels of splenic IL10 relative to both pregnant non infected and pregnant WT infected mice. These data suggest a model wherein the virulent pathogen (WT) failed to induce sufficient expression of cytokines such as IL10, that can down-regulate inflammation and promote survival. In contrast, attenuated pathogen *aroA* induced high levels of IL10, which may have efficiently down modulated overt inflammation and promoted survival.

Next, consistent with the serum data, non pregnant and pregnant WT infected mice showed an increased trend of splenic IL6 expression. TGFβ levels were similar in the non pregnant and WT infected groups. However *aroA* infected non pregnant and pregnant groups showed significantly lower levels of TGFβ (Fig. 13). It is unclear whether this bears any significance in the pathogenesis of *aroA* infection, or is a result of altered bacterial interaction with the host resulting in qualitative differences in cytokine production.

Looking at the inflammatory cytokines, levels of splenic IFNγ increased in the non pregnant mice in response to WT and *aroA* infection in comparison to naïve suggestive of

a beneficial increase in this cytokine in response to infection. Indeed, an IFNγ response has been indicated to be important for control of both ST WT and ST *aroA* infection (54, 186). However, splenic IFNγ levels did not show any differential pattern of expression among the pregnant mice, suggesting that it was not the lack of production of this cytokine that was causative in the severe systemic disease in ST WT infected pregnant animals.

Similar to IFNγ, the splenic TNFα levels showed an increased trend in non pregnant WT and *aroA* infected mice as compared to the naïve mice again favoring a beneficial role for this cytokine in clearing infection. In lieu with this, the pregnant mice infected with ST WT ST showed a significant decrease in splenic TNFα levels as compared to non pregnant WT infected mice (Fig. 14). TNFα has been shown to be critical for resistance against *Salmonella*. Administration of anti-TNFα antibody in mice makes them highly susceptible to ST infection (187). TNF receptor p55 KO mice succumb more rapidly to ST (188). Thus, it appears that at the systemic lymphoid compartment, an appropriate inflammatory response to ST WT is lacking in the pregnant hosts. As opposed to pregnant WT infected mice, pregnant *aroA* infected mice expressed TNFα levels comparable to non pregnant *aroA* infected mice. However, some studies have suggested that TNFα induction is not critical for clearance of *aroA* infection (188). Taken together, it is possible that the lack of sufficient inflammation in systemic lymphoid compartments and chiefly splenic TNFα production in the pregnant ST WT infected hosts may have contributed to adverse maternal outcome. As it has been suggested that activation of a limited number of pro-inflammatory cytokines suffices for control of infection with ST *aroA* mutants (189), it is also possible that pregnancy-induced weakening in the

lymphoid inflammatory response to infection, may have borne little consequence to the host to combat *aroA*.

A dichotomy in the cytokine responses is however revealed by our data, wherein the inflammatory response was profoundly elevated at the feto-maternal interface of pregnant ST WT infected animals. Indeed overt inflammation rather than absolute bacterial burden appears to have led to the adverse fetal outcome. For example, we found that the placental expression levels of G-CSF and IL6 were significantly higher in the pregnant WT infected mice as compared to pregnant non infected mice, and this increase was not seen in the case of *aroA* infection despite high bacterial burden (Fig. 15). Furthermore, placental TNFα levels were considerably higher (~100 fold) in the pregnant WT infected animals whereas it was contrastingly very low in the *aroA* infected group. TNFα is a toxic cytokine that when produced in excessive amounts has been associated with immunopathology and mortality due to LPS-associated septic shock (190). The excessive inflammation evoked by ST WT in the placental milieu may be envisaged as having influenced not only placental pathology, but also contributing to the elevated serum inflammatory cytokine/chemokine profile observed in pregnant ST WT infected mice. Thus the immune response evoked by attenuated strains such as *aroA* appeared qualitatively different, in particular not requiring or resulting in TNFα production (189), thus beneficial for the pregnant host.

Overall our results highlight a divergence and compartmentalization of the response to ST WT in pregnant hosts: while the systemic lymphoid organ remains relatively quiescent, overt placental inflammation leads to feto-maternal immunopathology.

5.5 Inflammation and placental pathology

There are several reports which suggest that infection induced inflammation can result in premature birth (191, 192). In fact we have also observed that if pregnant mice were infected with ST WT towards late pregnancy (~15-16 days), premature delivery ensued (data not shown). Nearly two decades ago it was noted that administration of inflammatory cytokines such as IFN-γ, TNF, and IL-12 can induce resorptions during pregnancy (193). Indeed it was suggested that immunomodulation during normal pregnancy leads to a Th2/anti-inflammatory bias to counteract the detrimental effects of inflammatory cytokines. Placental pathology associated with conditions such as preeclampsia, chorioamnionitis and preterm delivery have also been attributed to excessive inflammation (107, 118, 194). Although we observed that the WT infected mice do produce anti-inflammatory cytokines such as IL10 and TGFβ at the fetomaternal interface, their levels might not be sufficient to control the excessive inflammation triggered by the virulent pathogen.

The cytokines/chemokines such as G-CSF, I-309 and IL6 are also potent chemoattractants for neutrophils and monocytes suggesting that these innate immune cell types may be a critical component of the faulty inflammatory response seen in the case of ST WT infection. Our histology studies reiterate this rationale; as the placenta was heavily infiltrated with polymorphonuclear lymphocytes. Moreover, the accumulation of neutrophils deep down into the labyrinth region, indicates that the bacteria had invaded the outer layers of the placenta and breached the labyrinth to ultimately infect the fetus (Fig 19, 20). In contrast to this, in the *aroA* infected placentas the labyrinth layer looked healthy. Only few neutrophils were seen, with restricted signs of necrosis localized to the

decidual region. Taken together, it appears that neutrophils primarily drive the necrosis in the case of ST WT infection.

While MPO staining reiterates the abundance of neutrophils in ST WT infected placentas, we also noted some very large cells positive for MPO in the *aroA* infected samples. Morphologically these appear to be macrophages which may have engulfed dying neutrophils. Thus we speculate that *aroA* infection may be promoting a controlled inflammatory environment such that neutrophils are initially recruited to the placenta, however subsequent activation of macrophages results in their clearance. However, this needs further validation. Previously, it has also been reported in the context of *Listeria* infection that activation of macrophages and neutrophils are critical to clear *Listeria* infection (195). Absence of neutrophil clearing by macrophages results in heavy neutrophil accumulation in the systemic organs leading to microabscess formation (195). This strongly indicates the role of macrophages in regulating neutrophil influx to the site of infection. A similar increase in MPO has also been observed in the placenta and circulation of women suffering from preeclampsia (196). It has been reported previously that pro-inflammatory cytokines such as TNFα induce the expression of adhesion molecule ICAM-1 in the placenta. The trophoblastic cells normally do not express adhesion molecules at basal levels (197). The enormous amounts of pro-inflammatory cytokines produced in response to placental ST WT infection may have facilitated the recruitment and accumulation of neutrophils into the placenta by enhancing the expression of adhesion molecules on the trophoblast cells.

Uterine NK (uNK) cells are the most abundant lymphocytes present in the uterus and are a major source of IFNγ in the mouse uterus during the first half of pregnancy (198). IFNγ produced by uNK cells is essential for murine arterial remodellling and critical for implantation (86). However, excessive IFNγ is harmful to pregnancy (199) and significantly higher levels of IFNγ produced by NK cells during pregnancy is proposed to be a potential mechanism behind complications like preeclampsia (114, 200). Increased uNK cell cytotoxicity in response to LPS has been implicated in inflammation mediated fetal demise in IL10 deficient mice (201). In our current study we have uncovered the role of neutrophils in placental necrosis and fetal death in response to *Salmonella* infection. It may be worth examining the profile of other immune cell types such as NK cells in our ST infection model and their role if any in contributing to placental pathology found in the infected pregnant animals.

5.6 Pregnancy and concurrent infection

The data presented here provides strong evidence that it is not absolute bacterial burden but the nature of inflammation evoked by the pathogen which largely determines host outcome to infection. The pro-inflammatory cytokines/chemokines triggered by virulent ST WT prove to be detrimental to both the mother and the fetus. Alternatively, the attenuated ST *aroA* mutant, with a similar systemic and placental bacterial burden as WT, evokes restricted inflammation and the mother lives on to have a normal pregnancy. Fig. 21 depicts a potential scheme by which ST WT and *aroA* infections differentially modulate host immunity in the non pregnant or pregnant hosts. ST WT infection in the normally resistant non pregnant mice leads to optimum early activation of innate immune components (Fig. 21a) that control bacterial replication, and direct differentiation of T

cells (202). This leads to chronic but non-lethal infection. We speculate that in the pregnant host, even the small number of bacteria that reach the placenta, proliferate extensively due to an unknown pathogen-specific escape or molecular invasive mechanism. This results in a strong infection focus at the feto-maternal interface in the first few days after infection, with the systemic lymphoid compartment probably being quiescent and/or ignorant. The actively replicating virulent bacteria elicit excessive amounts of inflammatory cytokines/chemokines in the placenta. The overt inflammation triggers massive neutrophil infiltration and accumulation at the feto-maternal interface which leads to placental tissue injury and fetal death. Neutrophils are considered vital for defense against ST until adaptive immunity develops (203, 204). However, our observations indicate that excessive neutrophil permeation leads to rapid immunopathology in the pregnant host. The rampantly replicating bacteria and the inflammatory cytokines then spill over to the systemic compartments of the host leading to a breakdown of host resistance and death due to septic shock. In fact we have observed bacteraemia in the blood of pregnant animals on day 3 of infection with ST WT (data not shown) reiterating the generalized adverse systemic effects precipitated by initial feto-maternal infection. **Thus our model outlines a differential outcome for ST infection in the non pregnant and pregnant host dependant on the qualitative and quantitative cytokine responses (Fig.21-Model).**

Several studies have emphasized the increasing role of innate immune components in infection related pregnancy complications (205) (206) (207). The placenta expresses PRR's suggesting it has the potential to identify and respond to pathogen invasion (208). TLR-2 and TLR-4 are expressed in the first trimester human placental tissues (209).

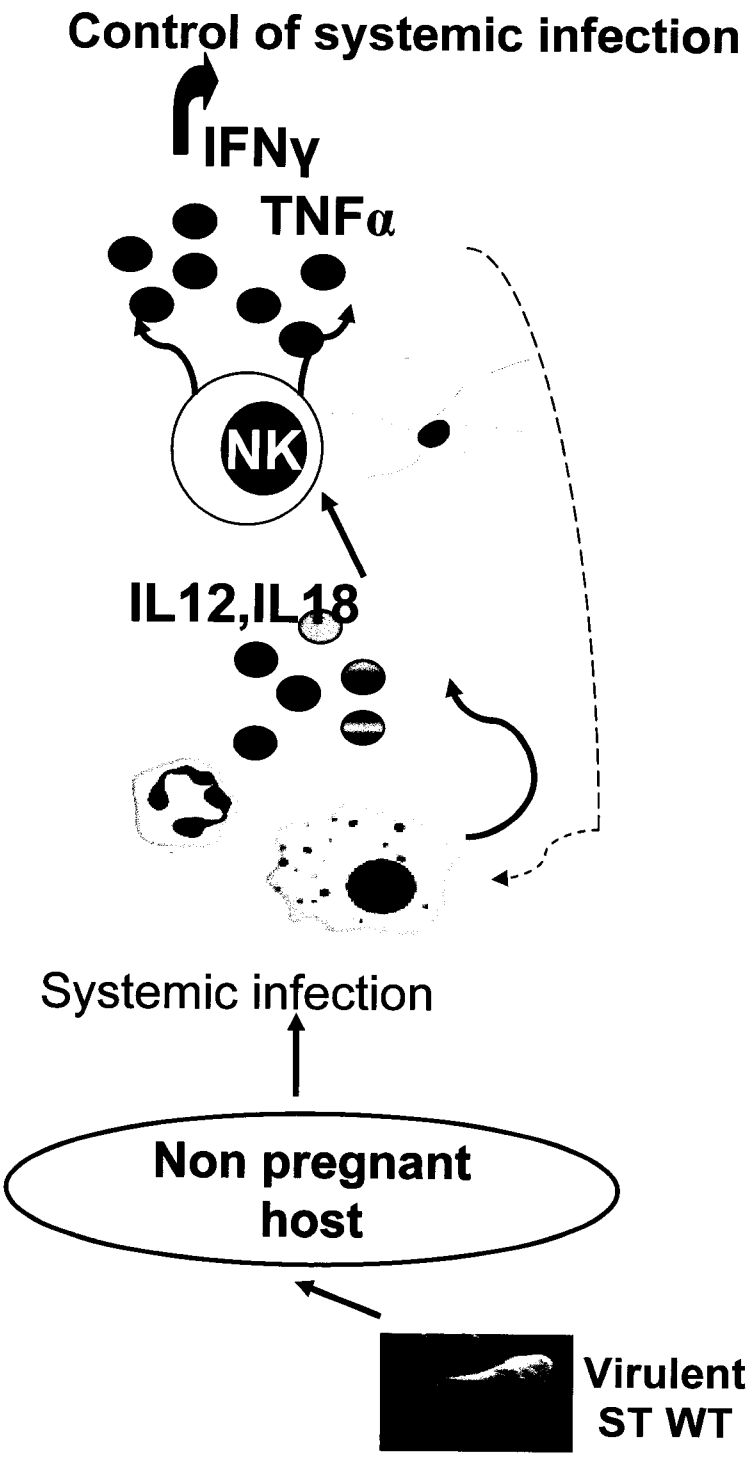


Figure 21a

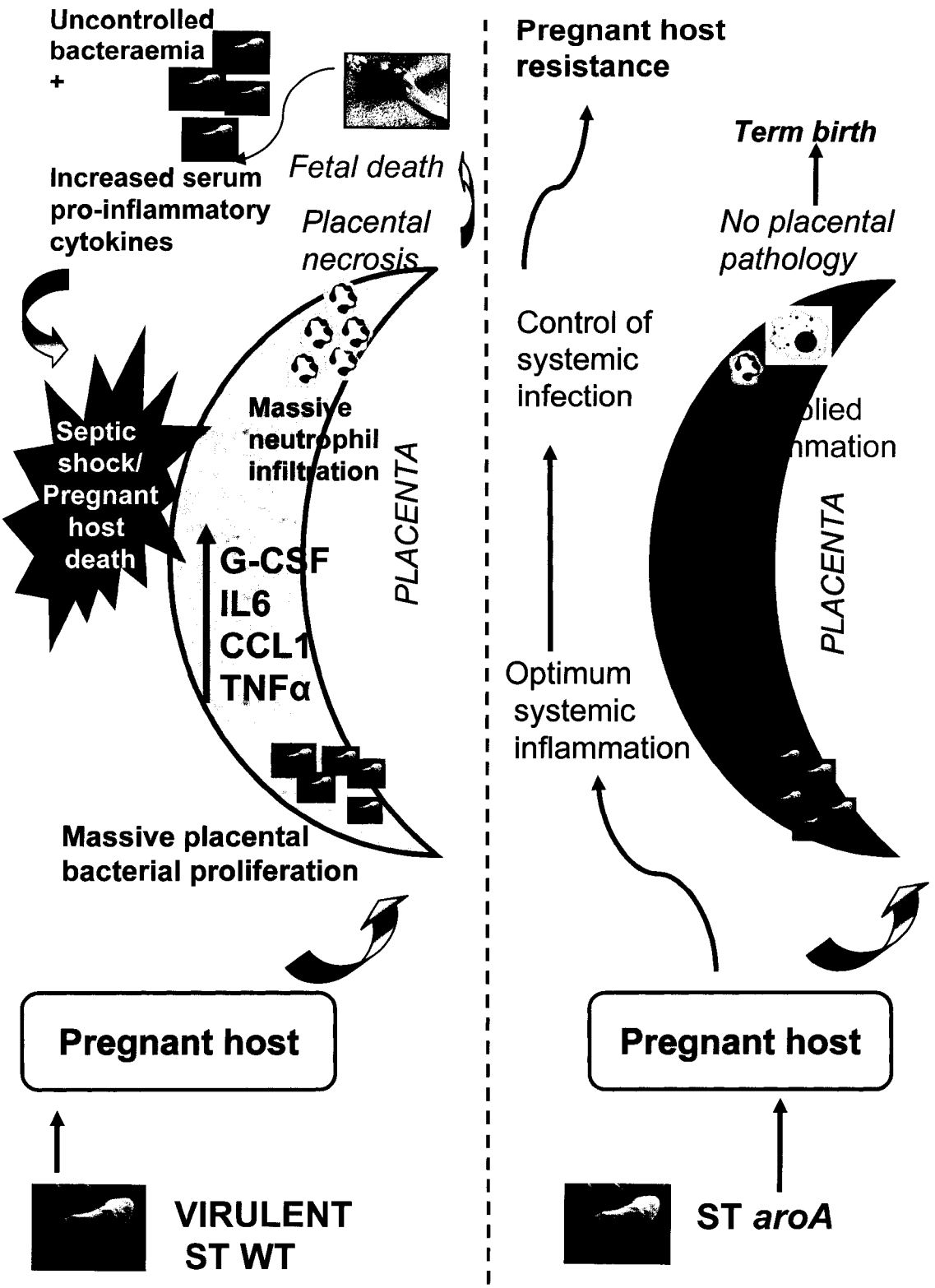


Figure 21b

Interestingly, in humans, the first trimester trophoblast cells express TLR's in the intermediate villous trophoblast and villous cytotrophoblast cells, whereas the outer layer of synctiotrophoblasts cells lack TLR expression (210). Thus the placenta acts as a specialized layer protecting the fetus against infectious agents and only if a pathogen breaches the outer trophoblast layer and invades deeper into the decidual compartment, immune responses are initiated. Previously it has been shown in the mice pregnancy model that LPS administration, via TLR-4 engagement leads to secretion of high levels of TNFα and IFNγ resulting in trophoblast apoptosis (211, 212). So it is possible that in ST infection, *Salmonella* LPS recognition by the placental TLR-4 induced aberrant production of inflammatory cytokines. Other than the TLR's, several other PRRs have also been implicated in modulating host recognition of pathogens. For example, NOD-like receptors such as NACHT, LRR and PYRIN domain containing proteins (NALP-1, NALP-3), Neural apoptosis inhibitory protein 5 (NAIP5), ICE protease activating factor (IPAF) act as sensors for bacterial RNA, peptidoglycan, flagellin and bacterial toxins (213). It is unclear how such PRRs may be differentially expressed by virulent versus attenuated strains, resulting in divergent effects such as our observations with ST WT and *aroA* in pregnant hosts. Recently, it has been reported that the *aro* mutation may result in defects in cell envelope biosynthesis as this strain is highly susceptible to complement killing and EDTA suggesting a defect in outer membrane integrity (39). Poor availability of aromatic compounds due to mutation in the *aro* gene may partly be responsible for the defects in cell wall structure observed in *aro* mutants. Studying the structural differences between *aroA* and WT cytoskeleton structures may reveal the molecular mechanisms that

modulate the qualitative differences in cytokine responses evoked by pathogens (213, 214).

Pregnancy is largely believed to be biased toward Th2 or humoral responses (215, 216). Concentrations of Th1 cytokines at the feto-maternal interface as well as in the circulation have been found to be elevated in women with unexplained recurrent abortions (217). Administration of cytokines like TNFα, IFNγ, and IL2 into pregnant mice causes abortions (193), whereas, IL10 administration has been found to reduce fetal loss in an abortion prone mice mating combination (82). Pregnancy related complications such as abortions and pre term labor have often been associated with an increase in maternal inflammatory and Th1 cytokines or a reduction in the expression of anti-inflammatory cytokines (218). *P. gingivalis* infection in mice leads to augmented maternal TNFα production while suppressing maternal IL10 leading to increased fetal resoptions (219). *T. cruzi* infection in mice induces high levels of maternal TNFα resulting in increased fetal mortality (220). In a *Leishmania* infection model it has been shown that Th1 responses (increased IFNγ, TNF and IL2 produced by placental cells) against the parasite resulted in decreased implantation and increased fetal resorptions in infected mice (101). Similar to the above findings, in our ST infection model, it appears that ST infection induces a shift towards inflammatory cytokines in the placenta. This disruption of balance between pro- and anti-inflammatory cytokines correlates to deadly maternal and fetal outcomes. The excessive inflammation during ST WT infection is triggered at the feto-maternal interface as contrarily the systemic organs do not seem to produce enough inflammation to curb the infection. Overall, my study highlights the complex cross-talk that may occur between concurrent infection and placenta, and the

critical balance that often needs to be maintained in the mother to allow for some beneficial inflammation to combat infection, while avoiding its overt side effects.

6. Summary and Conclusions

The main findings of this study are:

a) Pregnancy renders normally resistant mice highly susceptible to ST infection.

b) Placental tissue facilitates rapid bacterial expansion. ST WT shows profound proliferation in the placenta, leading to rapid loss of placental integrity.

c) Mere high bacterial burden is not responsible for such lethal fetal and maternal outcome because the avirulent *aroA* mutant does not induce fetal loss or maternal illness, despite massive placental infection.

d) Virulent ST triggers extreme inflammation characterized by high placental expression of pro-inflammatory cytokine/chemokines along with heavy infiltration of neutrophils into the placenta.

e) This results in massive increase of serum pro-inflammatory cytokine responses leading to septic shock.

Thus, virulence, but not pathogen burden, triggers a unique innate immune signature and inflammatory response at the feto-maternal interface, accelerating pathogenesis in the maternal host.

Our data supports the hypothesis that pro-inflammatory cytokines are detrimental to pregnancy. It has been previously shown that in case of ST infection adaptive immunity is substantially delayed (73). Consequently, innate immune responses may be solely responsible in controlling the infection untill specific adaptive immunity is activated and any deviation or deregulation in the innate immune arm especially in a pregnant host may lead to lethal host consequences.

We still do not know the mechanisms which enable *Salmonella* to proliferate unabatedly in the placenta of the pregnant animals. It will be interesting to investigate where the pathogen hides or sequesters itself within the placenta to replicate unrestrained? Does the pathogen utilize a specialized receptor to gain entry into the placental cells or does the usual type III secretion system characteristic of ST allow it to invade intestinal epithelial as well as placental cells? Is there any particular cell type which the bacterium exploits to proliferate within the placenta? Making longitudinal sections of the placenta for histology studies can aid in deciphering which layers of the placenta the pathogen preferentially invades and/or proliferates in. Further cell specific staining in the placenta needs to be done to identify other lymphocyte populations which may be recruited to the feto-maternal interface during WT infection.

These studies also bring to light the precise interactions of ST with a pregnant host. This can also aid in deciphering the mechanisms behind other pregnancy complications such as preeclempsia and pre-mature birth. The understanding that certain pathogens can exploit the altered immune status of the pregnant host resulting in deleterious maternal and fetal outcome is vital for designing vaccines which will be efficacious for pregnant women, efficient management of disease epidemics, increased reproductive success and improved women's health in general.

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