GROUP B STREPTOCOCCUS INFECTION OF THE HUMAN EXTRAPLACENTAL MEMBRANES

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Toxicology)
in The University of Michigan
2014

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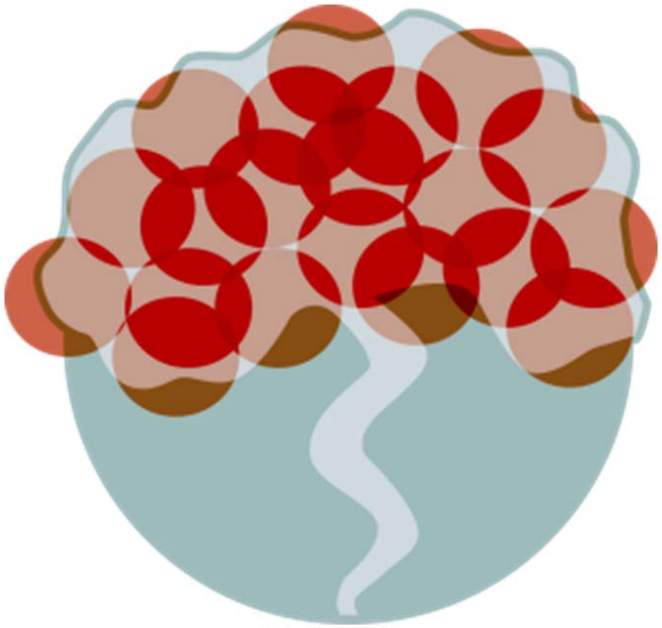

Designed by Natalie Wiersma

DEDICATION

To my family and friends

ACKNOWLEDGMENTS

Many people have graciously provided support to me throughout my time at the University of Michigan. I am indebted to my thesis advisor, Dr. Rita Loch-Caruso who has mentored me with patience, kindness, and constructive criticism. This dissertation would not have been possible without the many thoughtful discussions we've had over the years. Thank you to my committee members, Dr. David Aronoff, Dr. Peter Mancuso, Dr. Chaunwu Xi, and Dr. Carl Marrs, for their mentorship and advice.

Thanks to my whole family. A special thanks to my parents, Dr. Ronald and Laurel Boldenow for their endless love, support, and encouragement. They knew what a PhD meant physically, mentally, and spiritually, and supported me anyways. And for my siblings: Katherine and Karl Boldenow for sharing many laughs, tears, and adventures while attending school together.

I also wish to express my gratitude to my past and present lab members: Iman Hassan, Hae-Ryung Park, Dr. Kelly Hogan, Dr. Lauren Tetz, Dr. Casandra Korte, Dr. Sarah Jones, Adrienne Cheng, Kevin Sun, Josephine Tan, Sara Liao, Anjana Kumar, and Faith Bjork. Thank-you for asking endless thought provoking questions, teaching me new lab methods, and fostering a sense of exploration and collaboration. Also thanks to Dr. Shannon Manning, Dr. Hui Jiang, and Dr. Richard Lieberman for providing GBS strains, statistical and histology advice.

Finally, I would like to thank my roommates, friends, and classmates. Natalie Wiersma, Allyson Green, Jennie Heidmann, Samantha Miller, Karen Van Dyke, Andrew Van Winkle, and

Allison Schepers: You have seen the best and worst in me, thanks for all your help and sharing fun times with me.

This work would not have been possible without funding from the University of Michigan Horace H. Rackham School of Graduate Studies and research grants provided to Dr. Rita Loch-Caruso from the National Institute of Environmental Health (NIEHS)/National Institute of Health (NIH), Superfund Research Program of the NIEHS, and the Global Alliance for Preterm Prematurity and Stillbirth (GAPPS)/Bill and Melinda Gates Foundation.

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ABSTRACT

Streptococcus agalactiae (GBS) is the leading cause of infectious neonatal morbidity and mortality in the United States. GBS infections in the gravid female reproductive tract are associated with adverse birth outcomes. The ascending pathway of infection begins with GBS colonization of the vagina, passes through the cervix and uterine cavity where it can cross the extraplacental membranes and infect the fetus. However, the mechanisms by which GBS colonizes and infects the extraplacental membranes remain poorly understood. In addition, environmental toxicant interaction with the innate immune system during pregnancy-related infections remains to be elucidated.

In the present thesis, extraplacental membranes cocultured with GBS increased secretion of the antimicrobial peptide human beta defensin (HBD)-2 and killed GBS over time ($P < 0.05$). Notably, a pattern of localized increased HBD-2 in the amnion of GBS-infected membranes was observed. Interleukin (IL)-1α and IL-1β secreted from choriodecidual tissue was essential for stimulating HBD-2 in the amnion cells. Direct stimulation of amnion cells with live GBS, lipoteichoic acid (LTA), or lipopolysaccharide (LPS) did not increase HBD-2 release. Increases in cytokine release were GBS strain dependent ($P < 0.05$). GBS recovery from membranes was also GBS strain dependent, with colonizing strains persisting on the choriodecidual side of the membranes. The trichloroethylene (TCE) metabolite *S*-(1,2)-dichlorovinyl-L-cysteine (DCVC) significantly inhibited pathogen (LTA, LPS, and GBS)-stimulated TNF-α release from

extraplacental membranes. Both TNF-α mRNA expression and protein secretion were inhibited as early as 4 h after initiating co-treatment of tissue punches with DCVC and LTA ($P < 0.05$). A different TCE metabolite, trichloroacetic acid (10-500 μM), failed to inhibit LTA-stimulated cytokine release from extraplacental membranes.

In summary, extraplacental membranes in culture mounted a robust immune response to GBS. Cell-to-cell signaling from the choriodecidua to the amnion was critical for GBS-stimulated HBD-2 in amnion. Host response in the extraplacental membranes was GBS strain specific. The results from the present study provide new insight into the mechanisms of host defense during GBS infection and need to be considered for future treatment and prevention strategies. In addition, pathogen-toxicant interactions should be considered in the current paradigm for increased risk for intrauterine infection.

CHAPTER 1. INTRODUCTION

GBS Infection during Pregnancy as a Public Health Problem

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a gram positive bacterium. GBS remains the leading cause of infectious neonatal morbidity and mortality and is associated with adverse birth outcomes (MMWR) (Verani, McGee et al. 2010). Although GBS disease is reported in non-pregnant adults, pregnancy increases the risk of invasive GBS disease (Verani, McGee et al. 2010). Genital GBS colonization of pregnant women is associated with early term births and low birth weight (Mitchell, Brou et al. 2013). In addition, GBS is associated with neonate sepsis and meningitis, and GBS-infected infants born preterm have increased odds of infant mortality compared to infants delivered at term (Goldenberg, Hauth et al. 2000, Jordan, Farley et al. 2008). GBS was the most common microorganism isolated from maternal and fetal tissues of women with midgestation spontaneous abortion (McDonald and Chambers 2000). Likewise, histological chorioamnionitis and preterm birth at less than 32 weeks gestation were associated with GBS isolation from extraplacental membranes (Hillier, Krohn et al. 1991). Despite knowledge that GBS is a public health issue, little is known about the mechanisms by which GBS interacts with the host. Understanding the pregnant women's immune response to GBS is essential for developing intervention and treatment strategies prior to neonatal disease.

Prevalence of GBS Colonization and Incidence of Infection

The prevalence of GBS recto-vaginal colonization varies across the world. Approximately 10-30% of women are colonized recto-vaginally with GBS. Despite recommendations to screen all women for GBS during pregnancy (Verani, McGee et al. 2010), many countries still do not routinely screen for GBS. Based on available reports at the time, Stoll et al. estimated worldwide GBS colonization in 1998 to be approximately 12.7% (Stoll and Schuchat 1998). Since that publication, additional reports of GBS rates (summarized in Table 1.1) suggest that maternal GBS colonization continues to be a global concern.

In the United States, the incidence of early-onset neonatal GBS disease (with clinical symptoms within the first week of life) is 0.34 cases per 1000 live births (Jordan, Farley et al. 2008). The incidence of late-onset (> 1 week of age) neonatal GBS disease in the United States is 0.35 cases per 1000 live births. With prophylactic antibiotic treatment given to GBS-positive women, the incidence of early-onset neonatal GBS disease has decreased approximately 80% since the early 1990s. However, the incidence of late onset of disease has remained relatively stable, suggesting that treatment and intervention strategies need to expand beyond screening and antibiotics. In a systematic review and meta-analysis of papers reporting incidence worldwide, Edmond et al. found a mean incidence of 0.53 cases per 1000 live births (Edmond, Kortsalioudaki et al. 2012). Early-onset disease predominated with an average incidence of 0.43 cases per 1000 live births, suggesting that early-onset GBS disease is more prevalent worldwide, compared to the United States. Global differences in early-onset GBS disease during pregnancy may reflect differences in GBS screening and antibiotic prophylaxis.

GBS Strain Differences

GBS are classified into ten different serotypes based on capsular polysaccharides thought to be related to virulence (Slotved, Kong et al. 2007). GBS serotype distribution in pregnant women and neonates with disease varies widely across reports, but the most common serotypes appear to be III, Ia, and V. Edmond et al. reported serotype distribution isolated from normally sterile sites of neonates with disease (e.g., sepsis, meningitis, etc.) as: III (48.9%), Ia (22.9%), 1B (7.0%), II (6.2%), and V (9.1%) (Edmond, Kortsalioudaki et al. 2012). Similarly, Jordan et al. found that the serotype distribution isolated from normally sterile sites of neonates with disease was predominantly III (53%), Ia (24%), and V (13%) in the United States (Jordan, Farley et al. 2008). Even though serotype III appears more prevalent in neonatal disease cases, the distribution suggests GBS virulence is not solely reliant on serotype.

In addition to serotype, GBS are categorized into sequence types (ST) based on genetic variation in seven conserved genes (Manning, Springman et al. 2009). ST-17 and ST-19 are more commonly associated with neonatal disease compared to other sequence types (Manning, Springman et al. 2009). Limited studies have examined the mechanisms by which GBS serotypes and strain types alter virulence and even fewer studies have evaluated host response to different GBS serotypes and strain types.

Role of the Extraplacental Membranes in Ascending Infection

The current dogma for the progression of infection during pregnancy is the ascending pathway by which bacteria first colonize the vagina and cervix, migrate to the placenta and maternal-fetal membranes, cross the extraplacental membranes, and then colonize the amniotic cavity and fetus

(Figure 1.1) (Goldenberg, Hauth et al. 2000). Healthy extraplacental membranes (also called gestational membranes or maternal-fetal membranes), composed primarily of decidual cells, chorionic trophoblasts, fibroblasts, and amnion epithelial cells, provide a barrier that protects the fetus from infection. The extraplacental membranes also include a small number of resident innate immune cells (macrophages and monocytes) (Osman, Young et al. 2003, Osman, Young et al. 2006). Macrophage and monocyte cell responses during GBS infection have been reviewed elsewhere (Wennekamp and Henneke 2008). Macrophages and monocytes recognize GBS through pathogen-associated molecular patterns (PAMPs) and GBS promotes a rapid and robust inflammatory response in these cells.

Role of Antimicrobial Peptides in Host Response of the Gestational Tissues

GBS is capable of invading primary human chorion cells and can move through both ME180 (human cervical epithelial cell line) and primary chorion cell monolayers without disrupting the monolayer integrity (Winram, Jonas et al. 1998, Soriani, Santi et al. 2006). In contrast, GBS has not been shown to invade primary human amnion cells (Winram, Jonas et al. 1998). Several studies have shown that *ex vivo* full thickness extraplacental membranes are not penetrated by GBS and are capable of killing the bacteria (Kjaergaard, Helmig et al. 1999, Kjaergaard, Hein et al. 2001, Boldenow, Jones et al. 2013), though the ability for GBS to invade and persist in the whole extraplacental membranes may depend on GBS virulence factors such as pigmentation of GBS (Whidbey, Harrell et al. 2013).

The mechanisms by which extraplacental membranes kill bacteria are not fully established, but likely include antimicrobial peptides (AMPs) (Zaga-Clavellina, Ruiz et al. 2012, Boldenow,

Jones et al. 2013) and inflammatory mediators such as chemokines that recruit immune cells to the site to help resist infection (Stock, Kelly et al. 2007). AMPs are thought of as a first line of defense against infectious microorganisms during pregnancy because many are constitutively expressed and can kill bacteria through membrane disruption, pore formation in the membrane wall, and depolarization. Furthermore, AMPs can promote chemotaxis of T and B cells, providing an important link between the innate and adaptive immune systems. AMPs are found throughout the reproductive tract during pregnancy, including in amniotic fluid and extraplacental membranes (Horne, Stock et al. 2008, Frew and Stock 2011). Furthermore, AMPs such as human beta defensin (HBD)-2 are increased in amniotic fluid during intrauterine infection (Soto, Espinoza et al. 2007). HBDs have a broad spectrum of activity and are effective at killing both gram positive and gram negative bacteria (Chen, Niyonsaba et al. 2005).

Both our laboratory and Zaga-Clavellina et al. have shown increases in secreted HBD-2 and HBD-3, but not HBD-1, using a transwell tissue culture model of human extraplacental membranes cocultured with $1x10^6$ CFU/mL live GBS (Zaga-Clavellina, Garcia-Lopez et al. 2012, Boldenow, Jones et al. 2013). Our lab also demonstrated through immunohistochemistry that amnion cells are particularly critical to the HBD-2 response (Chapter 2).

The mechanism by which AMPs are increased in GBS-stimulated extraplacental membranes has not been fully elucidated. However, in other cell types, inflammatory cytokines are important for increased AMP expression and secretion (Liu, Destoumieux et al. 2002, Moon, Lee et al. 2002, McDermott, Redfern et al. 2003, Pioli, Weaver et al. 2006). Consistent with those studies, Stock,

et al. has shown that IL-1β directly increases HBD-2 in amnion epithelial cells (Stock, Kelly et al. 2007), and could be an important host response in GBS disease.

Role of Inflammation during GBS Infection in Humans and Animal Models

Intrauterine infection has been well established as a cause of preterm birth and other adverse birth outcomes. Infection increases inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) in the amniotic fluid (Hillier, Witkin et al. 1993, Basso, Gimenez et al. 2005, Challis, Lockwood et al. 2009). High levels of inflammatory cytokines in amniotic fluid have also been associated with meningitis and fetal brain damage (Dammann and Leviton 1997, Burd, Balakrishnan et al. 2012). Few studies associating infection with elevated cytokine and AMP levels have interrogated bacterial species or strain.

A recent report by Mitchell et al. found that maternal recto-vaginal GBS colonization increased the odds of early term birth and lower birth weight, and was associated with increased IL-1β in both maternal and fetal plasma (Mitchell, Brou et al. 2013). In addition, IL-2 levels were slightly elevated in maternal but not fetal plasma in GBS cases, and no differences were observed in IL-8, IL-6, and TNF-α. In another study two infants with GBS infections had increased plasma IL-1β, IL-2, and TNF-α compared to neonates without infection (Hodge, Hodge et al. 2004). Another study measuring IL-6 and TNF-α in cerebrospinal fluid of 62 infants found that three of the 22 infants with meningitis were GBS positive by blood culture (Dulkerian, Kilpatrick et al. 1995). All three GBS meningitis infants had elevated IL-6 levels compared to control infants without meningitis. Two of the GBS meningitis infants also had elevated TNF-α levels. These

studies, though limited, highlight differences between maternal and infant immune responses, and suggest that pathogen-specific responses occur.

Gravett and colleges developed a non-human primate model in rhesus monkeys (*Macaca mulatta*) to study GBS infection (Gravett, Witkin et al. 1994). Following GBS inoculation ($1x10^6$ CFU) in the amniotic compartment, cytokines IL-1β, IL-6, and TNF-α increased in the amniotic fluid, followed by the onset of contractions and preterm parturition. In subsequent primate experiments with similar GBS inoculation, ampicillin given in combination with dexamethasone and indomethacin suppressed IL-1β, IL-6, TNF-α, PGE_2, and $PGF_{2\alpha}$, and delayed delivery (Gravett, Adams et al. 2007). Ampicillin alone eliminated the GBS but did not reduce the cytokine levels, signifying that the current routine intervention strategy of antibiotics may not be sufficient. In a more recent study, inoculation of the choriodecidua of non-human primates with GBS increased amniotic fluid concentrations of IL-1β, IL-6, IL-8, and TNF-α; however, preterm labor and chorionic infiltration with neutrophils were not observed in all inoculated animals compared to control animals (Adams Waldorf, Gravett et al. 2011). Interestingly, lung injury in the neonates was observed even though no GBS was detected in the amniotic fluid, emphasizing the importance of the maternal immune response in the gestational tissues and suggesting that GBS crossing of the membranes does not have to occur to result in infant injury. In addition, intra-amniotic GBS infection in the non-human primate was associated with infant bacteremia and early onset sepsis (Rubens, Raff et al. 1991).

In a limited number of studies with mice, GBS infection had similar results as with the non-human primate model. Pregnant mice delivered within 18 hours following intraperitoneal (i.p.)

or intrauterine inoculation with GBS, with increased apoptosis in the placenta and membranes (Equils, Moffatt-Blue et al. 2009). Another study using non-pregnant mice injected i.p. with GBS showed increased cytokines IL-1β, TNF-α, IL-2, IFN-γ in spleen cells (Rosati, Fettucciari et al. 1998). In the latter study, IL-4, IL-5, and IL-10 were not detected or did not change during infection.

Role of Inflammation in Host Response of the Gestational Tissues

Gestational tissues stimulated with GBS also produce unique inflammatory cytokine profiles (studies summarized in Table 1.2). Using full thickness human *ex vivo* extraplacental membranes treated with GBS, Menon et al. found increases in secreted IL-1β, IL-6, IL-10, and TNF-α in the media (Menon, Peltier et al. 2009). Differences in IFN-γ or IL-8 were not observed. Using a similar extraplacental membranes model, Peltier et al. found increased IL-1β and IL-8 protein in the medium (Peltier, Drobek et al. 2012), but no changes were observed for IL-2, IL-10, and TNF-a. Using extraplacental membranes attached to transwells, Zaga et al. found increases in IL-1β and TNF-α in the choriodecidual compartment when the choriodecidual compartment or both compartments were stimulated with $1x10^6$ CFU live GBS (Zaga, Estrada-Gutierrez et al. 2004).

In addition to tissue culture models using full thickness extraplacental membranes, several studies have been conducted using human isolated cells. One study using human villous trophoblasts treated with GBS found concentration-dependent increases in IL-1β IL-6, IL-8 and IL-10 (Griesinger, Saleh et al. 2001). Kaplan et al. found that live GBS induces death in trophoblasts isolated from the membranes (Kaplan, Chung et al. 2008). Another study treated amnion cells from the extraplacental membranes with GBS found concentration-dependent

increases of IL-6 and IL-8 measured in the medium, but no apparent changes were observed in IL-1β or TNF-α secretion. However, no statistical analysis was run because the authors pooled samples (Reisenberger, Egarter et al. 1997). Primary isolated decidual cells treated with five GBS strains had strain-dependent increased secretion of MIP-1 and IL-8 (Dudley, Edwin et al. 1997). In addition, human primary chorion cells treated with the five different GBS strains had increased secreted MIP-1 with all but one GBS strain, again illustrating that host response may be dependent on GBS strain differences (Dudley, Edwin et al. 1996).

Consistent across the studies mentioned above, IL-1β increased with GBS infection. However, results varied for other cytokines such as IL-6, IL-8, and TNF-α. These differences could be due to different GBS strains used, which are rarely reported (Table 1.2). Strain differences are known to affect adherence and invasion, and could impact host response (Manning, Springman et al. 2010). In the few studies of GBS infections of gestational tissues that did include multiple strains, differences in host response across strains were noted. In addition, differences in host response could be due to use of live versus heat-killed GBS because heat killing of bacteria causes denaturation of key surface molecules and proteins that could affect the innate immune response. In particular, hemolysins (from *Staphylococcus*) that normally increase IL-1β secretion from host cells can be denatured by heat (Strunk, Richmond et al. 2011). The results of the prior study likely apply to GBS because hemolysin is particularly important to GBS-induced host responses as well (Doran, Liu et al. 2003, Whidbey, Harrell et al. 2013).

Role of Toll-like Receptors during GBS Infection of Gestational Tissues

Toll-like receptors (TLRs) reside in host cell membranes and detect bacterial components to signal the host cell response. To date, eleven mammalian TLRs have been identified, each recognizing different PAMPs (Takeda and Akira 2001). Following ligand recognition, TLRs recruit MYD88 (adaptor protein) which then initiates a signaling cascade that involves NF-κB and leads to host response including proinflammatory cytokine production (Pioli, Amiel et al. 2004, Koga, Aldo et al. 2009).

TLRs are found throughout the gestational compartment, and increases in TLRs have been noted with intrauterine infection and chorioamnionitis (Kim, Romero et al. 2004, Gillaux, Mehats et al. 2011). TLR-2 recognizes gram positive bacteria and has been shown to be important in GBS disease (Henneke, Morath et al. 2005). Lipoteichoic acid (LTA), a cell wall component of GBS and other gram positive bacteria, engages TLR-2 to promote an inflammatory response in primary blood monocyte cultures characterized by increased release of cytokines IL-8 and TNF-α (Henneke, Morath et al. 2005). Non-pregnant TLR-2 knockout mice inoculated intravenously with GBS have higher mortality rates, higher microbial loading in the blood, kidneys, and joints, and increased serum concentrations of IL-1β, IL-6, and TNF-α compared to wild-type animals (Puliti, Uematsu et al. 2009). Similarly, Mancuso et al. found that both adult and neonatal TLR-2 or MYD88 knockout mice inoculated with a low dose of GBS exhibited decreased survival, increased bacterial counts in blood, spleen, and kidney, and increased TNF-α and IL-6 in serum compared to controls (Mancuso, Midiri et al. 2004). In contrast, lack of TLR-2 and MYD88 had a protective effect in mice inoculated with higher GBS concentrations. TLR-2 knockout mouse macrophages treated with live GBS have diminished TNF-α secretion, and impaired mRNA

expression of IL-1β, IL-6, and lipocalin 2 compared to wild-type (Draper, Bethea et al. 2006). Together, these studies illustrate the importance of TLR-2 during GBS infection. However, a role for TLR-2 has yet to be established during pregnancy.

Interestingly, human extraplacental membranes treated *ex vivo* with GBS do not show increases in TLR mRNA expression, whereas TLR expression increases in extraplacental membranes exposed to other bacteria, suggesting a TLR-independent mechanism for GBS in the extraplacental membranes (Abrahams, Potter et al. 2013). TLR-independent mechanisms of host immune activation have been reported. Macrophages isolated from TLR-2 and TLR-6 knockout mice treated with GBS still showed normal TNF-α secretion compared to macrophages from wild-type mice (Henneke, Takeuchi et al. 2001). Using Chinese hamster ovary cells transfected with TLR-2, TLR-2 and CD14 inhibitors did not block TNF-α production, further supporting a TLR-2-independent mechanism (Flo, Halaas et al. 2000).

The inflammasome provides an additional mechanism for increases in inflammation during infection. Both *in vivo* and *ex vivo*, GBS-stimulated increases in IL-1β appear as a common finding and suggest an important role for the inflammasome during GBS infection. The inflammasome is a multi-protein complex composed in part of caspase-1, which is responsible for cleaving intracellular pro IL-1β to the active form, which can then be secreted from the cell. Costa et al. has demonstrated the importance of the inflammasome pathway for increasing IL-1β and IL-18 during GBS infection using mouse dendritic cells (Costa, Gupta et al. 2012).

Although TLRs and the inflammasome have been linked to cytokine secretion in gestational tissues stimulated with GBS, few studies have looked at these pathways in human tissues. Furthermore, no reports have examined the role of TLRs or the inflammasome in antimicrobial peptide responses in gestational tissues.

Toxicant-Pathogen Interactions

Increasingly, exposure to environmental contaminants have been associated with increased risk of adverse pregnancy outcomes (Ferguson, O'Neill et al. 2013). However, little is known about environmental toxicant interactions with bacteria, and potential impacts of such interactions on infectious disease. Recent articles have highlighted the need to examine toxicant-pathogen interactions in the etiology of disease (Birnbaum and Jung 2010, Dietert, DeWitt et al. 2010, Feingold, Vegosen et al. 2010). Given the likelihood that pregnant women are exposed to pollutants in their workplaces, homes, and outdoor environment, understanding the mechanisms involved in potential toxicant-pathogen interactions during pregnancy is essential. To date, only one study has examined toxicant-pathogen interactions in the pregnant woman using placental explants treated with *E.coli* and flame retardants (Peltier, Klimova et al. 2012).

Trichloroethylene as an Environmental Pollutant

Trichloroethylene (TCE; Figure 1.2A) is an industrial solvent primarily used for metal degreasing. TCE is also a widespread environmental contaminant of concern found in 852 of 1416 superfund sites (ATSDR 2011). In 2011, the Environmental Protection Agency (EPA) ranked TCE #16 on the Agency for Toxic Substances and Disease Registry (ATSDR) Priority List of Hazardous Substances (ATSDR 2011). TCE has recently been reclassified as a known

human carcinogen (U.S. Environmental Protection Agency 2011), and is also implicated in developmental, reproductive, and immune toxicity (U.S. Environmental Protection Agency 2011, Chiu, Jinot et al. 2013). TCE is metabolized through one of two pathways (Chiu, Okino et al. 2006), and metabolism is important for modulating toxicity (Griffin, Gilbert et al. 2000). The first pathway metabolizes TCE to trichloroacetic acid (TCA; Figure 1.2B) through cytochrome P450-mediated oxidation (Bradford, Lock et al. 2011). The second metabolic pathway conjugates TCE with glutathione to form *S*-(1,2)-dichlorovinyl glutathione (DCVG). DCVG is further metabolized to the bioactive metabolite *S*-(1,2)-dichlorovinyl-L-cysteine (DCVC; Figure 1.2C) (Kim, Kim et al. 2009). TCA and DCVC have been detected in human and rodents serum following exposure to TCE (Lash, Putt et al. 1999, Lash, Putt et al. 2006, Kim, Collins et al. 2009, Bradford, Lock et al. 2011). Furthermore, TCE has been detected in rodent placentas (Withey and Karpinski 1985). Given that the placenta is a highly perfused organ, it should be exposed to circulating TCE and its metabolites. Moreover, the human placenta expresses key enzymes capable of metabolizing TCE to bioactive forms, including CYP2E1, which is important for TCA formation (Hakkola, Raunio et al. 1996, Collier, Tingle et al. 2002), and glutathione-*S*-transferase (GST) (Nogutii, Barbisan et al. 2012), needed to generate DCVC.

Trichloroethylene as a Reproductive/ Immune Toxicant

Recent reports have implicated TCE in worsening infection in a rodent model. Mice co-treated with TCE and *Streptococcus zooepidemicus* had increased mortality, decreased bacterial clearance and decreased alveolar macrophage function. Increased odds of neural tube defects, oral clefts, and cardiac defects in humans are associated with TCE-contaminated drinking water (Bove, Shim et al. 2002). In addition, TCE exposure during pregnancy is associated with

intrauterine growth restriction (IUGR) (Windham, Shusterman et al. 1991). Despite evidence demonstrating TCE as a potential reproductive and immune toxicant, as well as the potential for TCE to interact with host defense during infection, no studies have examined TCE-pathogen interactions in the gestational tissues to date.

Research Objectives of this Thesis

GBS remains a serious public health issue and yet few studies have examined mechanisms by which GBS promote host responses in relevant human tissues. Furthermore, potential toxicant-pathogen interactions during pregnancy are unexplored. A better mechanistic understanding of how GBS interacts with gestational tissues is essential for designing future prevention and intervention strategies. This dissertation characterizes the innate immune response of human extraplacental membranes to Group B *Streptococcus* and tests the following hypotheses: 1) **that human extraplacental membranes resist GBS infection through increased expression of antimicrobial peptides; 2) that GBS-stimulated expression of the antimicrobial peptide HBD-2 involves paracrine cytokine signaling across the extraplacental membranes; and 3) that a metabolite of the common environmental contaminant TCE modifies the cytokine response of extraplacental membranes to GBS**. The specific aims of this research are to: 1) Determine the role of antimicrobial peptides in GBS infection of the extraplacental membranes; 2) Elucidate the signal transduction mechanisms across extraplacental membranes for HBD-2 secretion in amnion epithelial cells; 3) Examine the impact of GBS strain on the host response in the extraplacental membranes; and 4) Evaluate potential toxicant-pathogen interactions in the extraplacental membranes using TCE metabolites and GBS. The findings from this dissertation have the potential to expand our current paradigm of environmental impacts on pregnancy and

aid in development of improved intervention and treatment strategies for GBS infection prior to adverse neonatal disease.

Table 1.1. GBS Colonization Rates.

Author (Year)	Country/ Region	Sampling Method	Percent Colonized
El-Aila, et al. (2009)	Belgium	Swab culture	24
Rocchetti, et al. (2011)	Brazil	Vaginal swab culture, third trimester	25.4
Shabayek, et al. (2013)	Egypt	Vaginal Swab Culture, 264 preg. women, 100 non-preg. women	27.4
Mohammed, et al. (2013)	Ethiopia	Recto-vaginal swab culture	20.9
Tsui, et al. (2009)	Hong Kong	Swab culture	10.4
Javanmanesh and Eshraghi (2013)	Iran	Recto-vaginal swab culture, third trimester	22.76
Fatemi, et al. (2010)	Iran	Swab culture and PCR	20.6
Kim, et al. (2011)	Korea	Recto-vaginal swab culture	8.3
Hong, et al. (2010)	Korea	Unreported	10.0
Uh, et al. (1997)	Korea	Recto-vaginal swab culture	3.9
Al-Sweih, et al. (2005)	Kuwait	Recto-vaginal Swab culture at delivery	14.6
Joao, et al. (2012)	Latin America	Recto-vaginal swab cultures during preg.	8.3
Barcaite, et al. (2012)	Lithuania	Recto-vaginal swab culture, preg women	15.3
Brzychczy-Wloch, et al. (2012)	Poland	Recto-Vaginal Swab Culture (CDC guidelines), third trimester	30
Kwatra, et al. (2013)	South Africa	Swab culture	37.1
Turner, et al. (2012)	South East Asia Thai-Myanmar boarder	Recto-vaginal swab culture (and PCR), during labor	12.0 (8.6% positive for both culture and PCR)
Ma, et al. (2012)	South Taiwan	Recto-Vaginal Swab with PCR	13.25
Rausch, et al. (2008)	Switzerland	Swab culture	21
Joachim, et al. (2009)	Tanzania	Swab culture	23
Hassan, et al. (2011)	UK	Recto-vaginal swab culture	19
Mavenyengwa, et al. (2010)	Zimbabwe	Swab culture across pregnancy	47, 24.2, 21
Stoll, et al. (1998)	Middle East/North Africa	Adequate methods	22
Meta-analysis	Asia/Pacific	Most recto-vaginal culture methods	19
	Sub-Saharan Africa		19
	India/Pakistan		12
	Americas		14
			Total culture rate = 12.7%

Table 1.2. Host Response to GBS in Gestational Tissues.

Reference	Model	GBS Strain (Serotype)	Concentration	Results/ Outcome
Mitchell et al. (2013)	Human maternal and fetal plasma	N/A		Increased IL-1β and IL-2 in plasma from GBS colonized mothers, no differences in IL-6, IL-8, TNF-α
Hodge et al. (2004)	Human neonatal serum	N/A		Increased IL-1β, IL-2, TNF-α in GBS positive neonate serum with sepsis
Dulkerian et al. (1995)	Human neonatal blood	N/A		Increased IL-6 and TNF-α in neonate blood
Gravett et al. (1994)	Primate	Unspecified (III)	$1x10^6$ CFU	Increased IL-1β, IL-6, and TNF-α in amniotic fluid, PGE2 and PGF2a
Adams Waldorf et al. (2011)	Primate	COH-1 (III)	$1x10^6$ CFU	Increased IL-1β, IL-6, IL-8, TNF-α in amniotic fluid
Equils et al. (2009)	Mouse	Unspecified	$1x10^9$ CFU	Apoptosis in placenta and membranes
Rosati et al. (1998)	Mouse (non-pregnant)	090 (Ia)	$5x10^3$ CFU	Increased IL-1β, IL-2, IL-6, IL-12, IFN-γ, and TNF-α in spleen and peritoneal exudates cells. No change in IL-4, IL-5, IL-10
Menon et al. (2009)	Human extraplacental membranes	BAA-25	$1x10^7$ CFU	Increased IL-1β, IL-6, IL-10, TNF-α, no change in IFN-γ or IL-8
Peltier et al. (2012)	Human extraplacental membranes	BAA-25	Unspecified	Increased IL-1β and IL-8, No change in IL-2, IL-10, or TNF-α
Zaga et al. (2004)	Human extraplacental membranes	Unspecified (III)	$1x10^6$ CFU/mL	Increased IL-1β and TNF-α
Griesinger et al. (2001)	Human placental trophoblast cells	CCUG7742	$1x10^6$ CFU/mL	Increased IL-1β, IL-6, IL-8, and IL-10
Kaplan et al. (2008)	Human extraplacental membranes, trophoblasts, placental fibroblasts, and JEG3 cells	COH-1(III) and NCTC 10/84 (V)	$5x10^4$-$1.5x10^6$ CFU	Increased LDH release
Reisenberger et al. (1997)	Human amnion cells	Unspecified	$1x10^{5,6,7}$ CFU/mL	Increased IL-6 and IL-8, no change in IL-1β or TNF-α
Dudley et al. (1997)	Human decidual cells	Cr (I), Ha (II), Fo (II), Co (III), Ru (III)	$1x10^6$ CFU/mL	GBS strain specific increases in MIP-1 and IL-8
Dudley et al. (1996)	Human chorion cells	FX, CZ, HN, RL, CK	$1x10^7$ CFU/mL	GBS strain specific increases in MIP-1
Zaga-Clavellina et al. (2012)	Human extraplacental membranes	Unspecified (III)	$1x10^6$ CFU/mL	Increases in HBD-2 and HBD-3, No change in HBD-1
Boldenow et al. (2013)	Human extraplacental membranes	A909 (Ia)	$1x10^6$ CFU/mL	Increased HBD-2, no change in HBD-1

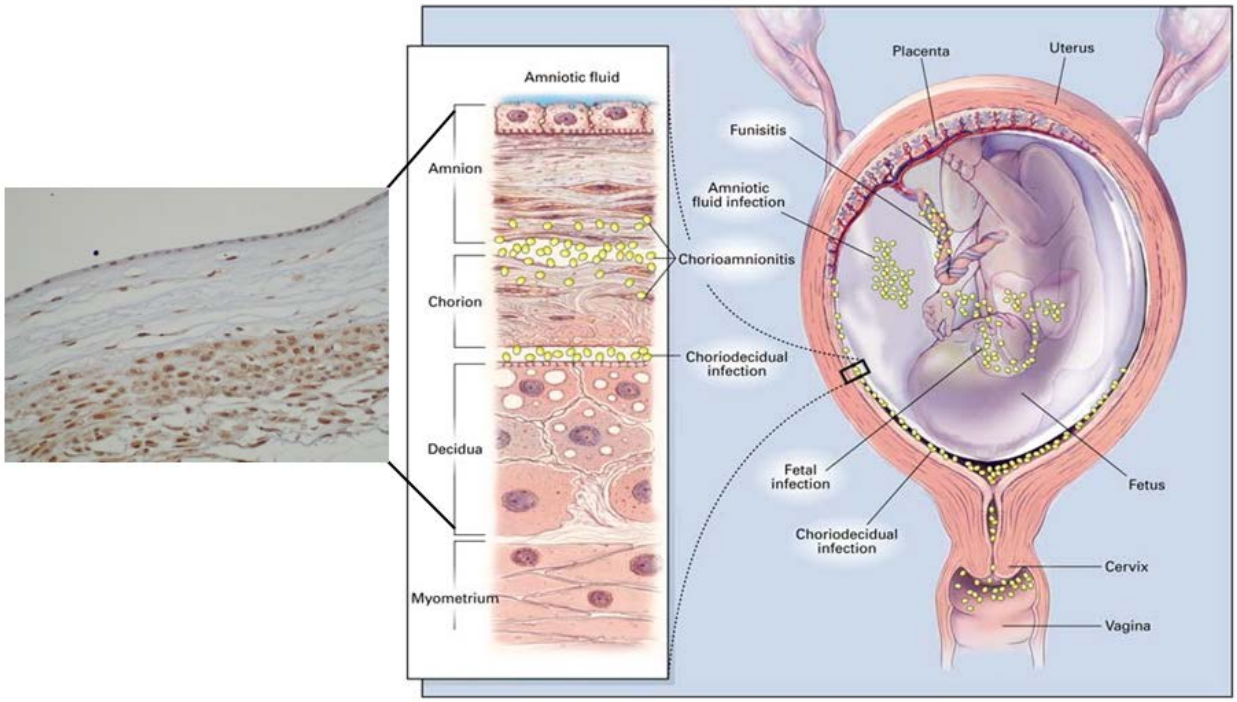

Figure 1.1. Ascending uterine infection.
Image modified from Goldenberg, et al. 2000.

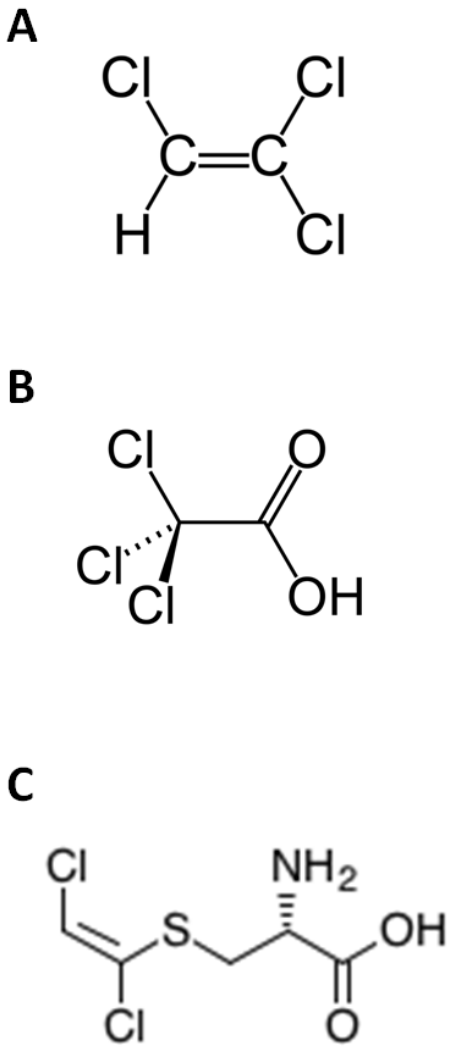

Figure 1.2. Structure of trichloroethylene (TCE; A), trichloroacetic acid (TCA; B), and *S*-(1,2)-dichlorovinyl-L-cysteine (DCVC; C).

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CHAPTER 2. ANTIMICROBIAL PEPTIDE RESPONSE TO GROUP B STREPTOCOCCUS IN HUMAN EXPTRAPLACENTAL MEMBRANES IN CULTURE[*]

Abstract

Objective: *Streptococcus agalactiae* (GBS) is an important cause of chorioamnionitis. This study characterizes GBS colonization and stimulation of antimicrobial responses in human extraplacental membranes using an *ex vivo* transwell two-compartment system of full-thickness membranes and live GBS.

Study design: Human extraplacental membranes were affixed to transwell frames (without synthetic membranes). Live GBS was added to the decidual side of membranes in transwell cultures, and cocultures were incubated for 4, 8 and 24 h. GBS recovery from homogenized membranes and culture medium was determined by enumerating colony forming units (CFU) on blood agar. Antimicrobial peptide expression was identified using immunohistochemistry and ELISA. GBS killing by HBDs was assessed *in vitro* by incubating GBS with different human beta defensins (HBDs) for 3 h, then enumerating CFU.

Results: GBS recovery from membranes markedly decreased over time ($P < 0.05$). The antimicrobial peptides HBD-1, HBD-2, HBD-3, and lactoferrin were expressed in both GBS-exposed and non-exposed tissues. Notably, a pattern of localized increased HBD-2 in the amnion

[*] Boldenow, E., S. Jones, R. W. Lieberman, M. C. Chames, D. M. Aronoff, C. Xi and R. Loch-Caruso (2013). "Antimicrobial peptide response to group B Streptococcus in human extraplacental membranes in culture." Placenta **34**(6): 480-485.

of GBS-infected tissue was observed. Moreover, GBS-treated membranes released increased amounts of HBD-2 into the amniotic and decidual compartments of the transwell cultures after 24 h ($P < 0.05$). In bacterial cultures, HBD-2 decreased GBS viability in a concentration-dependent manner ($P < 0.05$).

Conclusion: Innate immune responses in *ex vivo* human extraplacental membranes suppress GBS growth. HBD-2 was implicated in this GBS suppression with evidence of signal transduction across the tissue. Antimicrobial peptides may be important for innate immune defense against intrauterine GBS infections during pregnancy.

Introduction

Streptococcus agalactiae or Group B *Streptococcus* (GBS) is associated with adverse pregnancy and neonatal outcomes (Verani, McGee et al. 2010). Genital GBS colonization occurs in up to 30% of pregnant women (Verani, McGee et al. 2010) and is associated with neonatal sepsis and meningitis. Moreover, preterm infants with GBS disease have increased risk of mortality compared to GBS-infected infants delivered at term (Goldenberg, Hauth et al. 2000, Jordan, Farley et al. 2008). In one study, GBS was the microorganism most commonly isolated from maternal and fetal tissues in women with midgestation spontaneous abortions (McDonald and Chambers 2000). Likewise, histological chorioamnionitis and preterm birth at less than 32 weeks gestation were positively associated with GBS isolation from extraplacental membranes (Hillier, Krohn et al. 1991). In nonhuman primates, GBS induces preterm labor (Gravett, Haluska et al. 1996). Moreover, GBS adheres to and infects human extraplacental membranes and cells (Galask, Varner et al. 1984, Gravett, Haluska et al. 1996, Winram, Jonas et al. 1998).

Despite evidence linking GBS infection with adverse birth outcomes, the processes of GBS

colonization and infection of extraplacental membranes are poorly understood. Here we adapted an *ex vivo* transwell two-compartment system of full-thickness human extraplacental membranes for coculture with live GBS to characterize GBS colonization of membranes and test the hypothesis that antimicrobial peptides mediate tissue bactericidal activity.

Materials and Methods

Reagents and Materials

The GBS used in this study was strain A909, initially isolated from a septic newborn (Lancefield, McCarty et al. 1975) and transformed with plasmid encoding genes for Green Fluorescent Protein and erythromycin resistance (construct RS020, a gift from Amanda Jones, University of Washington). GBS was grown at 37 ºC in planktonic culture using Todd Hewitt Broth (THB, Becton-Dickinson, Franklin Lakes, NJ) or on sheep's blood agar plates (Blood Agar Base #2, Remel, Lenexa, KS and BBL defibrinated sheep blood, Franklin Lakes, NJ) with 5 μg/mL erythromycin (Acros Organics, Geel, Belgium). Media, buffers, fetal bovine serum (FBS) and penicillin/streptomycin (pen/strep) were from GIBCO (Grand Island, NY).

Culture of Extraplacental Membranes

Human extraplacental extraplacental membranes were collected from healthy pregnancies undergoing scheduled cesarean delivery prior to onset of labor at the University of Michigan Birth Center. Only healthy, non-smoking, singleton mothers were included. Women were excluded if they had collagen vascular disease, evidence of bacterial vaginosis, cervical cerclage, third trimester bleeding, multifetal pregnancy, immunocompromised conditions, major medical conditions (e.g., chronic renal disease, sarcoidosis, hepatitis, HIV), or if pathological evaluation

of the placenta or membranes was warranted. Except for pre-operatively administered antibiotics, women were excluded if prescription antibiotics were used during the two weeks preceding delivery. The University of Michigan Institutional Review Board approved this research (IRBMED#HUM0013915). No evidence of necrosis or infection was observed histologically.

Extraplacental membranes were cultured in a two-compartment transwell system as described previously published (Zaga, Estrada-Gutierrez et al. 2004, Thiex, Chames et al. 2009). Briefly, membranes were dissected from placenta immediately following delivery and transported to the lab in Dulbecco's phosphate-buffered saline (DPBS). Membranes were rinsed with medium and blood clots were removed. The membranes were then mounted on sterile transwell frames that had no synthetic membrane (gift from Corning, NY) and held in place with sterile latex elastic bands (ORMCO, Orange, CA). The membranes were affixed with the choriodecidua facing the inner chamber of the transwell and the amnion facing the outer chamber. The transwell inserts with membranes were placed in wells of 12-well culture plates containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% FBS and pen/strep. Our laboratory previously demonstrated that 1% FBS was sufficient to maintain tissue health and no benefit was added with higher FBS concentration (11). To maintain equal medium levels between the inner and outer transwell chambers, and thereby avoid potential confounding of results due to hydrostatic pressure, 0.5 mL medium was added to the smaller inner chamber and 1.5 mL medium was added to the larger outer chamber. Cultures were incubated at 37 ˚C and 5% CO_2. After 4 h, the medium was exchanged for DMEM/1% FBS without antibiotics. Sample size was based on power estimates using data generated in prior experiments (with cytokines) in our laboratory.

GBS Coculture with Extraplacental Membranes

GBS in early exponential growth phase was diluted with DMEM/1% FBS to $1x10^6$ colony forming units/mL (CFU/mL). Inoculant concentrations were validated by overnight growth on 5% sheep blood agar with erythromycin. Following a 24-h acclimation, the medium of the transwell choriodecidual compartment was replaced with 0.5 mL GBS inoculant ($1x10^6$ CFU/mL) or fresh DMEM/1%FBS without GBS (controls). Amnion compartment medium was also replaced with DMEM/1%FBS. Cocultures were then incubated for 4, 8, or 24 h. Coculture experiments were conducted in triplicate using extraplacental membranes from five women.

At designated time points, medium from each transwell chamber was collected. An aliquot of 100 µL medium was diluted for CFU determination on 5% sheep blood agar with incubation for 12-24 h at 37 ˚C, and the remainder was stored at -80 ˚C. Transwell inserts with attached tissue were transferred to fresh wells with DPBS in both chambers. The plate was gently rocked for 5 min to rinse away non-adherent bacteria. Two 3-mm biopsy punches were taken from each transwell-mounted tissue piece, placed in 1 mL PBS, and homogenized on ice with two 40-sec pulses. Tissue homogenates were serially diluted 1:10 in PBS, plated on 5% sheep blood agar in triplicate, and grown 12-24 h at 37 ˚C. Colony counts of viable bacteria in the membrane ($CFU/mL/cm^2$) were determined.

To control for altered GBS growth in tissue culture medium (used for coculture of tissue and GBS), GBS growth was observed using the above protocol in transwell culture wells with intact polycarbonate membranes but without extraplacental membranes . Medium from the upper and

lower compartments was plated on 5% sheep blood agar in triplicate and incubated overnight at 37 ˚C.

Immunohistochemistry

While still attached to the transwell insert, extraplacental membranes were fixed with 10% phosphate-buffered formalin (Fisher, Waltham, MA) for 24-48 h at 4 ˚C. The fixed tissue was gently removed from the transwell insert and a strip of membrane was cut ≤ 4 mm in width. Processing and staining were performed by the University of Michigan's Comprehensive Cancer Center Tissue Core. The tissue strips were embedded in paraffin "on edge", sectioned, and mounted on slides. For antimicrobial peptide staining, heat-induced antigen retrieval was performed in citrate buffer (pH 6.0). Immunoperoxidase staining was completed on a DAKO AutoStainer at room temperature using the LSAB+ System-HRP kit from DAKO. Briefly, peroxidase block was followed by a 30-min incubation with primary antibody at the dilutions indicated for the following antimicrobial peptides: human beta defensins (HBD)-1 (1:25, rabbit polyclonal, Santa Cruz), HBD-2 (1:25, goat polyclonal, Santa Cruz), HBD-3 (1:100, rabbit polyclonal, Novus), HBD-5 (1:100, goat polyclonal, Santa Cruz), elafin (1:200, rabbit polyclonal, Santa Cruz), or lactoferrin (1:400, rabbit polyclonal, Abcam). Samples were then incubated sequentially with biotinylated LINK (30 min), streptavidin-HPR (30 min), and 3, 3' diaminobenzidine (DAB+) chromogen solution (5 min), before being counterstained with hematoxylin.

Microscopy image capture and analysis were done using Nikon Elements Software. For amnion cell size analysis, images were captured from slides stained for HBD-2 after 24 h in culture. We

analyzed 7 images in 6 tissues, but one tissue had the amnion tear off in a way that allowed us to get only one image from that tissue. Approximately 5-7 amnion cells were measured per image and cell size was averaged for each tissue (N=5).

HBD ELISAs

HBD-1 and HBD-2 release from the extraplacental membranes into medium was measured using a commercially available enzyme linked immunosorbant assay (ELISA) kit according to manufacturer's instructions (Peprotech, Rocky Hill, NJ). The HBD-1 ELISA detection range was 4-1000 pg/mL and the HBD-2 ELISA detection range was 16-2000 pg/mL. Samples were not diluted. The HBD values are reported as pg protein/mL medium.

Antimicrobial Activity of HBDs to GBS

Antimicrobial activity assays were done according to previously published methods (Aronoff, Hao et al. 2008). Briefly, HBDs (Peprotech, Rocky Hill, NJ) were resuspended according to manufacturer's directions in 10 mM acetic acid and diluted in 0.1% BSA in PBS. GBS were grown for 2.5 h to exponential log phase at 37 ˚C with shaking in THB, diluted 1:1000 (approximately 1 x 10^4 CFUs) in 10 mM sodium phosphate buffer (pH 7.4), and then treated with 0-20 µg/mL of HBD-1, HBD-2, or HBD-3 for 3 h at 37 ˚C in a 96 well plate. Samples were serially diluted and 10 µL of each diluent was plated on 5% sheep blood agar in duplicate. Following overnight incubation at 37 ˚C, CFUs were enumerated. Data are expressed as bacterial survival relative to vehicle control (0.1 mM acetic acid, 0.01% BSA).

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). For transwell coculture GBS CFU quantification and HBD antimicrobial activity, ANOVAs with Tukey's post hoc test were performed. For HBD ELISA and cell size analysis, Student's paired *t*-test was used for each time point. Data were considered significant if the p-value was < 0.05.

Results

GBS Growth in Extraplacental Membranes

To simulate an ascending intrauterine infection, GBS was applied to the choriodecdiual compartment of the transwell-mounted extraplacental membranes. No GBS colonies formed from membranes of unexposed control tissues after 4, 8 or 24 h of coculture (Figure 2.1). GBS-infected tissues initially yielded increased CFUs after 4 h of coculture, but CFUs subsequently decreased in GBS-exposed membranes at 8 and 24 h (Figure 2.1; $P < 0.05$). A similar time-dependent pattern was observed for CFU recovery from medium of the cultures (data not shown). No GBS was recovered from the amnion compartment medium (not shown), suggesting that GBS did not cross the extraplacental membranes. Furthermore, GBS growth was robust in standard transwells (with synthetic membrane) containing DMEM tissue culture medium but lacking gestational tissue (Figure 2.2), indicating that the decreased CFU recovery from coculture with gestational tissues was dependent on the presence of the tissue and not an artifact of the DMEM medium or transwell system.

Immunohistochemical Detection of Antimicrobial Peptides in Extraplacental Membranes

Immunohistochemial staining assessed expression of AMPs in GBS-exposed and unexposed extraplacental membranes. Although HBD-2 antibody stained throughout the membranes in both the nucleus and the cytoplasm, HBD-2 staining increased in GBS-treated membranes after 24 h, especially in the amnion epithelium (Figure 2.3). In addition, amnion cells in GBS-treated tissues were significantly larger (8.3 μm) in diameter compared to amnion cells in untreated tissue (6.7 μm) ($P < 0.05$) at 24 h. Regardless of whether membranes were exposed *in vitro* to GBS or not, the amnion epithelial cells, chorionic trophoblasts, and decidual cells stained positive for HBD-1, HBD-3, and lactoferrin at all time points (Figure 2.4). Extraplacental membranes did not show staining for HBD-5 or elafin in control tissues or those exposed to GBS (Figure 2.4).

HBD-2 Secretion from Extraplacental Membranes

Extraplacental membranes in transwell culture released increased amounts of HBD-2 into the medium of the choriodecidual (1.65-fold) and amniotic compartments (1.59-fold) after 24 h of exposure to GBS (Figure 2.5; $P < 0.05$ compared with controls). There were no statistically significant differences in HBD-2 at 4 h and 8 h. Likewise, a 3.6-fold difference of HBD-1 between GBS and control tissues in amniotic chamber culture medium at 24 h was not statistically significant (Figure 2.6), though this negative result should be interpreted with caution due to the modest sample size.

HBD Bactericidal Activity against GBS

To investigate whether HBDs are capable of killing GBS directly, GBS was incubated with HBD-1, HBD-2, or HBD-3 in sodium phosphate buffer. HBD-2 killed GBS in a concentration-dependent manner, with nominal CFU recovered at 7.5 μg/ml and no GBS surviving exposure to

the highest concentration of at 10 µg/mL of HBD-2 (Figure 2.6; $P < 0.05$). In contrast, HBD-1 and HBD-3 did not completely kill GBS at 10 µg/mL (data not shown).

Discussion

Although GBS remains the leading cause of infection-related neonatal morbidity and mortality, the mechanisms by which it interacts with and crosses extraplacental membranes as an ascending infection are poorly understood (Verani, McGee et al. 2010). Using a two-compartment tissue culture model, the present study provides evidence that GBS did not readily cross healthy term extraplacental membranes. Notably, antimicrobial innate immune responses, in particular HBD-2, were stimulated in amnion following GBS inoculation on the decidual side of the membranes, suggesting signal transduction across the tissue.

Although previous studies noted antimicrobial properties of extraplacental membranes to live bacteria (Trelford and Trelford-Sauder 1979, Kjaergaard, Hein et al. 2001, Gomes, Romano et al. 2005, Insausti, Alcaraz et al. 2010), few studies quantified bactericidal activity of the tissue. The extraplacental membranes exhibited robust resistance to infection *in vitro* in our experiments. Viable GBS recovery from inoculum medium was reduced by at least half at 4, 8, and 24 h when cultured with extraplacental membranes, in agreement with Kjaergaard et al. who found reduced CFU counts of GBS recovered from inoculum medium over a 20-h incubation using a different two-compartment model of extraplacental membranes (Kjaergaard, Helmig et al. 1999). In addition, we provide new information that GBS recovered from the tissue itself decreased over time, indicating that GBS adherence to and penetration into extraplacental membranes was inhibited.

HBDs are small cationic antimicrobial peptides thought to permeabilize microbial membranes (King, Paltoo et al. 2007). We show, for the first time to our knowledge, that extraplacental membranes express the antimicrobial peptide lactoferrin in the amniotic, chorionic trophoblast, and decidual cell layers, independent of exposure to GBS. Furthermore, similar to findings in extraplacental membranes of women delivering at term (King, Kelly et al. 2007, King, Paltoo et al. 2007), we demonstrate that the antimicrobial peptides HBD-1, HBD-2, and HBD-3 were localized to the amnion, chorionic trophoblasts, and decidua of cultured extraplacental membrane explants, regardless of exposure to GBS. In particular, HBD-1 was highly expressed in all cell layers of extraplacental membranes irrespective of GBS treatment, consistent with other studies that suggest HBD-1 is constitutively expressed (Krisanaprakornkit, Weinberg et al. 1998, Valore, Park et al. 1998). Moreover, we found that secretion of HBD-1 into the medium did not change significantly with GBS stimulation. Our findings with HBD-1 are in agreement with reports by Garcia-Lopez et al. and Zaga-Clavellina et al. who had similar results with a different strain of GBS and *E. coli*. (King, Paltoo et al. 2007, Garcia-Lopez, Flores-Espinosa et al. 2010, Zaga-Clavellina, Garcia-Lopez et al. 2011). No evidence of necrosis or tissue degradation was observed in histological H&E slides, supporting evidence from the GBS killing and HBD-2 production data that the explants remained viable in culture throughout the experimental period.

GBS inoculation at the choriodecidual face of the membranes stimulated increased expression of HBD-2 in the amnion as visualized by immunostaining. The latter finding, in conjunction with reduced CFU recovery over time from the membranes, provides support that GBS stimulates signaling across the layers of the extraplacental membranes to increase HBD-2 production in

amnion cells. Moreover, our observation that GBS stimulated an increase of amniotic cell size further supports the occurrence of cross-tissue signaling. Although novel with respect to HBD-2, previous reports suggest similar cross-tissue signaling in release of cytokines and prostaglandins by extraplacental membranes stimulated with LPS (Zaga, Estrada-Gutierrez et al. 2004, Thiex, Chames et al. 2009, Thiex, Chames et al. 2010).

In addition to increased HBD-2 expression in amniotic cells, we observed increased HBD-2 in medium of the amniotic and choriodecidual compartments following choriodecidual stimulation with GBS, similar to findings reported by Zaga-Clavellina et al. (Zaga-Clavellina, Garcia-Lopez et al. 2011). In contrast to the latter study, we used a different GBS strain and no antibiotics in our culture system, allowing us to quantify the bacterial death directly related to the extraplacental membranes innate immune response. In addition, this is the first report of the time-dependent increase of HBD-2 in both amniotic and choriodecidual compartments with GBS stimulation.

Recently, Garcia-Lopez and colleagues suggested that IL-1β may be a key mediator in the tissue-specific HBD-2 responses that they observed with *Escherichia coli* stimulation of extraplacental membranes in a similar transwell system (Garcia-Lopez, Flores-Espinosa et al. 2010). Just as GBS stimulation of the choriodecidua produced a tissue-specific increase of IL-1β secretion from the choriodecidua (Zaga, Estrada-Gutierrez et al. 2004), *E. coli* stimulation of choriodecidua produced the same tissue-specific IL-1β secretion effect by the choriodecidua (Zaga-Clavellina, Garcia-Lopez et al. 2007). A similar mechanism within the innate immune system of extraplacental membranes may be employed to protect against infection by Gram positive

bacteria like GBS or Gram negative bacteria like *E. coli*. The cross-tissue signaling underlying the tissue-specific HBD responses observed in extraplacental membranes is not yet well characterized and warrants further study.

In studies comparing direct microbicidal activities of HBD-1, HBD-2 and HBD-3, we found that GBS strain A909 was most sensitive to killing by HBD-2 compared to HBD-1 and HBD-3, suggesting that increased HBD-2 secretion is linked to the GBS killing observed by us in extraplacental membranes. It is important to note that most *in vitro* bacterial viability studies (including ours) use synthetic or recombinant antimicrobial peptides at significantly higher levels than found *in vivo* (Starner, Agerberth et al. 2005), suggesting that antimicrobial peptides may be working synergistically *in vivo* (Chen, Niyonsaba et al. 2005).

Finally, formalin fixation of extraplacental membranes while mounted on the transwell frame, followed by "on edge" paraffin embedding of tissue strips, produced improved images for immunohistochemical assessment. The common technique for formalin fixation of extraplacental membranes is the "membrane roll," which can produce histological sections with artifacts such as amnion shearing, in which amnion separates from choriodecidua. In the present study, amnion shearing was minimized and the sections better approximated a true cross section of the tissue.

In conclusion, this study utilized a two-compartment transwell extraplacental membrane tissue culture system to show that the antimicrobial response to GBS stimulation of extraplacental membrane explants was sufficiently robust to inhibit GBS colonization. We show for the first time signal transduction across the tissue increased HBD-2 production in the amnion epithelial

cells in response to GBS. Moreover, we demonstrated that HBD-2 directly decreased GBS viability in a tissue-free system, suggesting a major role for HBD-2 in killing GBS in the extraplacental membranes.

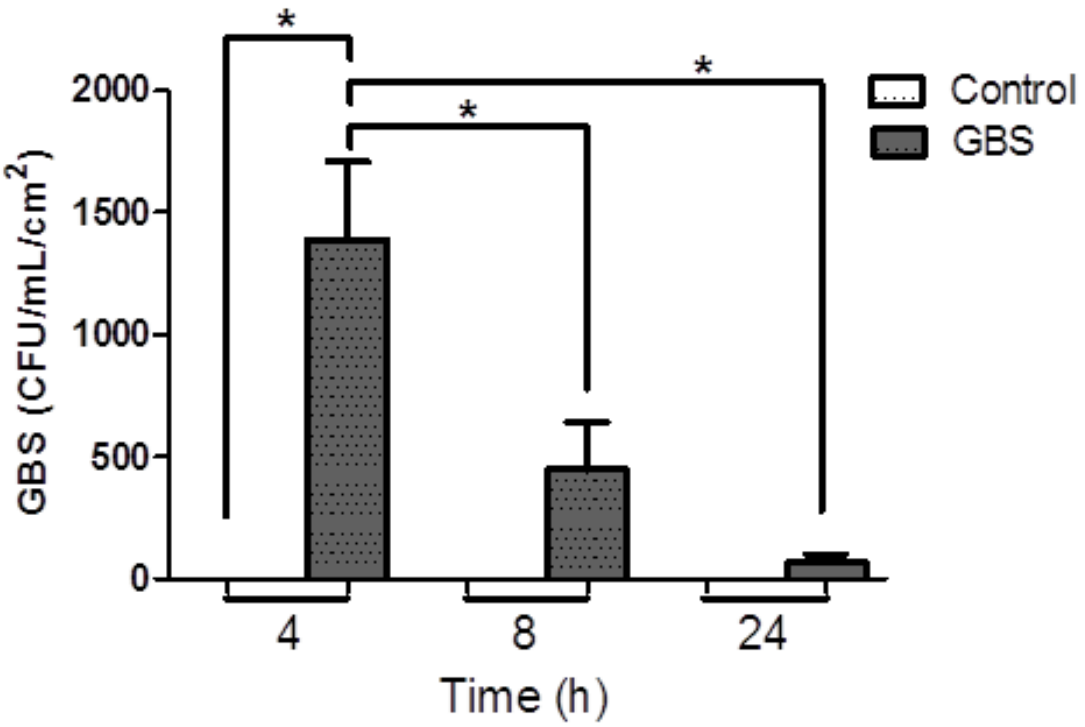

Figure 2.1. Recovered GBS from extraplacental membranes.
Recovered GBS (expressed as CFU) from homogenized tissue punches of the extraplacental membranes following coculture with $1x10^6$ CFU/mL. GBS inoculants for 4, 8, and 24 h. Control tissues were not exposed to GBS. Columns are mean ± SEM (N=4-5 women, 3 replicates per woman). Asterisks (*) represent significant differences when compared by Tukey's post-hoc test following ANOVA ($P < 0.05$).

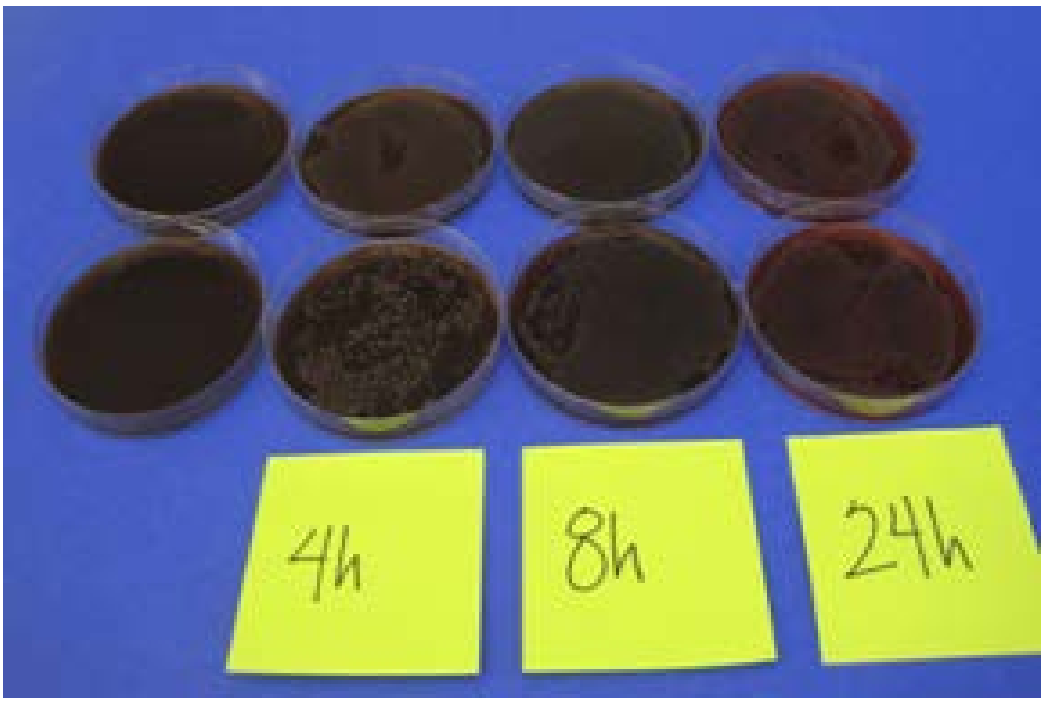

Figure 2.2. GBS growth on agar.
GBS was recovered from DMEM medium in transwells lacking extraplacental membranes. GBS ($1x10^6$ CFU) was grown in transwell inserts lacking extraplacental membranes with DMEM/1%FBS, incubated for 4, 8, and 24 h, and plated on 5% sheep blood agar. Plates in the left-most column are derived from transwells with no GBS. Plates in adjacent columns are representative of bacterial growth at 4, 8 and 24 h. The top row shows growth of bacteria recovered from medium of the inner transwell chamber, and the bottom row shows growth of bacteria recovered from medium of the outer transwell chamber. In most cases, the vigorous bacterial growth formed a lawn, such that individual colony forming units (CFUs) could not be distinguished. These images show that GBS exhibited robust growth in the transwell system in medium used for culturing extraplacental membranes but lacking extraplacental membranes.

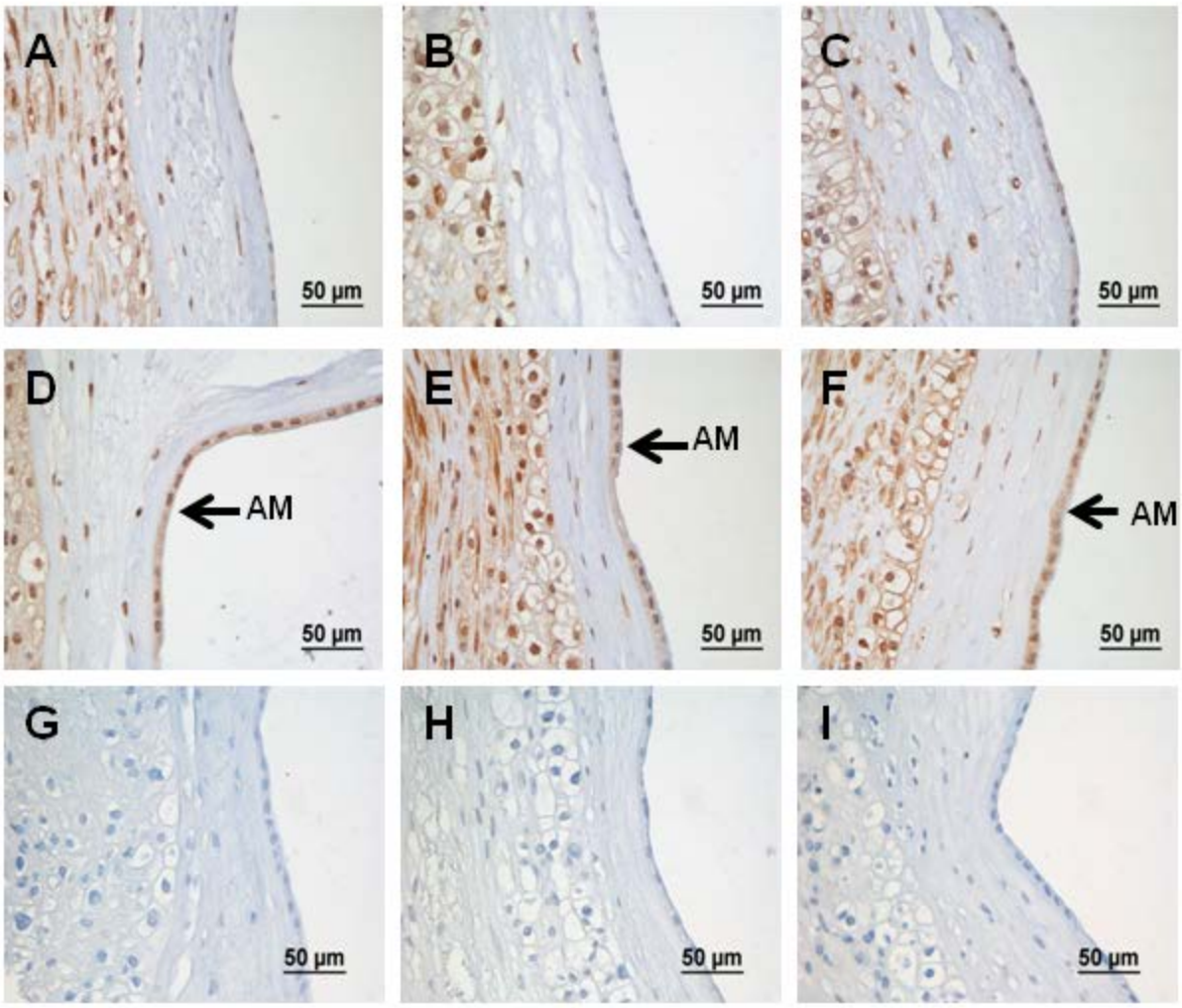

Figure 2.3. Immunohistochemical staining for HBD-2 in human extraplacental membranes. Extraplacental membranes were cocultured with GBS added to the choriodecidual chamber of the Transwell cultures. The top row (A, B, and C) shows representative images for no treatment controls. The middle row (D, E, and F) shows representative images for GBS-treated tissues (decidual side only). The bottom row (G, H, and I) shows representative images of negative control sections incubated with secondary antibody only. Tissues within a column were cultured from the same subject. Intense staining is visible in amnion epithelium (AM) of GBS-stimulated membranes (arrows), despite lack of direct contact of the amnion with GBS.

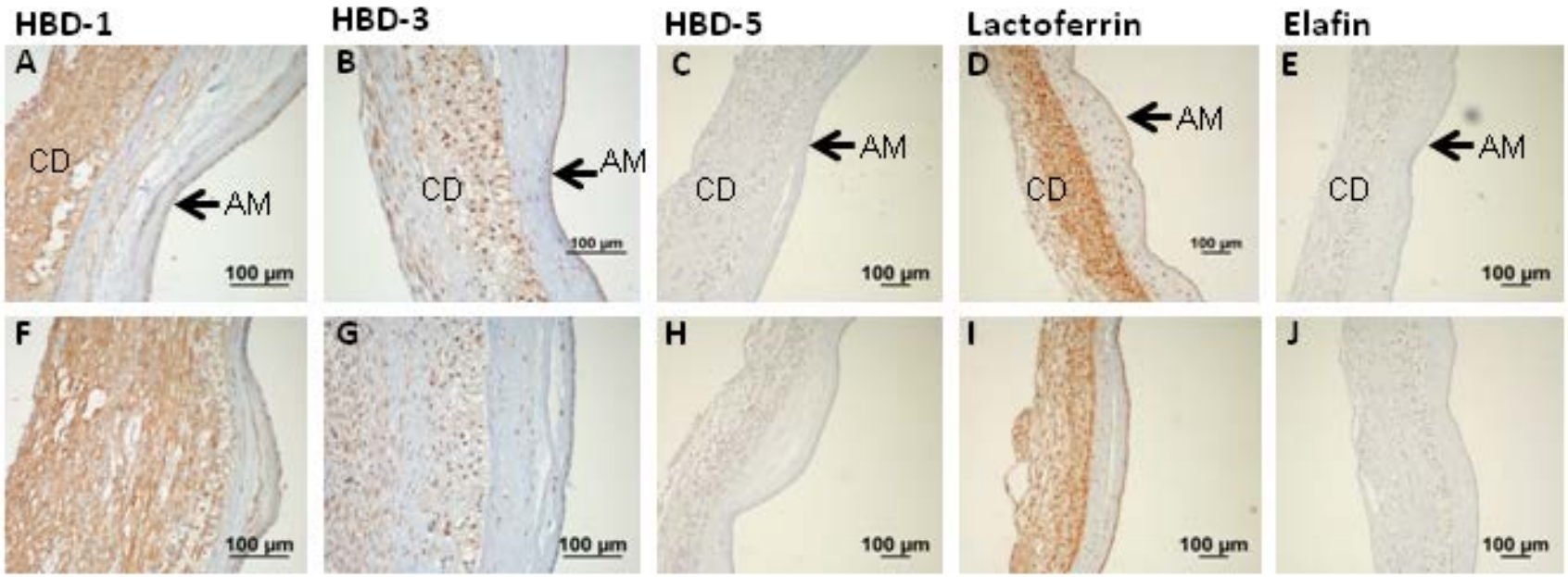

Figure 2.4. Immunohistochemical staining for antimicrobial peptides in human extraplacental membranes.
The top row (A-E) shows representative images from non-treatment controls. The bottom row (F-J) shows representative images from GBS treated tissues. A and F are stained for HBD-1. B and G are stained for HBD-3. C and H are stained for HBD-5. D and I are stained for lactoferrin. E and J are stained for elafin. Tissues within a column were cultured from the same subject. AM is amnion epithelial layer and CD is choriodecidua layer. No notable differences were seen between non-treatment controls and GBS treated tissues for the antimicrobial peptides shown. No staining was observed with no primary controls.

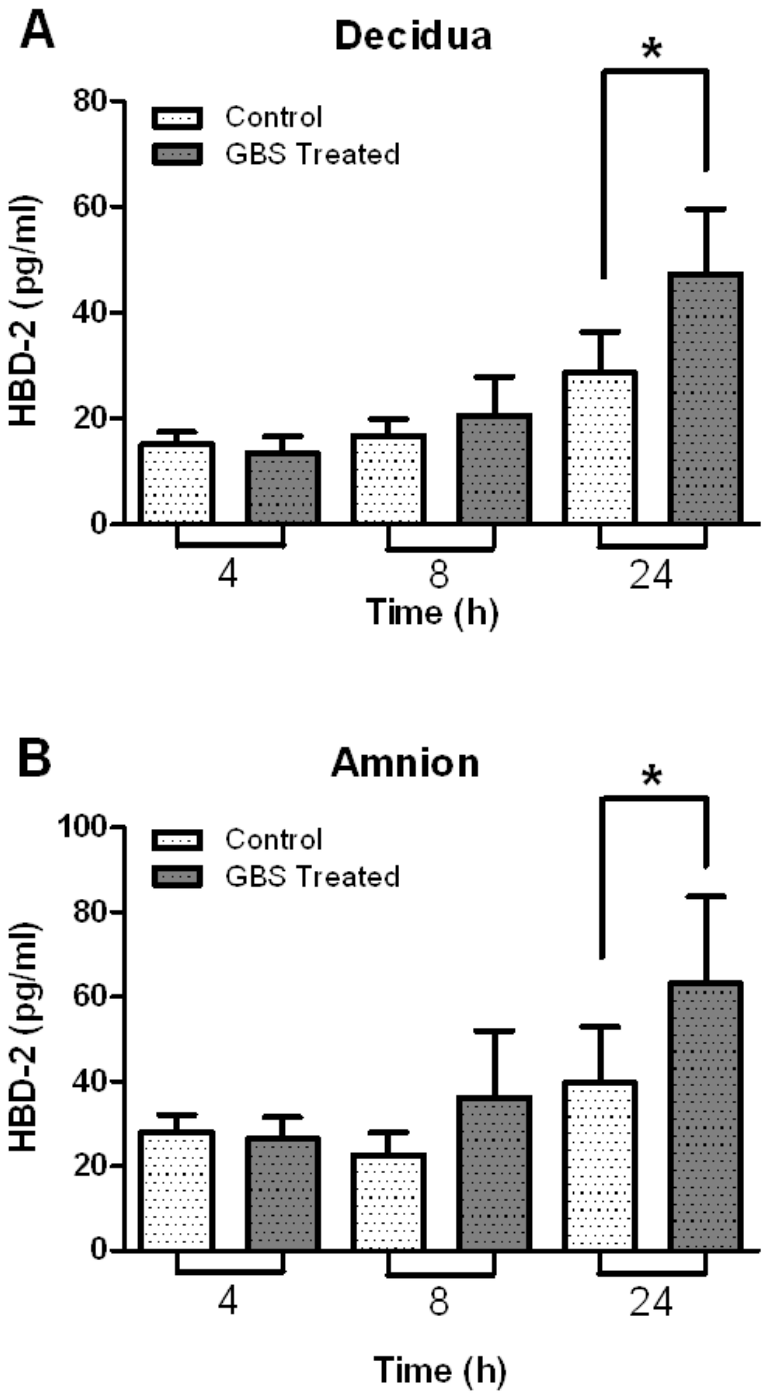

Figure 2.5. HBD-2 release into medium by extraplacental membranes.
HBD-2 release into medium by extraplacental membranes in transwell cultures with GBS (dark columns) and without GBS (light columns) exposure on the choriodecidual side. (A) Decidual compartment and (B) amniotic compartment. HBD-2 protein in the medium was measured by ELISA. Columns are mean ± SEM (N=4-5 women, 3 replicates per woman). Asterisks (*) represent significant differences ($P < 0.05$) by paired student t-test.

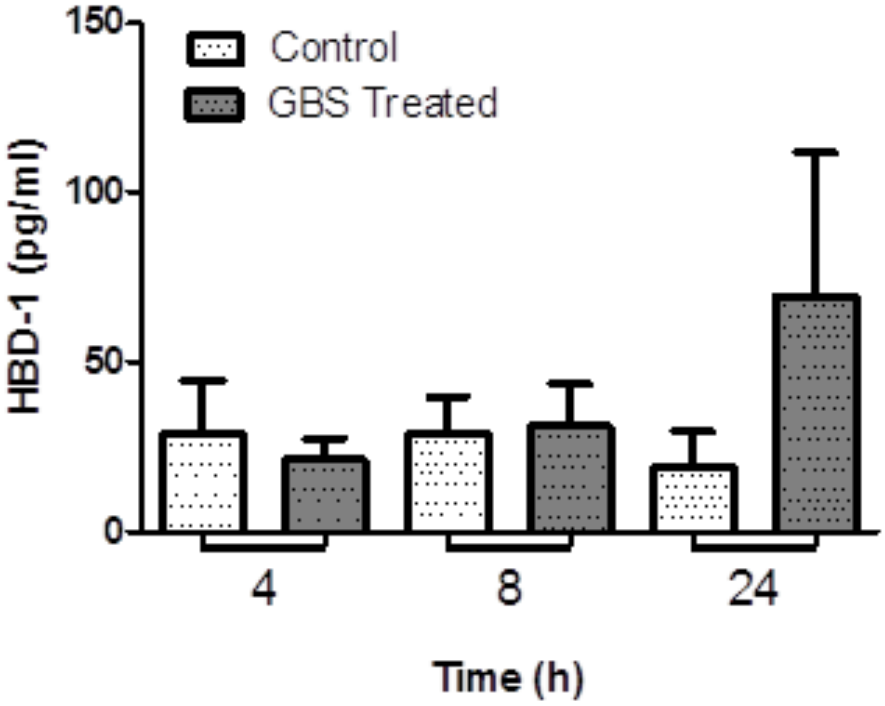

Figure 2.6. HBD-1 release by extraplacental membranes.
HBD-1 release into the amnion compartment medium by extraplacental membranes in transwell cultures with GBS (dark columns) and without GBS (light columns) exposure on the choriodecidual side. HBD-1 protein in the medium was measured by ELISA. Columns are mean ± SEM (N=5 women, 3 replicates per woman). No statistical significance was seen over 24 h using a paired student t-test.

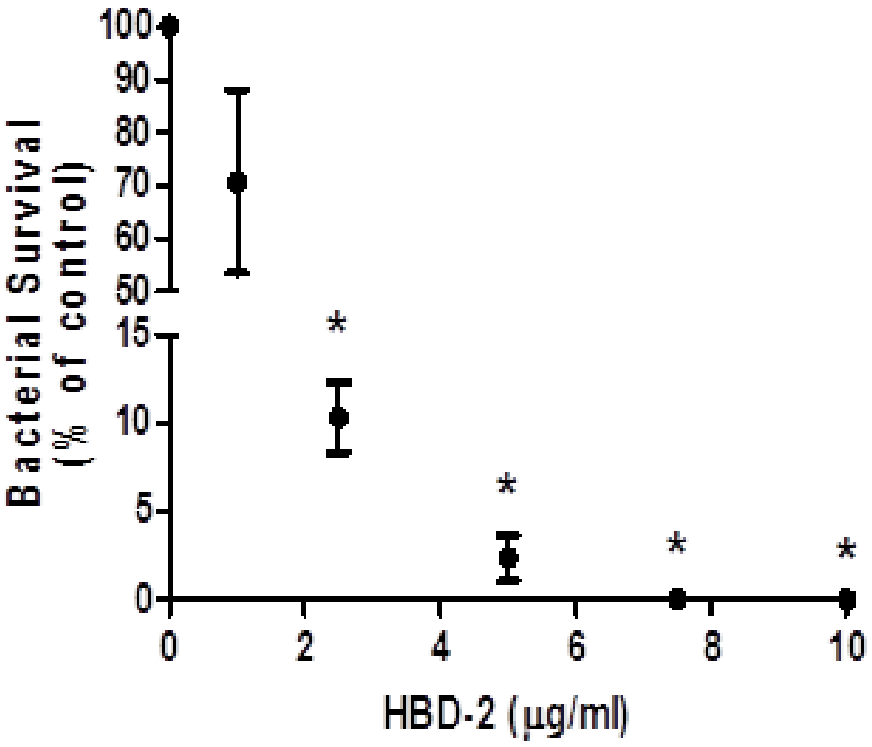

Figure 2.7. HBD-2 kills GBS.
GBS were incubated in the presence of different concentrations of HBD-2 for 3 h. Data shown are mean ± SEM (N=4 independent experiments), expressed as a percentage of bacterial survival relative to vehicle control. Asterisks (*) represent significant differences when compared by Tukey's post-hoc test following ANOVA ($P < 0.05$).

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CHAPTER 3. GROUP B STREPTOCOCCUS-STIMULATED HUMAN CHORIODECDUA RELEASES IL-1α and IL-1β TO INCREASE HUMAN BETA DEFENSIN-2 IN AMNION EPITHELIAL CELLS

Abstract

Problem: *Streptococcus agalactiae* or Group B *Streptococcus* (GBS) is the leading cause of infectious neonatal morbidity and mortality in the United States. Recently, we demonstrated in an *in vitro* two-compartment model of full thickness human extraplacental membranes that human beta defensin-2 (HBD-2) expression is stimulated in the amnion epithelial cells following GBS inoculation on the decidual side of the membranes. Here, we test our hypothesis that the choriodecidua plays a necessary role in GBS-stimulated HBD-2 increases in amnion epithelial cells through a secreted factor of choriodecidual origin.

Method of Study: Human extraplacental membranes from term cesarean sections were blunt dissected to separate amnion from choriodecidua. Choriodecidual tissue was cultured with GBS, lipoteichoic acid (LTA), or lipopolysaccharide (LPS) for 24 h to access responses and to generate choriodecidual conditioned medium. Human amnion epithelial cells were isolated and treated with the choriodecidual conditioned medium, live GBS, LTA, LPS, or recombinant cytokines with and without IL-1 inhibitors. Cytokines and HBD-2 in choriodecidual and amnion epithelial cell culture medium were determined by ELISA.

Results: GBS choriodecidual conditioned medium significantly stimulated release of HBD-2 in human amnion epithelial cell cultures. In contrast, direct stimulation of amnion epithelial cells with live GBS, LTA, or LPS did not increase HBD-2 release, implicating a critical role for choriodecidua. Choriodecidual tissue punches stripped of amnion released significantly increased amounts of IL-1α and IL-1β in response to GBS, and recombinant IL-1α and IL-1β stimulated HBD-2 release in amnion epithelial cell cultures in a concentration-dependent manner. Neutralizing antibody for IL-1β significantly inhibited, and IL-1 receptor antagonist (IL-1Ra) nearly abolished, the stimulated release of HBD-2 from amnion epithelial cells treated with GBS choriodecidual conditioned medium. Although both IL-α and IL-1β stimulate HBD-2 production in the amnion epithelial cells, they do not appear to work synergistically.

Conclusions: Increases in HBD-2 from the amnion epithelial cells are from secreted IL-1α and IL-1β from the choriodecidual tissue. These data demonstrate cell-cell communication critical for host defense during GBS infection in the extraplacental membranes. In addition, this study shows that IL-1 is vital for HBD-2 production in the amnion epithelial cells.

Introduction

Streptococcus agalactiae or Group B *Streptococcus* (GBS) is the leading cause of infectious neonatal morbidity and mortality in the United States (Verani, McGee et al. 2010). GBS infections in the gravid female reproductive tract are associated with adverse birth outcomes such as sepsis and meningitis. The ascending pathway of infection begins with colonization of the vagina. GBS then passes through the cervix and enters the uterine cavity where it can cross the extraplacental membranes and infect the neonate. Despite the importance of the

extraplacental membranes, the mechanisms by which GBS colonizes the extraplacental membranes and causes infection remain poorly understood.

Human beta defensins (HBDs) are an important part of the innate immune system and play critical roles responding to infectious microorganisms. HBDs are expressed throughout the reproductive tract including the extraplacental membranes. HBDs are considered a first defense during pregnancy because they can kill bacteria directly through membrane disruption, pore formation in the membrane wall, and polarization (King, Kelly et al. 2007, King, Paltoo et al. 2007, Lai and Gallo 2009, Frew and Stock 2011). Furthermore, HBDs can promote chemotaxis, providing an important link between the innate and adaptive immune systems. HBD-2 has been shown to be higher in amniotic fluid from women with intrauterine microbial infection compared to women without intrauterine infection (Soto, Espinoza et al. 2007). In addition, HBD-2 concentrations in second trimester amniotic fluid have been positively correlated with preterm premature rupture of the extraplacental membranes (Iavazzo, Tassis et al. 2010). However, infants born preterm had lower HBD-2 levels measured in cord blood compared to term neonates (Olbrich, Pavon et al. 2013). Infants that suffered from late onset sepsis tended to have lower levels of HBD-2 in cord blood suggesting HBD-2 is critical for effectively fighting infections. Despite the importance of HBD-2 for pregnancy- related infections, few studies have looked at potential stimuli and mechanisms of HBD-2 in the extraplacental membranes and amnion epithelial cells. Pathogens increase HBD-2 in *ex vivo* extraplacental membranes models yet little is known about how the pathogens are interacting with the tissue or which cells are primarily responsible for the HBD-2 production (Garcia-Lopez, Flores-Espinosa et al. 2010, Zaga-Clavellina, Martha et al. 2012, Zaga-Clavellina, Ruiz et al. 2012). In addition, recombinant IL-1β

has been shown to stimulate HBD-2 secretion in amnion epithelial cell culture (Stock, Kelly et al. 2007).

Recently, we demonstrated in an *in vitro* two-compartment model of full thickness human extraplacental membranes that HBD-2 is stimulated in the amnion epithelial cells following GBS inoculation on the decidual side of the membranes (Boldenow, Jones et al. 2013). No bacteria were observed invading or crossing the tissue, suggesting a trans-tissue signaling mechanism. Here, we utilized separated extraplacental membranes cocultured with GBS to test our hypothesis that the choriodecidua plays a necessary role in GBS-stimulated HBD-2 increases in amnion epithelial cells through a secreted factor of choriodecidual origin. Moreover, we provide evidence that IL-1α and IL-1β are the choriodecidual signaling molecules critical for the HBD-2 response in amnion epithelial cells.

Materials and Methods

Reagents and Materials

The GBS strain used in this study (A909, construct RS020, a gift from Amanda Jones, University of Washington), was initially isolated from a septic newborn (Lancefield, McCarty et al. 1975). GBS was grown at 37 ºC in culture using Todd Hewitt Broth (THB, Becton-Dickinson, Franklin Lakes, NJ) or on sheep's blood agar plates (Blood Agar Base #2, Remel, Lenexa, KS and BBL defibrinated sheep blood, Franklin Lakes, NJ) with 5 μg/mL erythromycin (Hemostat Labs, Dixon, CA). Media (DMEM catalog # 21063 and DMEM:F12 catalog #11039), buffers, fetal bovine serum (FBS; catalog #10438), trypsin-EDTA (catalog #25200), and penicillin/streptomycin (pen/strep; catalog #15140) were from GIBCO (Grand Island, NY).

Epidermal growth factor (EGF), and recombinant cytokines (IL-1β, IL-6, IL-8, IL-17, TNF-α) were from Peprotech (Rocky Hill, NJ). Lipoteichoic acid (LTA) from *Staphylococcus aureus*, IL-1β neutralizing antibody, and IgA isotype control were from Invivogen (San Diego, CA). Lipopolysaccaride (LPS) from *Salmonella typhimurium* was from List Biological Laboratories (Campbell, CA). IL-1Ra was from Sigma-Aldrich (Saint Louis, MO).

Culture of Extraplacental Choriodecidual Membranes

Human extraplacental membranes were collected from healthy, non-smoking, singleton pregnancies undergoing scheduled cesarean delivery prior to onset of labor at the University of Michigan Birth Center as previously described (Boldenow, Jones et al. 2013). The University of Michigan Institutional Review Board approved this research (IRBMED#HUM0037054).

Immediately following delivery the membranes were transported to the lab in Dulbecco's phosphate-buffered saline (DPBS). Membranes were rinsed with medium and blood clots removed. Membranes were then blunt dissected to separate the choriodecidua from the amnion. After dissection, the choriodecidua was punched using a 12-mm biopsy punch. Tissue punches were placed in 12-well plates with 1 mL medium containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% FBS and pen/strep. Cultures were incubated at 37 ˚C and 5% CO_2. After 4 h, the medium was exchanged for DMEM/1% FBS without antibiotics.

GBS Coculture with Extraplacental Choriodecidual Membranes

GBS in early exponential growth phase was diluted with DMEM/1% FBS to and estimated $1x10^6$ colony forming units/mL (CFU/mL). Inoculant concentrations were validated by overnight

growth on 5% sheep blood agar with erythromycin. GBS was heat killed by incubating bacterial culture at 70 ˚C for 15 min. Lack of viability was confirmed by plating GBS on 5% sheep blood agar. LTA and LPS treatments were made in DMEM/1% FBS. Following a 24-h acclimation, the medium of the choriodecidual punches was replaced with 1 mL GBS inoculant (~$1x10^6$ CFU/mL), LTA (1 µg/mL), LPS (100 ng/mL), or fresh DMEM/1%FBS (control). Following 24 h of incubation with GBS , medium from the choriodecidual punches was filtered through a 0.2 µm pore filter to remove the bacteria, and then stored at -80 °C: this medium is referred to as GBS conditioned choriodecidual medium in this study. Medium from choriodecidual punches treated with LTA or LPS for 24 h was also 0.2 µm filtered and then stored at -80 °C: this medium is referred to as LTA or LPS conditioned choriodecidual medium. Coculture experiments were conducted in triplicate using extraplacental membranes from a minimum of seven women.

Amnion Epithelial Cell Isolation

Amnion epithelial cells were isolated from the same membranes used for choriodecidual punch cultures, using methods adapted from three protocols (Ilancheran, Michalska et al. 2007, Liu, Cheng et al. , Pratama, Vaghjiani et al.). Briefly, blunt-dissected amnion was digested with 0.25% trypsin-EDTA at 37 °C for 30 minutes. Amnion tissue pieces were transferred to fresh trypsin-EDTA and the digestion was repeated. Following each digestion, the trypsin-EDTA was neutralized in the digest with medium (DMEM:F12) supplemented with 10% FBS and pen/strep. Cells were pelleted by centrifugation at 128 g for 5 minutes, suspended in medium, repelleted, and resuspended in medium containing EGF (DMEM:F12 supplemented with 10% FBS, pen/strep, and 10 ng/mL EGF). Amnion epithelial cells were plated at 500,000 cells/ well (12-

well plates) in 1 mL medium, and grown to 70-80% confluence. Medium was changed two days after seeding, and cells were treated on day 3.

Amnion epithelial cells were treated with either GBS choriodecidual conditioned medium, LTA (10 µg/mL), LPS (100 ng/mL), live GBS (~$1x10^6$ CFU/mL), or one of the following recombinant cytokines: IL-1α (1 ng/mL), IL-1β (1 ng/mL), IL-6 (100 ng/mL), IL-8 (100 ng/mL), IL-17 (100 ng/mL), or TNF-α (100 ng/mL). In addition, cells were untreated (controls), treated with increasing concentrations of IL-1α (12.5-1000 pg/mL), IL-1β (12.5-1000 pg/mL), or co-treated with IL-1α + IL-1β. Cells were incubated with treatments for 24 h. The medium used for these amnion epithelial cell treatments was the same as that used with choriodecidual punches (DMEM supplemented with 1% FBS and pen/strep), except that no pen/strep antibiotic was included for cultures with live GBS. No changes in cellular morphology, cytokine secretion, or HBD-2 secretion were noted with the medium change. Experiments were conducted in triplicate using extraplacental membranes from a minimum of five women.

IL-1 Inhibitors

To inhibit IL-1 activity, amnion epithelial cells were treated with GBS choriodecidual conditioned medium for 24 h with and without IL-1β neutralizing antibody (1000 ng/mL), IgA isotype control (1000 ng/mL), or IL-1 receptor antagonist (IL-1Ra; 100 ng/mL; 5.8 nM). IL-1β neutralizing antibody was incubated with the GBS choriodecidual conditioned medium for 30 minutes prior to incubation with the amnion epithelial cells. The concentration used for IL-1β neutralizing antibody and IL-1Ra was determined by concentration-response curves generated by treating amnion epithelial cells with 1 ng/mL IL-1α or IL-1β and increasing concentrations of IL-

1β neutralizing antibody or IL-1Ra (Figures 3.1 and 3.2). Reported Kd values for IL-1Ra range from 0.2 – 14 nM (Arend 1991, Symons, Young et al. 1995). Experiments were conducted in triplicate using amnion epithelial cells from five women.

Cytokine and HBD ELISAs

HBD-2 concentrations in GBS choriodecidual conditioned medium were measured using a commercially available enzyme linked immunosorbant assay (ELISA) kit according to manufacturer's instructions (Peprotech, Rocky Hill, NJ). The HBD-2 ELISA detection range was 16-2000 pg/mL. Cytokine concentrations in GBS choriodecidual conditioned medium were measured by the University of Michigan Immunology Core using commercially available ELISA kits (R&D Systems). Cytokine detection ranges were as follows: 7.81-500 pg/mL for IL-1α; 2.91-2500 pg/mL for IL-1β; 9.38-125,000 pg/mL for IL-6; 31.2-2000 pg/mL for IL-8; and 15.6-5000 pg/mL for TNF-α. Samples were diluted as necessary. Cytokine and HBD-2 concentrations are reported as pg or ng protein/mL medium.

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA) or SigmaStat 3.5 software (SigmaStat Software, San Jose, CA). ANOVAs with Tukey's post hoc test were performed. Data were considered significant if the p-value was < 0.05. When values were below the ELISA kit limit of detection (LOD), values were transformed to LOD/$\sqrt{2}$ prior to statistical analysis (Croghan and Egeghy 2003).

Results

HBD-2 Release in Amnion Epithelial Cell Cultures Stimulated with Choriodecidual Conditioned Medium

To determine if choriodecidua secretes factors that stimulate HBD-2 production in amnion epithelial cells, GBS was applied to choriodecidual punch cultures that had been stripped of the amnion, and the GBS choriodecidual conditioned medium was added to amnion epithelial cell cultures. Amnion epithelial cells exposed to the GBS choriodecidual conditioned medium for 24 h showed a robust (11.2 fold) increase in HBD-2 release (Figure 3.3; $P < 0.05$) compared to amnion epithelial cells exposed to control choriodecidual conditioned medium. In contrast, neither LTA choriodecidual conditioned medium nor LPS choriodecidual conditioned medium stimulated release of HBD-2 from amnion epithelial cells. Furthermore, amnion epithelial cells directly stimulated with live GBS, LTA, or LPS did not exhibit an increased HBD-2 response. HBD-2 was not detected in choriodecidual medium alone (data not shown), indicating that the HBD-2 increases observed with GBS choriodecidual conditioned medium were the result of HBD-2 secretion from amnion epithelial cells in culture.

Cytokine Release by Choriodecidual Punch Cultures

Cytokines were measured in the medium of cultured choriodecidual punches exposed to pathogenic stimuli, in order to identify cytokines that may contribute to the HBD-2 response in amnion epithelial cells. First, heat-killed GBS and live GBS were compared for stimulation of IL-1β and TNF-α release. Heat-killed GBS failed to stimulate secretion of IL-1β or TNF-α from choriodecidual punches, in contrast to live GBS which elicited a strong IL-1β (287.2 ± 44.7 pg/mL) and TNF-α (1969 ± 456.7 pg/mL) response (Figure 3.4; $P < 0.05$). Additional cytokines

were then probed to compare stimulated release by live GBS, LTA, and LPS. Live GBS significantly increased IL-1α (38.5 fold), IL-1β (71.1 fold), TNF-α (10.2 fold), IL-6 (4.5 fold), and IL-8 (2.4 fold) compared to controls (Figure 3.5; $P < 0.05$). Live GBS did not change IL-17 secretion from choriodecidual punches compared to control. LTA significantly increased only IL-8 (2.9 fold) and TNF-α (11.7 fold), and LPS significantly increased IL-6 (5.3 fold), IL-8 (3.3 fold), and TNF-α (33.4 fold). Neither LTA nor LPS stimulated increased release of IL-1α or IL-1β.

Effect of IL-1 Inhibitors on HBD-2 Release from Amnion Epithelial Cells

To test the hypothesis that IL-1α and IL-1β secreted from choriodecidua mediates amnion-secreted HBD-2, amnion epithelial cell cultures were treated with GBS choriodecidual conditioned medium containing IL-1β neutralizing antibody or IL-1Ra as inhibitors of IL-1. To determine the appropriate concentration of inhibitors, we established concentration-response curves for each inhibitor in amnion epithelial cells treated with 1 ng/mL IL-1α or 1 ng/mL IL-1β (Figures 3.1 and 3.2). Inhibitor concentrations were selected based on manufacturer recommendations and minimal inhibitory concentrations. The IL-1β neutralizing antibody partially suppressed HBD-2 secretion stimulated by GBS choriodecidual conditioned medium (1.8 fold reduction), whereas IL-1Ra almost abolished the response (9.7 fold reduction) (Figure 3.6; $P < 0.05$). IL-1β (10 ng/mL) was used as positive control for HBD-2 secretion from amnion epithelial cells.

HBD-2 Release from Amnion Epithelial Cells Stimulated with Recombinant Cytokines

Because experiments with IL-1 inhibitors suggested a role for IL-1α and IL-1β in the HBD-2 response in amnion epithelial cells, we treated amnion epithelial cells with recombinant cytokines (Figure 3.7). HBD-2 release was significantly increased in a concentration-dependent manner by either IL-1α or IL-1β (Figure 3.7 and 3.8; $P < 0.05$), demonstrating that IL-1α and IL-1β directly stimulate HBD-2 release. However, co-treatment with equal concentrations of each IL-1α and IL-1β did not produce an increased HBD-2 response compared with exposure to either recombinant cytokine alone (Figure 3.8). Treatment with recombinant cytokines, IL-6, IL-8, IL-17, or TNF-α had no significant effect on HBD-2 from amnion epithelial cell cultures (Figure 3.7).

Discussion

As one of the leading causes of neonatal morbidity and mortality, GBS infection during pregnancy remains a serious public health problem (Verani, McGee et al. 2010). The mechanisms by which GBS interacts with the host immune response have yet to be fully elucidated in gestational tissues. We previously reported that HBD-2 expression increases in amnion epithelial cells of full thickness extraplacental membranes following GBS inoculation on the decidual side of the membranes *in vitro*, in the apparent absence of direct contact with the amnion epithelial cells directly (Boldenow, Jones et al. 2013). Here, we show for the first time that secreted factors from GBS stimulated choriodecidual tissue are essential for secretion of HBD-2 from the amnion. In addition we show that LTA and LPS choriodecidual conditioned medium fail to stimulate HBD-2 release from amnion epithelial cell cultures. Likewise, live GBS failed to stimulate HBD-2 release from cultured amnion epithelial cells. In contrast to our study, other cell types demonstrate direct bacterial stimulated HBD-2 secretion, suggesting that our

observations reflect tissue specificity (O'Neil, Porter et al. 1999, Krisanaprakornkit, Kimball et al. 2000, Wehkamp, Harder et al. 2004, Pivarcsi, Nagy et al. 2005).

The large increase in IL-1α and IL-1β in the medium from GBS-treated choriodecidual tissue suggests that IL-1 is important for HBD-2 secretion in amnion epithelial cells. LTA and LPS treated choriodecidual tissues did not have increased IL-1α or IL-1β secretion. We further demonstrated the importance of IL-1 in the HBD-2 pathway by inhibiting HBD-2 secretion from amnion epithelial cells using an IL-1β neutralizing antibody and the IL-1Ra (non selective IL-1 receptor antagonist). We observed near complete inhibition of HBD-2 secretion when using IL-1Ra and only partial inhibition when using IL-1β neutralizing antibody suggesting that both IL-1α and IL-1β secreted from the choriodecidual tissue have a redundant function in HBD-2 secretion from amnion epithelial cells. A similar mechanism, demonstrating the necessity of IL-1β in stimulating HBD-2 secretion has been proposed for pulmonary epithelial cells (Tsutsumi-Ishii and Nagaoka 2003). Previous studies suggest that amnion epithelial cells are incapable of producing IL-1, which further implicates dependence of amnion epithelial cells on the choriodecidua for HBD-2 production (Menon, Swan et al. 1995, Reisenberger, Egarter et al. 1998).

Previous studies have shown that both IL-1α and IL-1β are present in placenta and extraplacental membranes of healthy women (Hu, Yang et al. 1992, Keelan, Marvin et al. 1999, Young, Thomson et al. 2002). We demonstrated that recombinant IL-1β is capable of stimulating HBD-2 in our amnion epithelial cell cultures, consistent with previous studies (Stock, Kelly et al. 2007). In addition, we show for the first time that recombinant IL-1α also stimulates HBD-2 secretion in

human amnion cells, and does so at concentrations similar to IL-1α concentrations we detected in medium of GBS-stimulated choriodecidual tissue punch cultures. Our results are consistent with increases in IL-1α reported in bladder tissue and urine of mice treated with pathogenic GBS (Ulett, Webb et al. 2010). Furthermore, IL-1α and IL-1β are implicated in stimulating HBD-2 secretion from other cell and tissue types including human keratinocytes, uterine macrophages, corneal epithelial cells and middle ear epithelial cells (Liu, Destoumieux et al. 2002, Moon, Lee et al. 2002, McDermott, Redfern et al. 2003, Pioli, Weaver et al. 2006).

Cytokines mediate parturition-activating pathways by increasing prostaglandins, matrix metalloproteinases, and recruitment of neutrophils and macrophages in the gestational tissues. In particular, IL-1β has been implicated in adverse birth outcomes. Recently, Mitchell et al. linked maternal recto-vaginal GBS colonization during pregnancy with increased IL-1β in maternal and fetal serum and early term births (Mitchell, Brou et al. 2013). Primates inoculated with GBS had increased amniotic fluid concentrations of IL-1β, as well as IL-6, and TNF-α, PGE_2 and $PGF_{2\alpha}$, prior to the onset of contractions and parturition (Gravett, Witkin et al. 1994). Furthermore, direct infusion with IL-1β stimulated the onset of contractions and preterm labor in primates (Sadowsky, Adams et al. 2006). Fewer studies have examined the role of IL-1α in adverse birth outcomes. One study found that IL-1α treated pregnant mice have increased prostaglandin E_2 (PGE_2) and fetal deaths (Silver, Edwin et al. 1997).

Although recombinant IL-1α and IL-1β each stimulated HBD-2 release in amnion epithelial cells, other cytokines (IL-6, IL-8, IL-17, and TNF-α) did not significantly increase HBD-2 secretion. The concentrations of IL-6, IL-17, and TNF-α we used to treat amnion epithelial cells

were higher than the concentrations we observed in the medium of the GBS-stimulated choriodecidual punch cultures. IL-17 is secreted from Th17 cells and implicated in HBD-2 secretion in pulmonary epithelium and keratinocytes (Kao, Chen et al. 2004, Liang, Tan et al. 2006, Pennino, Eyerich et al. 2010). Although IL-17 appears to produce a slight increase in HBD-2 (not statistically significant) in our amnion epithelial cells, no IL-17 was detected when choriodecidual punches were treated with GBS. Thus, IL-17 does not appear to be an important immune mediator of HBD-2 release in human extraplacental membranes *ex vivo* and is likely because of minimal immune cells present in the healthy tissue we collect. However, Th17 cells may play a role *in vivo*.

In our study, bacteria cell wall or membrane components LTA and LPS did not stimulate IL-1α or IL-1β secretion from the choriodecidual tissue. Other studies have observed an increase in IL-1β secretion from full thickness extraplacental membranes treated with LPS (Fortunato, Menon et al. 1996, Zaga, Estrada-Gutierrez et al. 2004, Miller and Loch-Caruso 2010, Hoang, Potter et al. 2014). In contrast to our study which used only choriodecidual tissue, these studies use full thickness membranes and the magnitude of the changes was modest and varied between studies. In agreement with our study, King, et al. found that LPS and LTA had no effect on antimicrobial peptide mRNA expression in human endometrial epithelial cells (King, Fleming et al. 2002). Likewise, we observed that heat-killed GBS failed to elicit IL-1β or TNF-α secretion from the choriodecidual tissue, suggesting that cytokine stimulation relies on either a heat-liable protein or internalization of the live bacterium. The latter results are similar to those showing that live *S. auerus* engages inflammatory pathways and cytokine secretion differently than heat-killed *S. auerus* in mononuclear cells (Strunk, Richmond et al. 2011). Although, not tested here, cellular

internalization of live GBS may be critical for cytokine secretion in our model, as has been demonstrated previously in mouse dendritic cells (Costa, Gupta et al. 2012).

Our data showing an integral role for IL-1β in the stimulation of HBD-2 secretion suggest a role for the inflammasome. The inflammasome is a multi-protein complex composed in part of caspase-1 which is responsible for cleaving pro IL-1β in the cell to the active form, which can then be secreted from the cell. One study has shown that caspase-1 is slightly elevated in the amniotic fluid of laboring women at term compared to non-laboring, at term women and of particular interest caspase-1 was significantly elevated in amniotic fluid from women who delivered preterm with an infection compared to women who delivered preterm without an infection (Gotsch, Romero et al. 2008). Additional components of the inflammasome: NLRP3 and ASC (apoptosis-associated speck-like protein) are important for mediating caspase-1 regulated IL-1β secretion during GBS infection in mouse dendritic cells (Costa, Gupta et al. 2012). Future studies will explore the role of the inflammasome in the IL-1β secretory pathway.

In conclusion, this study utilized human extraplacental membranes to show that GBS treated choriodecidual tissue secrete IL-1α and IL-1β. Furthermore, we show that IL-1α and IL-1β from the choriodecidual tissues are important for GBS stimulated HBD-2 secretion from the amnion epithelial cells.

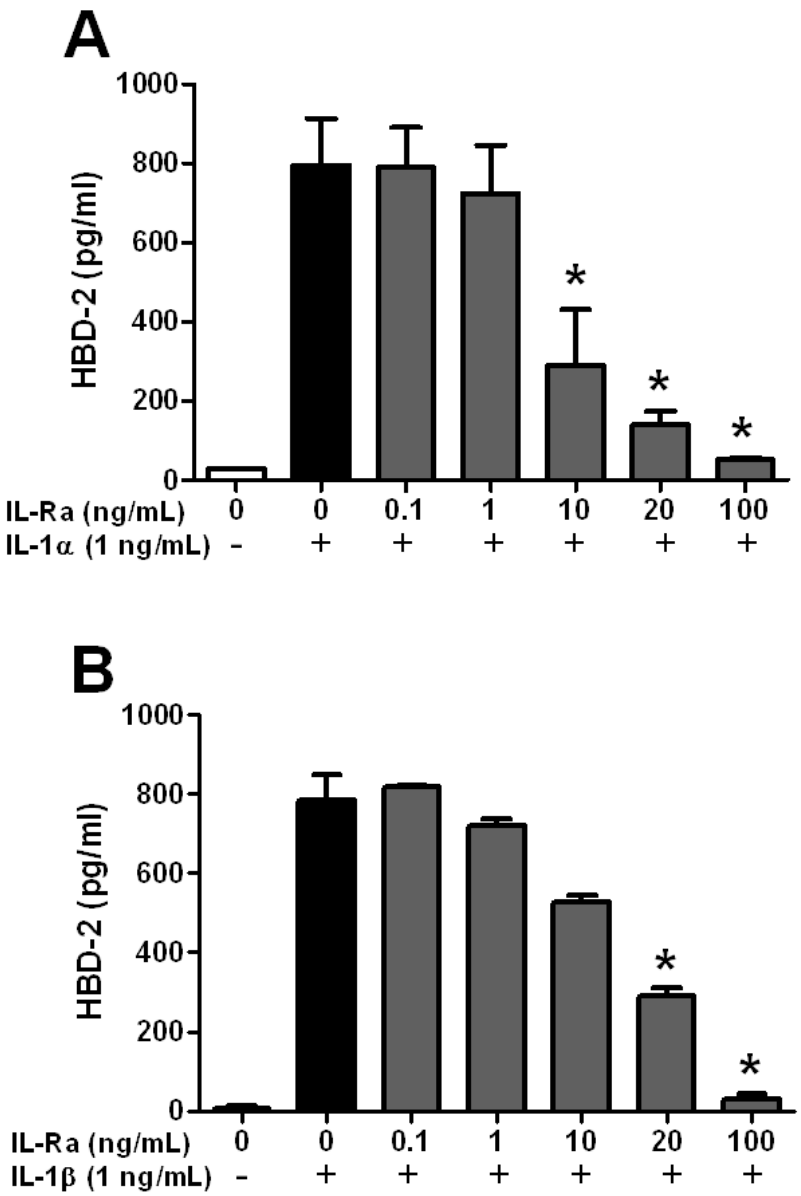

Figure 3.1. Effects of IL-1Ra on IL-1α and IL-1β stimulated HBD-2.
HBD-2 release into medium by primary amnion epithelial cells treated with IL-1Ra and recombinant IL-1α (A; 1 ng/mL) or IL-1β (B; 1 ng/mL) after 24 h. HBD-2 protein in the medium was measured by ELISA. Columns are mean ± SEM (N=3 women). Asterisks (*) represent significant differences between treatment and IL-1α or IL-1β (1 ng/mL) when compared by Tukey's post hoc test following ANOVA ($P < 0.05$).

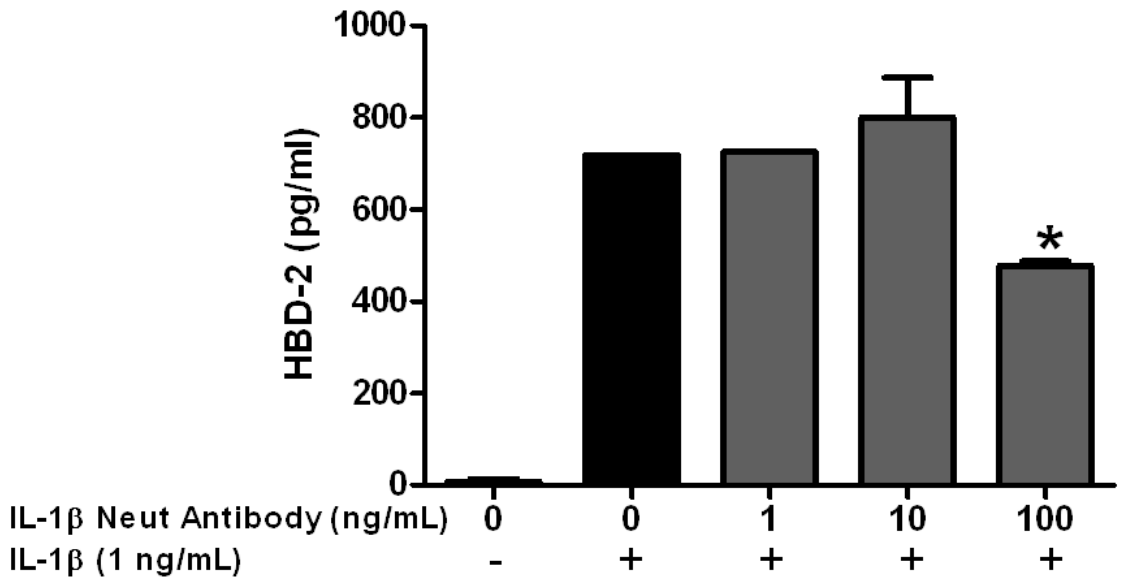

Figure 3.2. Effects of IL-1β neutralizing antibody on IL-1β stimulated HBD-2.
HBD-2 release into medium by primary amnion epithelial cells treated with IL-1β neutralizing antibody and recombinant IL-1β (1 ng/mL) after 24 h. HBD-2 protein in the medium was measured by ELISA. Columns are mean ± SEM (N=3 women). Asterisks (*) represent significant differences between treatment and IL-1β (1 ng/mL) when compared by Tukey's post hoc test following ANOVA ($P < 0.05$).

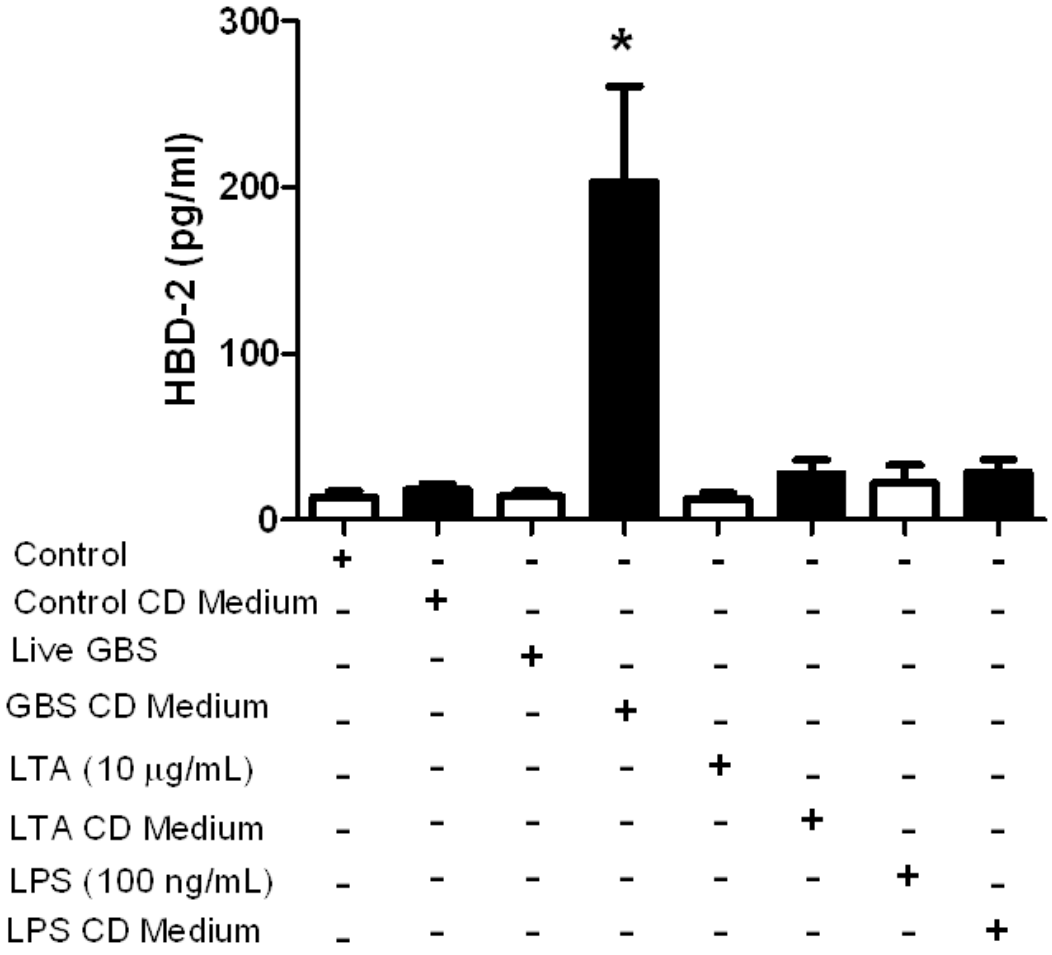

Figure 3.3. HBD-2 release into medium by amnion epithelial cell cultures.
HBD-2 release in human amnion epithelial cell cultures treated directly with pathogenic stimuli (live GBS, LTA, or LPS) or with choriodecidual (CD) conditioned medium generated by culturing CD tissue punches with live GBS, LTA, or LPS for 24 h. HBD-2 protein in the medium was measured by ELISA. Columns are mean ± SEM (N=24 women for GBS CD conditioned medium, N=5 women for live GBS, and N=7 women for remaining treatment groups). The asterisk (*) represents a significant difference between treatment and control when compared by Tukey's post hoc test following ANOVA ($P < 0.05$).

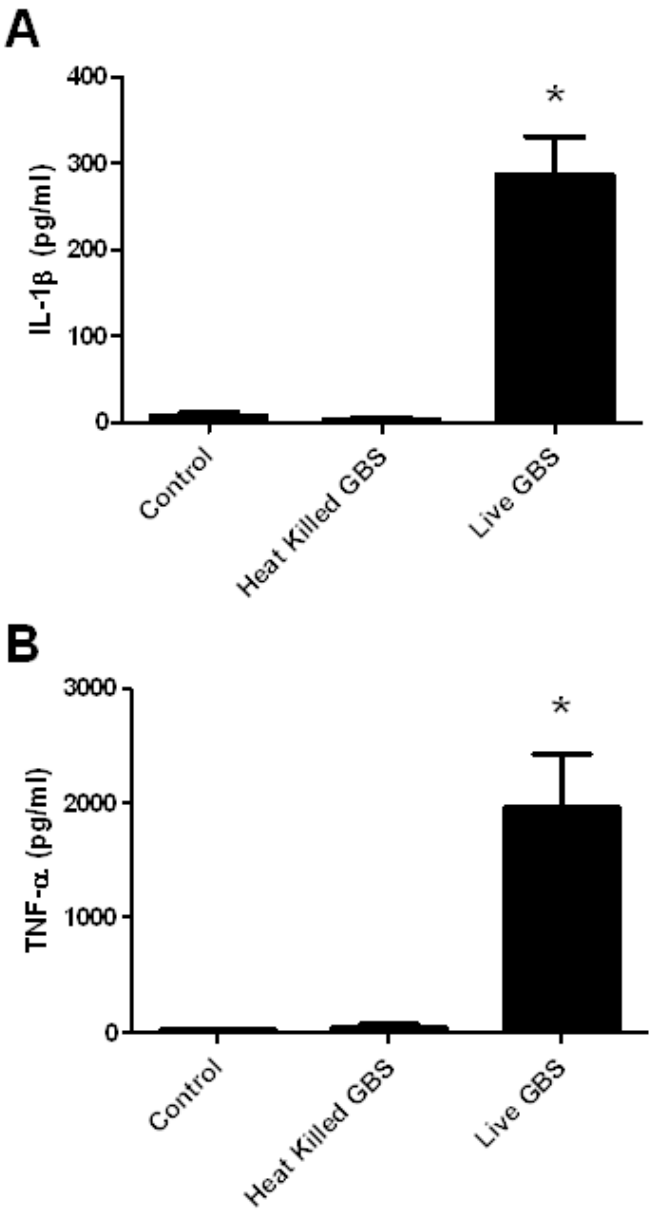

Figure 3.4. IL-1β and TNF-α release into medium by choriodecidual punch cultures.
L-1β and TNF-α release by choriodecidual punch cultures treated with medium alone (control), heat-killed GBS, or live GBS. IL-1β (A) and TNF-α (B) in the medium were measured by ELISA. Columns are mean ± SEM (N=5 women). Asterisks (*) represent significant differences between treatment and control when compared by Tukey's post hoc test following ANOVA ($P < 0.05$).

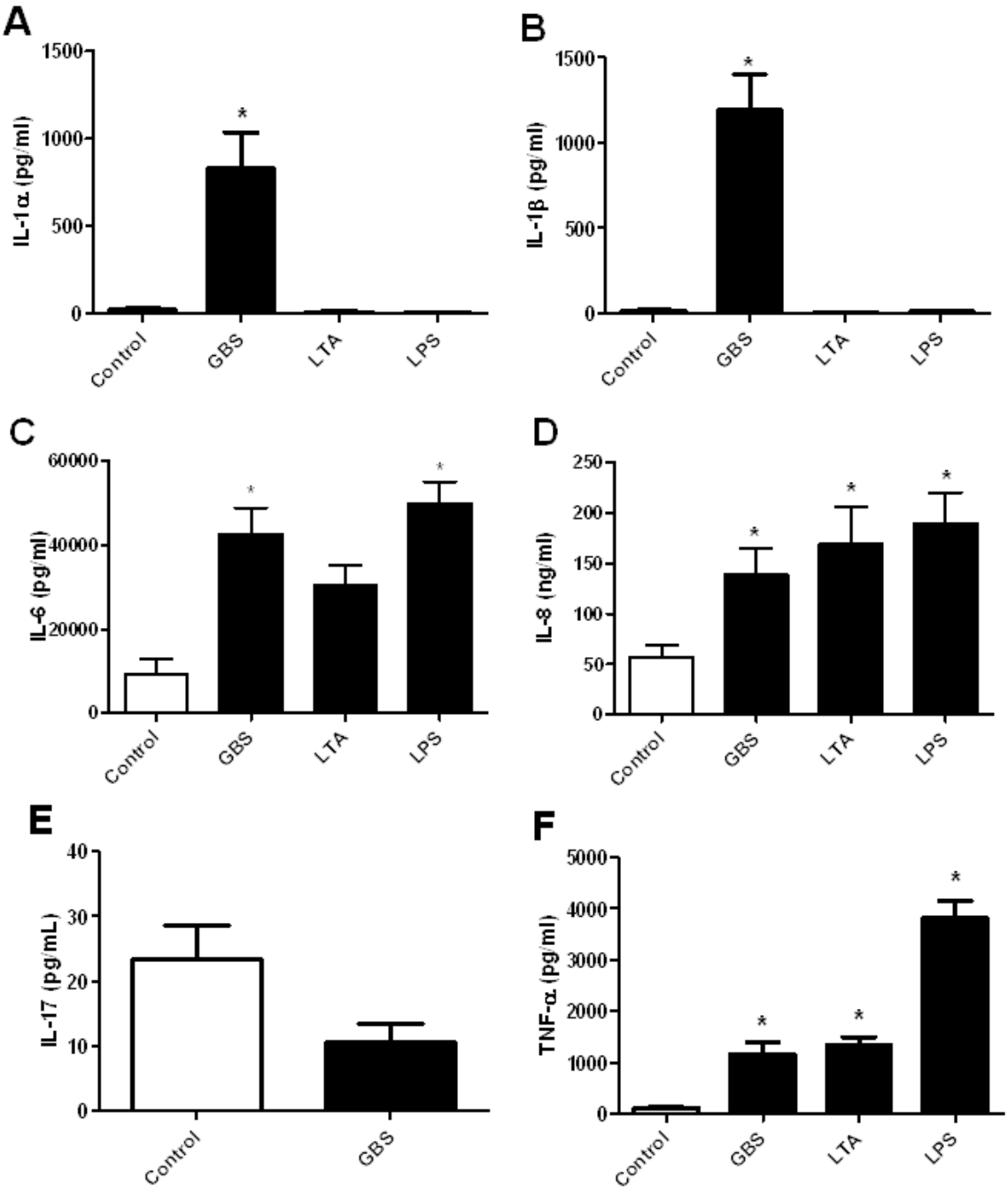

Figure 3.5. Cytokine release into medium by choriodecidual punch cultures.
Cytokine release into medium by choriodecidual punch cultures treated with medium alone (control), live GBS, LTA, or LPS. IL-1α (A), IL-1β (B), IL-6 (C), IL-8 (D), IL-17 (E), and TNF-α (F) in the medium were measured by ELISA. Columns are mean ± SEM (N=21 women for Control and live GBS treatment groups; N=6 women for LTA and LPS treatment groups). Asterisks (*) represent significant differences between treatment and control when compared by Tukey's post hoc test following ANOVA ($P < 0.05$).

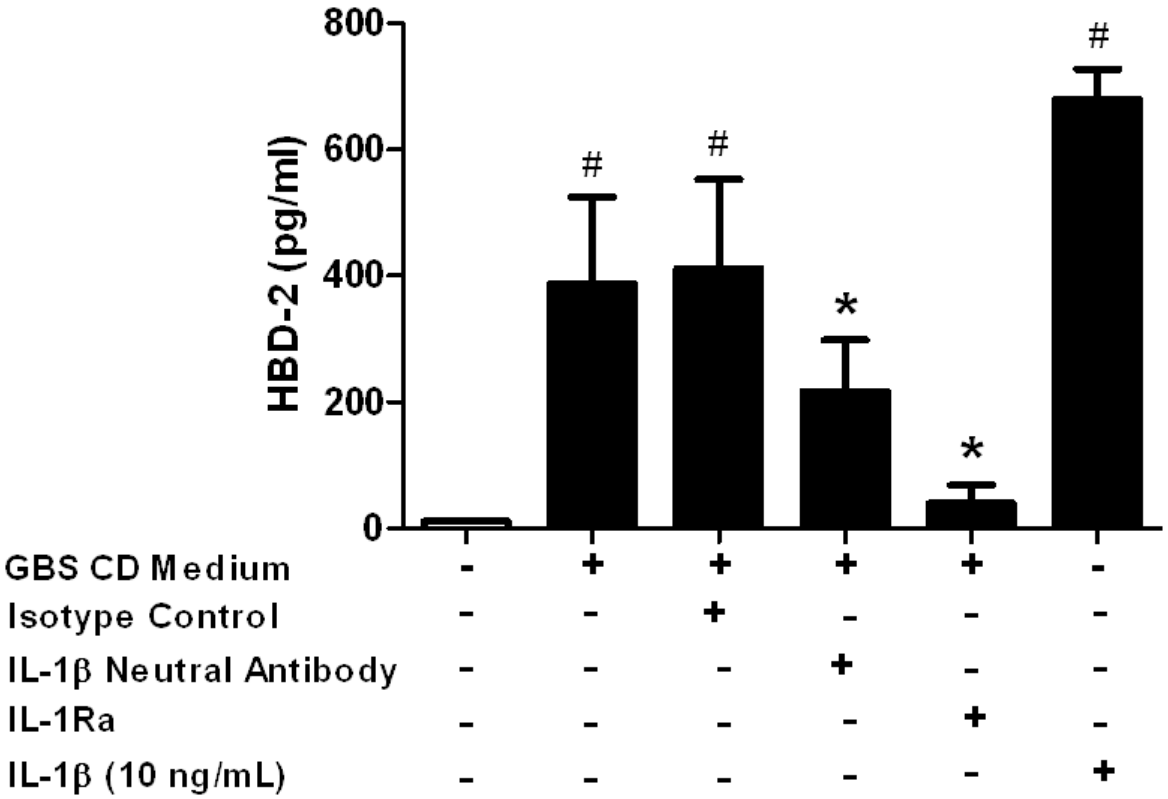

Figure 3.6. The effect of IL-1 inhibitors on HBD-2 release by amnion epithelial cells. Amnion epithelial cells were treated with choriodecidual (CD) conditioned medium with and without IL-1 inhibitors for 24 h. IL-1β (10 ng/mL) treatment for 24 h was used as a positive control for HBD-2 secretion from amnion epithelial cells. HBD-2 protein in the amnion cell culture medium was measured by ELISA. Columns are mean ± SEM (N=5 women). Pound symbols (#) represent significant differences between treatment and control (medium only) and asterisks (*) represent significant differences between treatment and GBS CD Medium when compared by Tukey's post hoc test following ANOVA ($P < 0.05$).

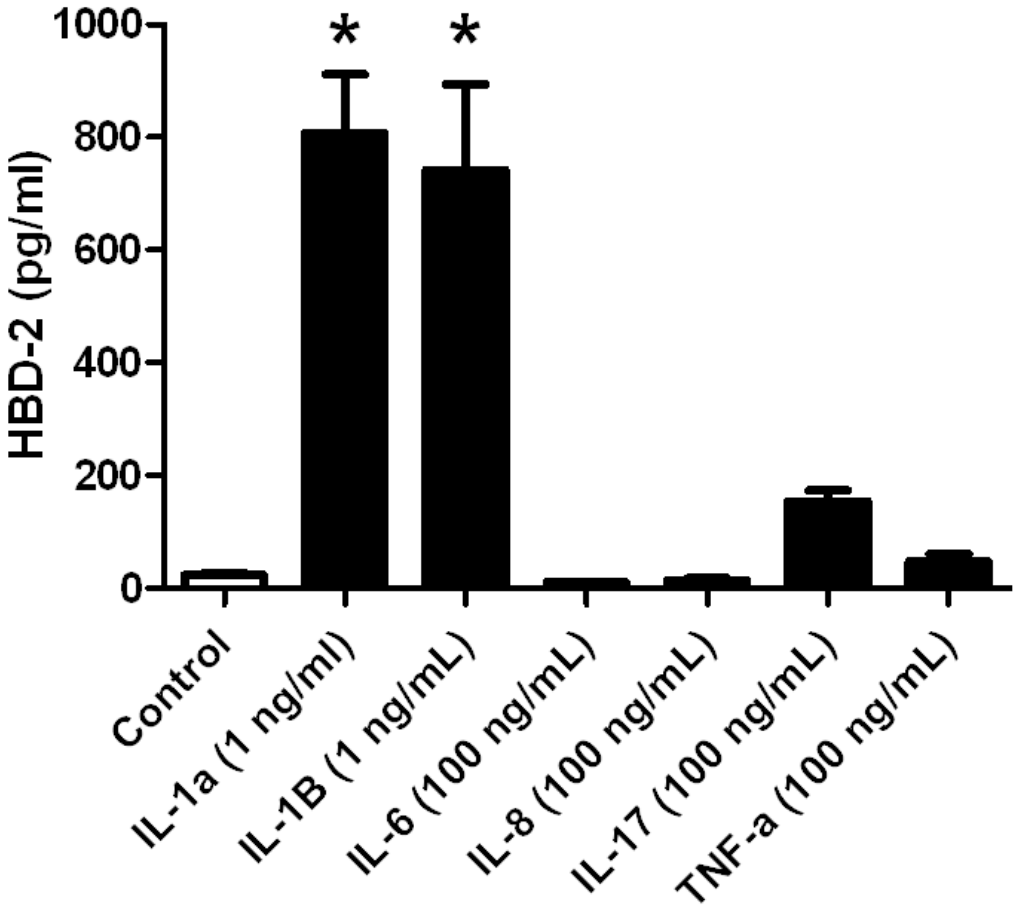

Figure 3.7. HBD-2 release by primary amnion epithelial cells treated with cytokines. HBD-2 release into medium by primary amnion epithelial cells treated with recombinant cytokines after 24 h. HBD-2 protein in the medium was measured by ELISA. Columns are mean ± SEM (N=3-6 women). Asterisks (*) represent significant differences between treatment and control when compared by Tukey's post hoc test following ANOVA ($P < 0.05$).

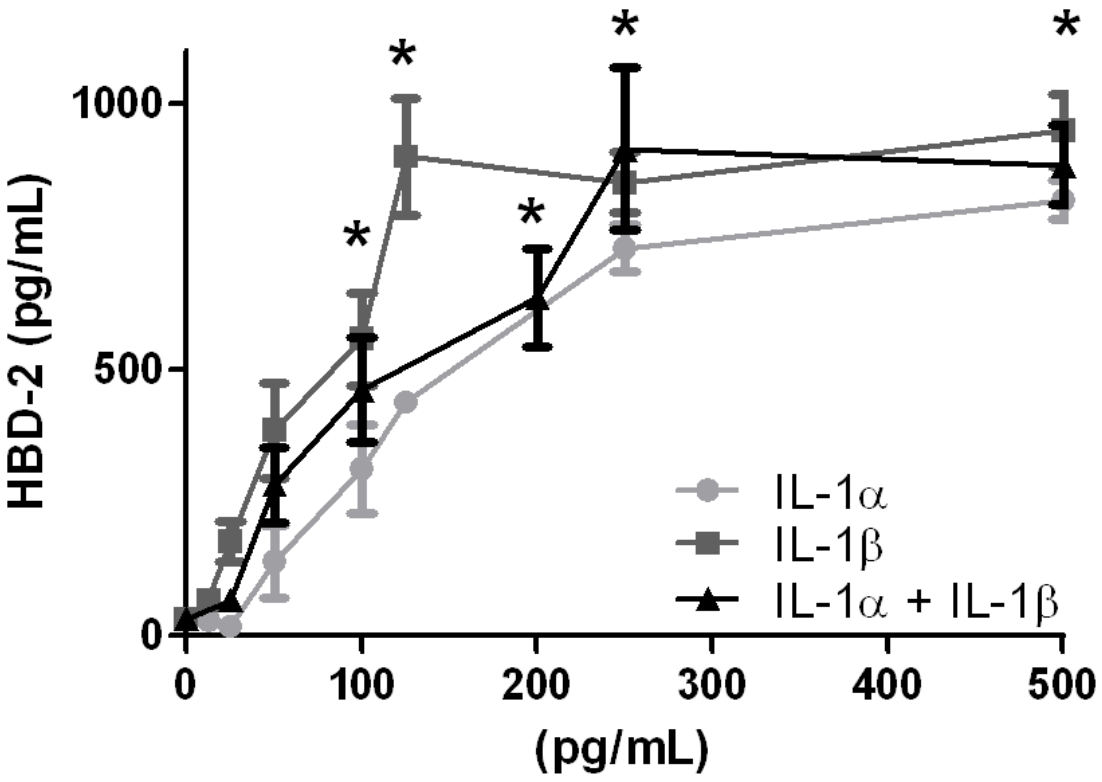

Figure 3.8. HBD-2 release by amnion epithelial cells treated with IL-1α and IL-1β.
HBD-2 release by amnion epithelial cells treated with recombinant IL-1α and IL-1β after 24 h. Equal concentrations of each cytokine were used for the IL-1α + IL-1β treatment group. HBD-2 protein in the medium was measured by ELISA. Columns are mean ± SEM (N=7 women). Asterisks (*) represent significant differences compared to medium only control when compared by Tukey's post hoc test following ANOVA ($P < 0.05$). No statistical differences were observed between cytokines treatments at any given concentration.

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CHAPTER 4. GROUP B STREPTOCOCCUS STRAIN VARIATION IMPACTS CYTOKINE AND CHEMOKINE HOST RESPONSE IN HUMAN EXTRAPLACENTAL MEMBRANES IN VITRO

Abstract

Problem: *Streptococcus agalactiae* (GBS) is an important cause of chorioamnionitis and neonatal disease, yet little is known about how GBS strain differences impact host response in the extraplacental membranes. This study characterizes colonization and stimulation of cytokines and chemokines in human extraplacental membranes by five GBS strains using an *ex vivo* transwell two-compartment culture system of full-thickness membranes.

Method of Study: Human extraplacental membranes were affixed to transwell frames (without synthetic membranes). Live GBS was added to the decidual side of membranes in transwell cultures, and cocultures were incubated for 24 h. Cytokines were identified in the medium using ELISA. Data were statistically analyzed using a linear mixed model. GBS recovery from homogenized membranes was determined by enumerating colony forming units (CFU) on blood agar. Formalin fixed tissues were Hucker-Twort stained to determine the presence of GBS within the membranes.

Results: GBS-treated membranes released increased amounts of the proinflammatory cytokines IL-1β, IL-6, TNF-α, and IL-8 into the amniotic and decidual compartments of the transwell cultures after 24 h ($P < 0.001$), regardless of GBS strain type. The magnitude of the cytokine

response was strain dependent. GBS recovery from membranes was observed for the two colonizing strains only, with both colonizing strains persisting on the choriodecidual side of the membranes.

Conclusions: Host cytokine and chemokine responses in human extraplacental membranes are GBS strain specific, suggesting that strain GBS strain variation should be considered for future treatment and prevention strategies. Because the observed GBS strain differences could be related to expression of virulence factors, future studies will explore how GBS virulence factors impact the extraplacental membranes host response.

Introduction

Streptococcus agalactiae or Group B *Streptococcus* (GBS) continues to be a serious public health problem and is associated with adverse pregnancy and neonatal outcomes (Verani, McGee et al. 2010) including neonatal sepsis and meningitis. Genital GBS colonization during pregnancy occurs in up to 30% of women and is the primary risk factor for neonatal GBS disease (Schuchat and Wenger 1994, Phares, Lynfield et al. 2008, Verani, McGee et al. 2010). Prior to neonatal infection, GBS colonizes the maternal vagina and can ascend to the uterine compartment through the extraplacental membranes (also called gestational or maternal-fetal membranes). However, GBS colonization of the vagina does not always translate to invasive GBS disease (Jones, Bohnsack et al. 2003, Luan, Granlund et al. 2005, Bohnsack, Whiting et al. 2008, Manning, Springman et al. 2009). Although the extraplacental membranes serve as an important barrier to infection, the mechanism by which GBS crosses the membranes during infection has not been fully elucidated. GBS has been shown to adhere to and infect human extraplacental membranes and cells (Galask, Varner et al. 1984, Gravett, Haluska et al. 1996, Winram, Jonas et al. 1998). In addition, the extraplacental membranes mount a robust host

immune response, including cytokine and antimicrobial peptide secretion to pathogenic organisms in culture (Menon, Swan et al. 1995, Zaga, Estrada-Gutierrez et al. 2004, Garcia-Lopez, Flores-Espinosa et al. 2010, Zaga-Clavellina, Martha et al. 2012, Zaga-Clavellina, Ruiz et al. 2012).

GBS virulence is thought to involve genomic variation and suppression or evasion of host immunity. To date, most studies have focused on GBS virulence mechanisms across strains with little attention on the host response. Conversely, host response studies have focused on the vaginal epithelium and few studies have examined multiple GBS strains within a single tissue to compare the differences in host response. Here we adapted an *ex vivo* transwell two-compartment system of full-thickness human extraplacental membranes for coculture with different strains of live GBS to test the hypothesis that GBS strain impacts the strength and nature of the extraplacental membranes cytokine response, which are an important for resisting infection by promoting chemotaxis and antimicrobial peptides secretion.

Materials and Methods

Reagents and Materials

Five GBS strains were selected for this study (Table 4.1) with differing capsular and sequence types. Invasive strains were associated with neonatal sepsis, while colonizing strains were from routine screening and were not associated with either maternal or neonatal disease. GBS strain A909 was initially isolated from a septic newborn (Lancefield, McCarty et al. 1975) and subsequently transformed with plasmid encoding genes for Green Fluorescent Protein and erythromycin resistance (construct RS020, a gift from Amanda Jones, University of Washington). GB37 and GB411 were isolated from newborn blood samples with early onset

disease (Spaetgens, DeBella et al. 2002) and GB112 and GB590 were isolated from pregnant or postpartum women during vaginal rectal screening (Davies, Raj et al. 2001) (gift from Shannon Manning, Michigan State University). All GBS strains were grown at 37 °C in planktonic culture using Todd Hewitt Broth (THB, Becton-Dickinson, Franklin Lakes, NJ) or on sheep's blood agar plates (Blood Agar Base #2, Remel, Lenexa, KS and BBL defibrinated sheep blood, Franklin Lakes, NJ). Media, buffers, fetal bovine serum (FBS; catalog #10438) and penicillin/streptomycin (pen/strep; catalog #15140) were from GIBCO (Grand Island, NY).

Culture of Extraplacental Membranes

Human extraplacental membranes were collected from healthy pregnancies undergoing scheduled cesarean delivery at term prior to onset of labor at the University of Michigan Birth Center as previously described (Boldenow, Jones et al. 2013). The University of Michigan Institutional Review Board approved this research (IRBMED#HUM0013915).

Extraplacental membranes were cultured in a two-compartment transwell system as described previously published (Boldenow, Jones et al. 2013, Zaga, Estrada-Gutierrez et al. 2004, Thiex, Chames et al. 2009). Briefly, membranes were dissected from placenta immediately following delivery and transported directly to the laboratory in Dulbecco's phosphate-buffered saline (DPBS). Membranes were rinsed with medium and blood clots were removed. The membranes were then mounted on sterile transwell frames that had no synthetic membrane (gift from Corning, NY) and held in place with sterile latex elastic bands (ORMCO, Orange, CA). The membranes were affixed with the choriodecidua facing the inner chamber of the transwell and the amnion facing the outer chamber. The transwell inserts with membranes were placed in wells

of 12-well culture plates containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% FBS and pen/strep. To maintain equal medium levels between the inner and outer transwell chambers, 0.5 mL medium was added to the smaller inner chamber and 1.5 mL medium was added to the larger outer chamber. Cultures were incubated at 37 °C and 5% CO_2. After 4 h, the medium was exchanged for DMEM/1% FBS without antibiotics.

GBS Coculture with Extraplacental Membranes

GBS in early exponential growth phase was diluted with DMEM/1% FBS to $1x10^6$ colony forming units/mL (CFU/mL). Inoculant concentrations were validated by overnight growth on 5% sheep blood agar. Following a 24 h acclimation, the medium of the transwell choriodecidual compartment was replaced with 0.5 mL GBS inoculant ($1x10^6$ CFU/mL) or fresh DMEM/1%FBS without GBS (controls). Amnion compartment medium was also exchanged with fresh DMEM/1%FBS. Cocultures were then incubated for 24 h. Coculture experiments were conducted in triplicate or quadruplicate using extraplacental membranes from seven or thirteen women.

After 24h, medium was collected and stored at -80 °C. In a subset of experiments (N=3 women), transwell inserts with attached tissue were transferred to fresh wells with DPBS in both chambers. The plate was gently rocked for 5 min to rinse away non-adherent bacteria. Two 3-mm biopsy punches were taken from each transwell-mounted tissue piece, placed in 1 mL PBS, and homogenized on ice with two 40-sec pulses. Tissue homogenates were serially diluted 1:10 in PBS, plated on 5% sheep blood agar in triplicate, and grown 12-24 h at 37 ˚C. Colony counts of viable bacteria in the membrane (CFU/mL/cm^2) were determined.

Gram Staining

While still attached to the transwell insert, extraplacental membranes were fixed with 10% phosphate-buffered formalin (Fisher, Waltham, MA) for 24-48 h at 4 ˚C. The fixed tissue was gently removed from the transwell insert and a strip of membrane was cut ≤ 4 mm in width. Processing and staining were performed by the University of Michigan's Comprehensive Cancer Center Tissue Core. The tissue strips were embedded in paraffin "on edge", sectioned, and mounted on slides. Hucker-Twort tissue gram staining was performed by the University of Michigan's Unit for Laboratory Animal Medicine. Two sections in three tissues were examined for all treatments. Microscopy image capture was done using Nikon Elements Software.

Cytokine ELISAs

Cytokine release from the choriodecidual membranes punches into medium was measured by the University of Michigan Immunology Core using commercially available enzyme linked immunosorbant assays (ELISA; R&D Systems). The ELISA detection range was: 7.81-500 pg/mL for IL-1α, 2.91-2500 pg/mL for IL-1β, 39.1-2000 pg/mL for IL-1Ra, 9.38-125,000 pg/mL for IL-6, and 31.2-2000 pg/mL for IL-8, 15.6-1000 pg/mL for IL-10, 15.6-1000 pg/mL for IL-17, 15.6-1000 pg/mL for MCP-1, and 15.6-5000 pg/mL for TNF-α. The IL-18 detection range was 20-5000 pg/mL using antibodies were purchased from MBL International (Woburn, MA). Samples were diluted as necessary. Values are reported as pg or ng protein/mL medium.

Statistical Analysis

Data presented in graphs and tables are expressed as mean ± SEM and were analyzed using

GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). For transwell coculture GBS CFU quantification, ANOVAs with Tukey's post hoc test were performed. Differences were considered significant if the p-value was < 0.05. Linear mixed models analyses were performed using R 3.0. with the nlme package. Data for IL-1β, IL-6, IL-8, and TNF-α were log transformed (on measurement +1). For each of the four cytokines, a linear mixed model was used to test for response differences among different GBS strains including control. Random effects were assumed for each woman, and interactions between GBS strain and transwell side were included in the model. The same analysis was performed among different GBS strains excluding control. In addition, pair wise comparisons across GBS strains were performed.

Results

Cytokine Secretion from Extraplacental Membranes

To simulate an ascending intrauterine infection, GBS was added to the choriodecidual compartment of the transwell-mounted extraplacental membranes. Inter- and intra-individual differences were noted, with approximately 30% of the variability attributable to differences among women. Cytokines IL-1β, IL-6, TNF-α, and IL-8 were positively correlated. All GBS strains significantly increased IL-1β, IL-6, IL-8, and TNF-α secretion from the extraplacental membranes compared to medium only control (Table 4.2). For all cytokines, concentrations were higher in the choriodecidual compartment compared to the amnion compartment (Table 4.2). Each GBS strain produced a unique cytokine profile and the magnitude of cytokine increases was strain dependent, also. Large increases in IL-1β (20-44 fold) and TNF-α (11-36 fold) were observed in the choriodecidual compartment after 24 h coculture with GBS (Figure 4.1). The amnion compartment also had increased IL-1β (2.2-5.9 fold) and TNF-α (2.2-9.8 fold). More

modest increases were noted for IL-6 (2.3-5.8 fold) and IL-8 (1.4-2.4 fold) in the choriodecidual compartment after 24 h.

Linear mixed models analysis, revealed statistically significant differences in cytokine concentrations between GBS strains for IL-1β (P = 0.005), IL-8 (P = 0.0280), and TNF-α (*P* < 0.001). Although not statistically significant, data suggested that IL-6 response was also GBS strain specific (P = 0.054). Overall, strain A909 promoted the strongest increases in IL-1β (38 fold), IL-6 (5.8 fold), IL-8 (2.4 fold), and TNF-α (36 fold) secretion in the choriodecidual compartment compared to medium only control (Table 4.2). Pairwise comparison (Table 4.3) demonstrated that A909 increases TNF-α significantly compared to all other GBS strains. Although, GB112 and GB411 have the same sequence type and serotype, extraplacental membranes treated with GB411 had increased IL-1β compared to membranes treated with GB112 (P = 0.016). We were interested in comparing GB37 and GB590 because mice treated with GB37 have increased mortality compared to GB590 (Rogers, Singh et al. 2014). GB590 stimulated greater TNF-α secretion compared to GB37 (P = 0.025). Although, not comparable in our model due to insufficient GBS strains, no obvious patterns emerged in cytokine secretion comparing invasive to colonizing strains (Figure 4.1).

Although, not included in the mixed model analysis because samples were measured in a subset of women (N=4), it appears that all GBS strains increase IL-1α (4-7 fold), IL-1Ra (2-5.5 fold), and IL-10 (2-7 fold) compared to no treatment control in the choriodecidual compartment (Table 4.2). These data should be interpreted with caution due to the smaller sample size measured (N= 4 women for IL-1α and IL-1Ra). In addition, IL-17, IL-18, and MCP-1 concentrations were very

low in the medium and did not show increased secretion with GBS treatment, regardless of strain (data not shown).

GBS Growth in Extraplacental Membranes

In a subset of experiments, CFUs were measured from homogenized tissue. No GBS colonies formed from membranes of unexposed control tissues after 24 h of coculture (not shown). GBS invasive strains A909, GB37, and GB411 had virtually no recoverable CFUs after coculture with extraplacental tissue for 24 h (Figure 4.2). GB112 was highly variable, but had recoverable CFUs in all experiments. GB590 was also highly variable, but had some CFUs after coculture with the extraplacental membranes. Furthermore, GBS growth of all strains was robust in DMEM/ 1% FBS, indicating that the decreased CFU recovery from coculture with extraplacental tissues was dependent on the presence of the tissue and not an artifact of the medium or transwell system (data not shown).

Tissue Gram Staining

Hucker-Twort staining was performed to identify GBS in the extraplacental membranes tissue following GBS treatment. No GBS was identified in extraplacental membranes without GBS coculture or in membranes exposed on the choriodecidual side to GBS strains A909, GB37, or GB411 (Figure 4.3 A, B, C, and E, respectively). Clusters of GB112 were identified on the choriodecidual side (Figures 4.3 D and Figure 4.4). GB590 was found both on the choriodecidual surface and within the tissue (Figure 4.3 F and Figure 4.5).

Discussion

This is the first study to examine multiple GBS strains in a full thickness extraplacental membranes model. This study showed that five GBS strains of differing capsular and sequence types stimulated cytokine release in human extraplacental membranes, and that GBS strain difference influenced the magnitude of the host response. Previous studies demonstrated increased cytokine release from extraplacental membranes and cells stimulated with GBS (Reisenberger, Egarter et al. 1997, Griesinger, Saleh et al. 2001, Zaga, Estrada-Gutierrez et al. 2004, Menon, Peltier et al. 2009). However, these former studies typically used only one or two GBS strains. Consistent with previous reports, the present study further shows that cytokine release from choriodecidual side of the membranes was greater than release from the amnion side (Zaga, Estrada-Gutierrez et al. 2004, Miller and Loch-Caruso 2010).

GBS are classified into ten different serotypes based on capsular polysaccharides which were originally proposed as the major contributing factor to virulence (Slotved, Kong et al. 2007). Among the few studies to examine host response to more than one GBS strain in gestational tissues, human decidual cells treated with five different heat-killed GBS had strain-specific increases of macrophage inflammatory protein (MIP)-1α and IL-8 (Dudley, Edwin et al. 1997), and chorion cells treated with five different heat-killed GBS had increased MIP-1α dependent on GBS strain (Dudley, Edwin et al. 1996). Although the authors of both the previous studies suggested that serotype may be partially responsible for the results, the reports failed to provide any description of the GBS strains used. Moreover, epidemiological data, indicate that different regions around the world have different serotype prevalence yet serotype alone does not account for disease incidence (Ippolito, James et al. 2010).

In addition to serotypes, GBS strains are classified into sequence types (ST) based on genetic variation in seven conserved genes. One particular ST (ST-17) is more commonly associated with neonatal disease compared to other sequence types (Manning, Springman et al. 2009). Of particular interest was comparing GB112 and GB411 because they are both the same serotype and sequence type (ST-17). However, as noted in Table 4.1, GB411 caused invasive GBS disease, while GB112 is a colonizing strain, suggesting that virulence goes beyond simple serotype and sequence type classification. In our study, GB411 caused significant increases in IL-1β compared to GB112. In addition, GB112 was recovered from the extraplacental membranes, whereas GB411 was not. Recently, we showed that IL-1β is important in the release of the antimicrobial peptide HBD-2 which is capable of killing GBS (Chapter 2 and 3). Although, not studied here directly, GB112 may be capable of persisting in the extraplacental membranes by not promoting a strong IL-1β increase, thereby not increasing HBD-2.

Recently, Aronoff and colleagues showed that strain GB37 is significantly more lethal compared to GB590 in a nonpregnant mouse model (Rogers, Singh et al. 2014). Furthermore, human monocyte THP-1 cells secreted more TNF-α treated with GB37 compared to GB590 (Rogers, Singh et al. 2014). In contrast, our study shows extraplacental membranes treated with GB590 secreted more TNF-α compared to membranes treated with GB37, illustrating potential host response differences across cell and tissue types. Additional studies are needed to determine how these differences in host response across cells and tissue types contribute to the entire immune response during infection.

In our study, the two colonizing strains persisted in the tissue whereas the three invasive strains did not. Clusters of GB112 were noted on the surface of the choriodecidual side of the membrane (the site of infection), yet GB112 did not appear to invade the tissue. The presence of clusters were not uniform throughout, which may have contributed to the variability observed in the CFU recovery. The bacterial clustering suggests biofilm formation. Biofilm formation has been identified in the vaginal epithelium and in amniotic fluid (Swidsinski, Mendling et al. 2005, Romero, Kusanovic et al. 2007, Romero, Schaudinn et al. 2008). Furthermore, GBS is capable of forming biofilms (Cucarella, Solano et al. 2001, Rinaudo, Rosini et al. 2010). In contrast to other GBS strains, GB590 was found both on the choriodecidua surface and within the tissue, suggesting invasion into the tissue is possible. Although not assayed in the present study, we previously recovered GBS strain A909 CFUs from transwell culture medium of human extraplacental membranes that were comparable to CFUs in homogenized tissue (Boldenow, Jones et al. 2013). It's important to note that the present study was done in tissue from healthy women. Women with poor health could be more susceptible to colonization or invasive infection.

In addition to serotypes and sequence types, GBS virulence may be related to adaptability. The GBS transcriptome changes after contact with human tissues (Mereghetti, Sitkiewicz et al. 2008, Mereghetti, Sitkiewicz et al. 2009, Sitkiewicz, Green et al. 2009). GBS changes its virulence gene expression patterns when grown in human amniotic fluid. These changes include adhesion, capsule, and hemolysin gene expression (Sitkiewicz, Green et al. 2009). Although we did not examine GBS gene expression or characteristics post coculture, GBS gene expression patterns in one GBS strain differed between human subjects after culture in human blood (Mereghetti,

Sitkiewicz et al. 2008, Mereghetti, Sitkiewicz et al. 2009, Sitkiewicz, Green et al. 2009). In addition, the host cytokine secretion differed among individuals. We did observe considerable variability in host response between women, consistent with inter-individual cytokine differences previously reported (Miller and Loch-Caruso 2010).

Furthermore, GBS are named for their beta hemolytic activity as assayed by their ability to completely lyse red blood cells. Hemolytic activity may contribute to differences observed in host response. Hemolysins are thought to cause cellular damage and aid in invasion (Doran, Liu et al. 2003, Maisey, Doran et al. 2008). Despite original reports that the extraplacental membranes and amnion epithelial cells provide a competent barrier to GBS (Winram, Jonas et al. 1998, Kjaergaard, Helmig et al. 1999), recent work shows that GBS invade extraplacental membranes in a hemolysin-dependent manner (Whidbey, Harrell et al. 2013). In addition, hyper-hemolytic activity significantly increased IL-1β release in amnion epithelial cells. Moreover, another study found that GBS strains lacking beta hemolysin and cytolysin resulted in a marked reduction in IL-8, GRO-α/β, IL-6, and GM-CSF gene expression in human brain microvascular endothelial cells (Doran, Liu et al. 2003). In our study, all GBS strains were hemolytic except GB37. Although not statistically significant across all GBS strains, GB37 tended to increase cytokine expression to a lesser extent compared to other GBS strains.

In summary, GBS regardless of strain increased cytokine secretion from the extraplacental membrane cytokines; however, we show for the first time that GBS strain impacts the magnitude of cytokine secretion from the extraplacental membranes. No single GBS virulence factor examined thus far can completely account for invasive GBS disease. The mechanisms of GBS

pathogenesis have not been fully elucidated and identifying molecular targets for vaccine development have progressed slowly. Future work will focus on evaluating host response with additional GBS strains and characterizing the virulence factors of the GBS strains used in this study.

Table 4.1. GBS Strain Variations.

Strain		Sequence Type	Serotype
A909	Invasive	ST-7	cpsIa
GB37	Invasive	ST-1	cpsV
GB112	Colonizing	ST-17	cpsIII
GB411	Invasive	ST-17	cpsIII
GB590	Colonizing	ST-19	cpsIII

Abbreviations: ST, sequence types, cps, capsular type

Table 4.2. Cytokine Concentrations in Choriodecidual and Amniotic Compartment Medium. Medium from transwell cultures of human extraplacental membranes treated with different GBS strains. Data are presented as mean (SEM).

		Choriodecidua Compartment		Amnion Compartment	
Interleukin-1α[c]	Control	< LOD[a]		< LOD	
(pg/mL)	A909	39.26[b]	(3.90)	< LOD	
	GB112	38.95	(5.26)	< LOD	
	GB411	60.89	(27.31)	< LOD	
	GB37	33.10	(6.24)	< LOD	
	GB590	33.82	(4.46)	< LOD	
Interleukin-1β	Control	12.81	(1.58)	12.19	(2.21)
(pg/mL)	A909	494.29	(150.28)	43.44	(7.91)
	GB112	263.73	(63.30)	34.76	(13.69)
	GB411	570.51	(162.67)	72.58	(27.42)
	GB37	275.85	(115.52)	26.93	(8.97)
	GB590	377.04	(189.17)	40.36	(13.81)
Interleukin-1Ra[c]	Control	313.30	(45.91)	701.20	(95.33)
(pg/mL)	A909	1743.00	(412.10)	972.70	(184.80)
	GB112	1061.00	(392.20)	970.80	(149.00)
	GB411	698.20	(88.49)	783.00	(165.50)
	GB37	713.50	(85.32)	718.60	(116.70)
	GB590	1088.00	(410.10)	801.80	(102.00)
Interleukin-6	Control	20.79	(3.51)	14.79	(3.38)
(ng/mL)	A909	122.17	(47.74)	28.82	(4.18)
	GB112	65.30	(8.42)	21.07	(3.53)
	GB411	70.09	(8.81)	23.52	(3.89)
	GB37	49.36	(8.94)	23.12	(5.80)
	GB590	48.61	(11.08)	24.14	(5.47)
Interleukin-8	Control	178.50	(29.76)	135.39	(25.61)
(ng/mL)	A909	437.32	(46.36)	164.41	(23.41)
	GB112	320.46	(33.60)	159.71	(24.90)
	GB411	364.63	(29.78)	158.51	(23.76)
	GB37	402.03	(73.84)	155.84	(31.54)
	GB590	259.46	(33.51)	167.21	(23.65)
Interleukin-10	Control	67.09	(29.40)	71.94	(34.27)
(pg/mL)	A909	469.17	(197.94)	121.73	(49.21)
	GB112	202.91	(84.98)	127.82	(67.40)
	GB411	143.37	(41.18)	59.70	(24.36)
	GB37	364.18	(147.59)	79.66	(45.02)
	GB590	162.66	(41.98)	72.84	(33.53)
Tumor Necrosis Factor-α	Control	49.90	(10.48)	31.83	(5.89)
(pg/mL)	A909	1818.28	(349.67)	313.24	(120.14)
	GB112	1113.46	(335.49)	290.01	(206.54)
	GB411	1164.23	(260.04)	229.98	(130.01)

	GB37	597.00	(124.01)	70.27	(16.35)
	GB590	1200.71	(329.05)	240.03	(111.54)

[a] Limit of detection(LOD) was 2.91 pg/mL for Interleukin-1α.

[b] Data are presented as mean (SEM). N=13 membranes for Control, A909, GB112, and GB411 treatment groups, N=7 membranes for the GB37 and GB590 treatment groups. Cytokines were measured 24 h after incubation with or without GBS ($1x10^6$ CFU/mL).

[c]IL-1α and IL-1Ra were measured in only measured in a subset of samples N=4 membranes for Control, A909, GB37, GB112, GB411, and GB590.

Table 4.3. P-Values for Linear Mixed Model Pairwise Comparisons of GBS Strains by Cytokine.

Interleukin-1β

	A909	GB37	GB112	GB411
A909				
GB37	**< 0.001**			
GB112	**0.003**	0.168		
GB411	0.642	0.151	**0.016**	
GB590	**0.027**	0.246	0.559	0.564

Interleukin-6

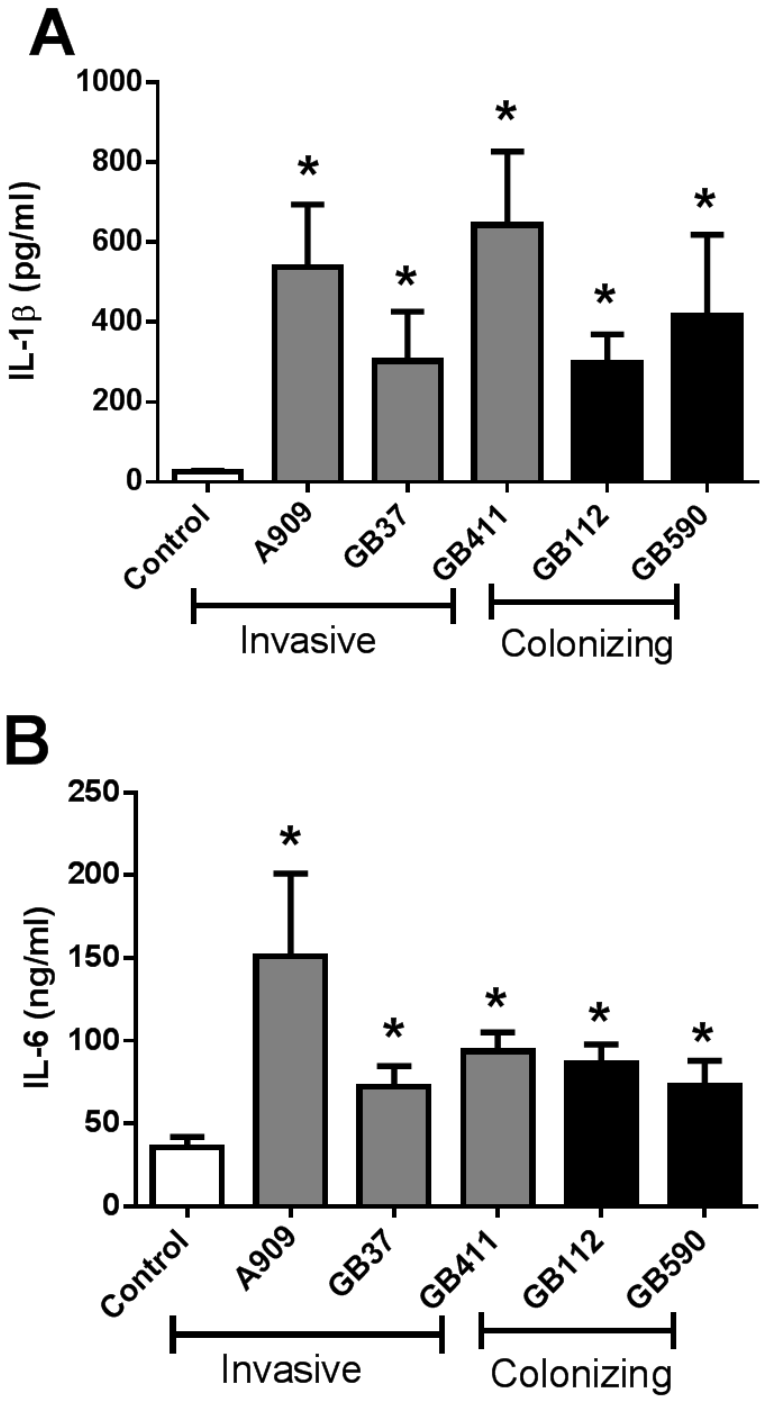
	A909	GB37	GB112	GB411
A909				
GB37	**0.028**			
GB112	0.148	**0.044**		
GB411	0.316	0.116	0.588	
GB590	**0.045**	0.931	**0.036**	0.136

InterleukinL-8

	A909	GB37	GB112	GB411
A909				
GB37	0.208			
GB112	**0.004**	0.379		
GB411	0.193	0.666	0.068	
GB590	**0.004**	0.214	0.411	0.411

Tumor Necrosis Factor-α

	A909	GB37	GB112	GB411
A909				
GB37	**< 0.001**			
GB112	**< 0.001**	**0.006**		
GB411	**< 0.001**	0.392	0.848	
GB590	**0.015**	**0.025**	0.658	0.074


A
IL-1β (pg/ml)
1000
800
600
400
200
0
Control
A909
GB37
GB411
GB112
GB590
Invasive
Colonizing
B
IL-6 (ng/ml)
250
200
150
100
50
0
Control
A909
GB37
GB411
GB112
GB590
Invasive
Colonizing

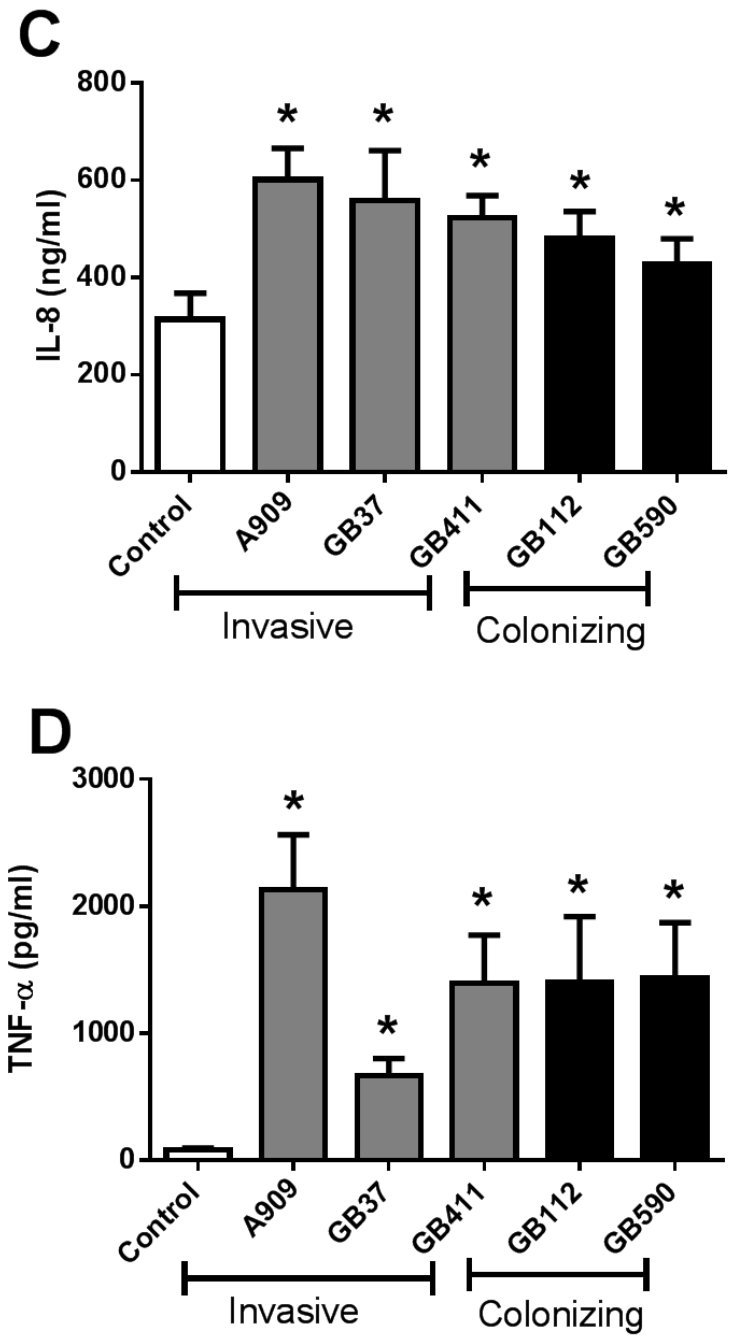


Figure 4.1. Cytokine release into medium by extraplacental membranes treated with GBS. Total cytokine release (from both the choriodecidua and amnion compartments) into medium by extraplacental membranes in transwell cultures with invasive (dark columns) and colonizing GBS (light columns) exposure on the choriodecidual side. IL-1β (A), IL-6 (B), IL-8 (C), and TNF-α (D) in the medium were measured by ELISA. Columns are mean ± SEM (N=13 women for control, A909, GB112, and GB411 treatment groups; N=7 women for GB37 and GB590 treatment groups). The asterisk (*) represents significant differences compared to medium only control analyzed by a linear mixed model ($P < 0.05$). Pairwise comparisons are reported in Table 3.

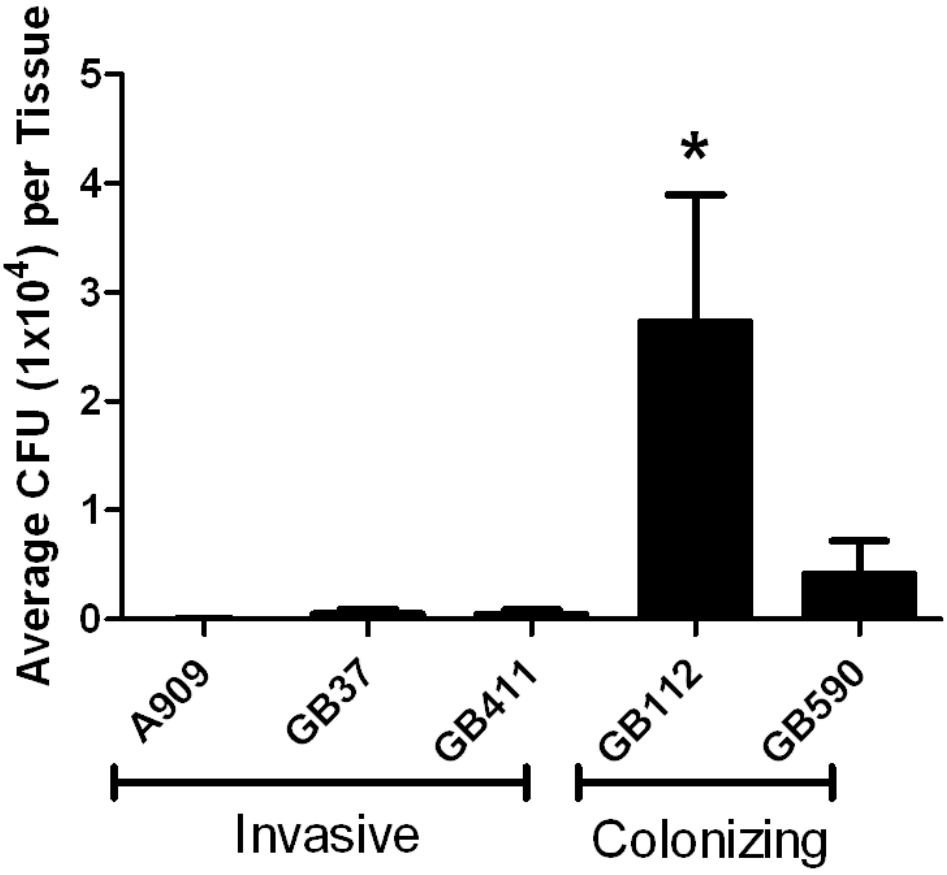


Figure 4.2. Recovered GBS from extraplacental membranes.
Recovered GBS (expressed as CFU) from homogenized tissue punches of extraplacental membranes following coculture with 1 x 10^6 CFU/mL GBS inoculants for 24 h. Columns are mean ± SEM. (N=3 women). The asterisk (*) represents significant differences compared to A909, GB37, and GB411 when compared by Tukey's post-hoc test following ANOVA ($P < 0.05$).

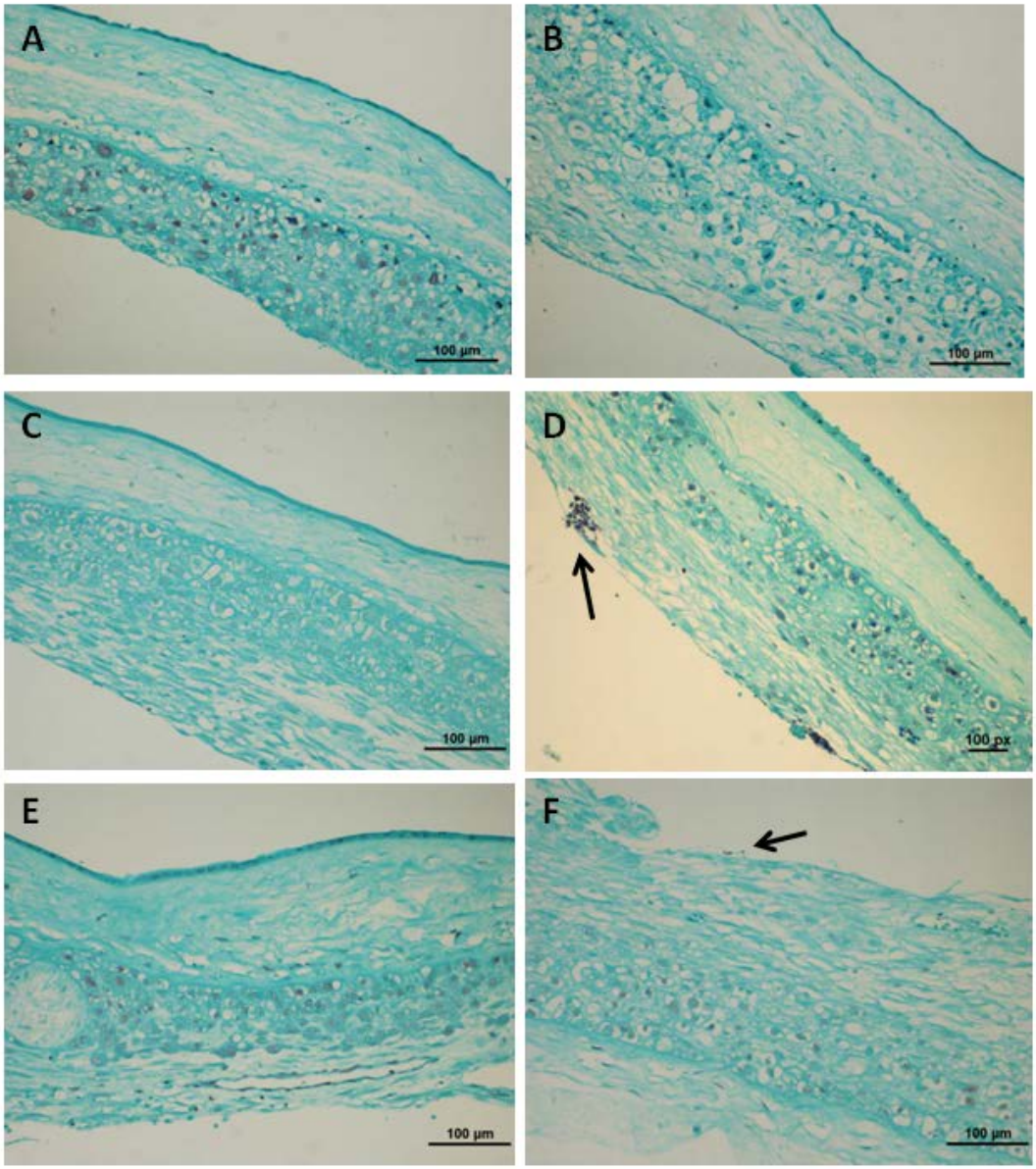


Figure 4.3. Hucker-Twort tissue gram straining in human extraplacental membranes. Membranes were cocultured with GBS added to the choriodecidual chamber of the transwell cultures (20x). Representative images for no GBS control (A), A909 (B), GB37 (C), GB112 (D), GB411 (E), and GB590 (F). Arrows indicate GBS clusters.

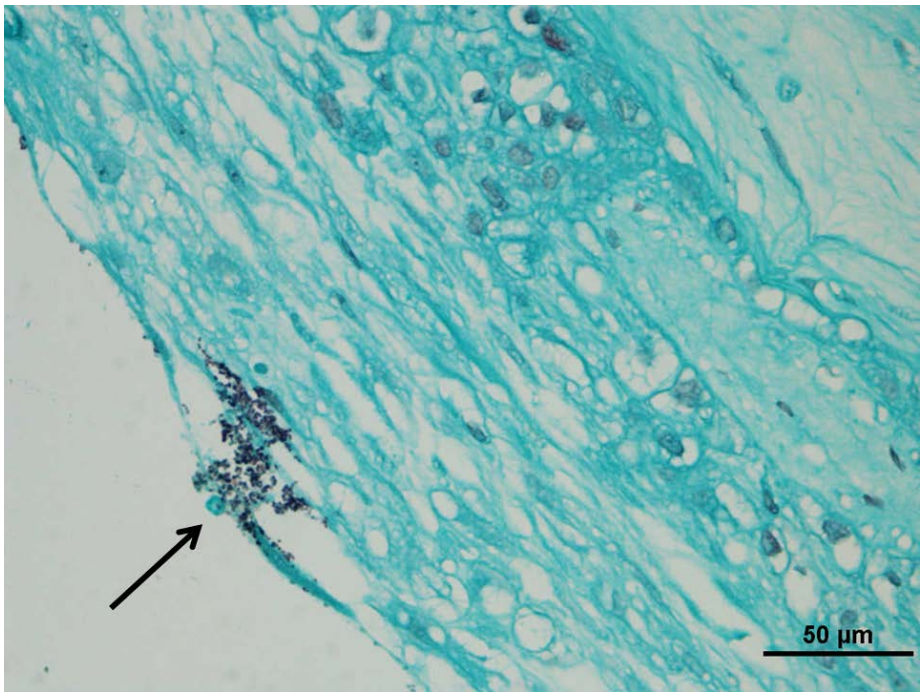


Figure 4.4. Higher magnification image of Hucker-Twort tissue gram stain.
Human extraplacental membranes were cocultured with GBS strain GB112 added to the choriodecidual chamber of the transwell cultures (40x). Arrow indicates GBS cluster identified on the choriodecidual side of the membrane.

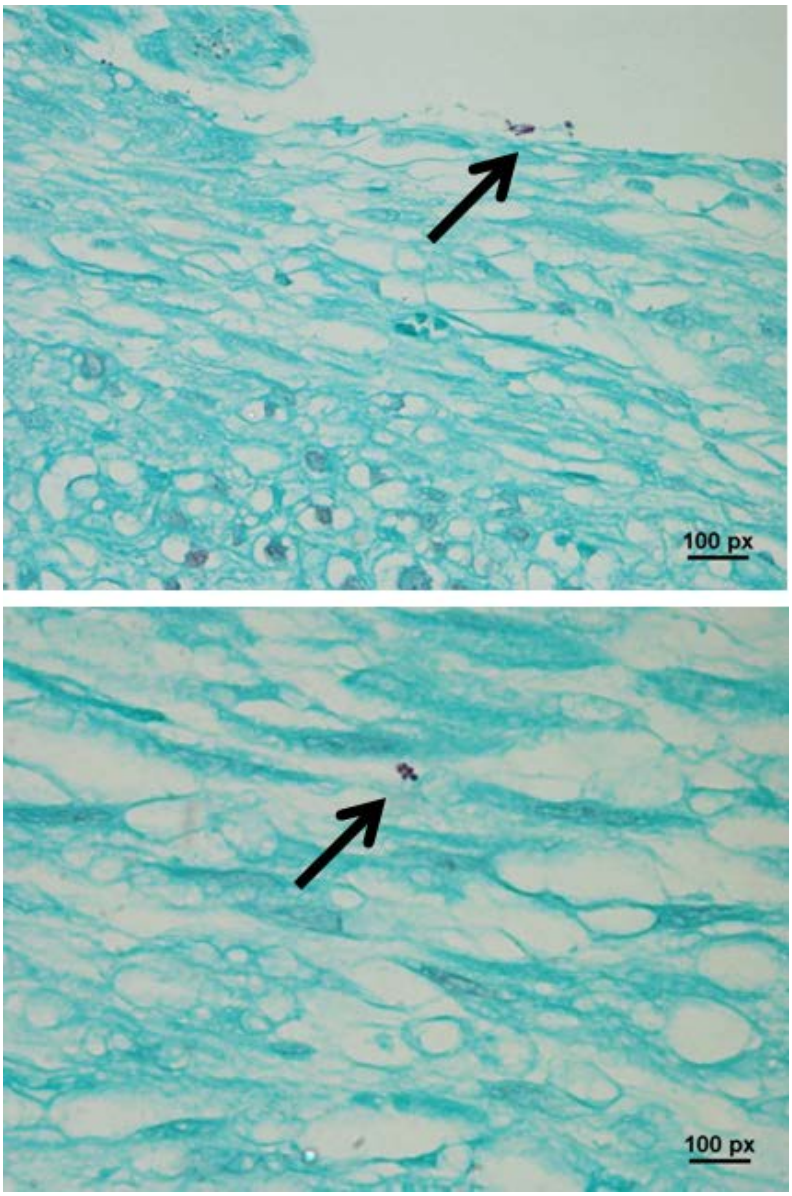


Figure 4.5. Higher magnification of Hucker-Twort tissue gram stain.
Human extraplacental membranes were cocultured with GBS strain GB590 added to the choriodecidual chamber of the transwell cultures. 40x (A) and 100x (B). Arrows indicate GBS clusters identified on the choriodecidual side and within the membranes.

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CHAPTER 5. THE TRICHLOROETHYLENE METABOLITE DICHLOROVINYL CYSTEINE INHIBITS PATHOGEN-STIMULATED TNF-α IN HUMAN EXTRAPLACENTAL MEMBRANES IN VITRO

Abstract

Problem: Infection in pregnancy increases risk for adverse birth outcomes such as preterm birth, neonatal sepsis, and neurodevelopmental disorders. Despite this clinical significance, the mechanisms by which pathogens interact with host tissues of the female gravid reproductive tract are poorly understood. Even less studied is the potential for environmental chemicals to modify susceptibility of gestational tissues to infection. TCE is a widespread environmental contaminant implicated in reproductive and immune system toxicity. Here we test our hypothesis that a bioactive metabolites of trichloroethylene (TCE), decreases the innate immune response of extraplacental membranes.

Method of Study: Full thickness term human extraplacental membranes were punched and cultured for 4, 8, and 24 h with the TCE metabolites trichloroacetate (TCA) or dichlorovinyl cysteine (DCVC) in the absence or presence of lipoteichoic acid (LTA; gram positive cell wall component) or lipopolysaccharide (LPS; gram negative cell membrane component) to simulate an infection. In addition, full thickness human extraplacental membranes were mounted on transwell inserts and cocultured with DCVC and live Group B *Streptococcus* (GBS) for 24 h. Cytokines from the medium were determined by ELISA. TNF-α mRNA expression was measured by PCR.

Results: LTA and LPS significantly increased TNF-α secretion from extraplacental membranes punch and transwell cultures ($p \leq 0.05$). DCVC (5-50 μM) significantly inhibited LTA- and LPS-stimulated TNF-α release from tissue punches of extraplacental membranes after 24 h ($p \leq 0.05$). Both TNF-α mRNA expression and protein secretion were inhibited as early as 4 h after initiating co-treatment of tissue punches with DCVC and LTA. DCVC also inhibited GBS-stimulated IL-1β, IL-8, and TNF-α release from extraplacental membranes in transwell cultures ($p \leq 0.05$). Among the cytokines analyzed, DCVC had the greatest inhibition on pathogen-stimulated TNF-α release. In contrast, TCA (up to 500 μM) did not inhibit LTA-stimulated cytokine release from tissue punches.

Conclusions: DCVC inhibits pathogen-stimulated TNF-α mRNA and protein expression. Because cytokines are important mediators for responding to infectious organisms and TNF-α is specifically involved in neutrophil recruitment and stimulation of phagocytosis in macrophages, these findings suggest that environmental contaminant exposure could potentially modify susceptibility to and severity of infection during pregnancy. In addition, this study demonstrates a new model for studying toxicant-pathogen interactions in the extraplacental membranes.

Introduction

The primary pathway of intrauterine infection is via the ascending pathway, progressing from the vagina and across the extraplacental membranes to enter the amniotic cavity (Romero, Mazor et al. 1991, Goldenberg, Hauth et al. 2000). Other pathways through maternal-fetal blood exchange in the placenta contribute to intrauterine infection to a lesser extent. Thus, the extraplacental membranes form a critical barrier to infection of the gestational compartment. The innate

immune response includes cytokines and antimicrobial peptides that are important components of host defense against microbial infection.

Although intrauterine infection, including infection of the extraplacental membranes, is recognized as a leading etiological factor associated with adverse birth outcomes (Dammann and Leviton 1997, Goldenberg, Hauth et al. 2000, Pararas, Skevaki et al. 2006, Romero, Espinoza et al. 2007, Goldenberg, Culhane et al. 2008), the ability of common environmental contaminants to modify susceptibility of the female reproductive tract to infection during pregnancy has scarcely been explored. Indeed, recent articles have highlighted the need for increased research on toxicant-pathogen interactions, in general (Birnbaum and Jung 2010, Dietert, DeWitt et al. 2010, Feingold, Vegosen et al. 2010). Given the likelihood that pregnant women are exposed to pollutants in their workplaces, homes, and outdoor environment, limited knowledge of toxicant actions on host defense of extraplacental membranes (also known as gestational and maternal-fetal membranes) to infection is a critical barrier to understanding the mechanisms of adverse pregnancy outcomes.

Trichloroethylene (TCE) is colorless volatile industrial solvent primarily used for metal degreasing. TCE is also a widespread environmental contaminant of concern, ranked #16 on the ATSDR 2011 Priority List of Hazardous Substances (ATSDR 2011) and recently reclassified as a known human carcinogen by the US EPA (U.S. Environmental Protection Agency 2011). Recent reports show increased mortality, decreased bacterial clearance and decreased alveolar phagocytosis in rodents co-treated with TCE and *Streptococcus zooepidemicus* (Aranyi, O'Shea et al. 1986, Selgrade and Gilmour 2010). Metabolism appears to be necessary for at least some of

TCE's immunomodulatory activity *in vivo* (Griffin, Gilbert et al. 2000). Most absorbed TCE is rapidly metabolized via one of two irreversible pathways (Chiu, Okino et al. 2006). The first pathway involves cytochrome P450-mediated oxidation, with trichloroacetic acid (TCA) the major bioactive metabolite (Bradford, Lock et al. 2011). The second TCE metabolic pathway involves conjugation with glutathione to form *S*-(1,2)-dichlorovinyl glutathione (DCVG), which is further metabolized to the bioactive metabolite *S*-(1,2)-dichlorovinyl-L-cysteine (DCVC) (Kim, Kim et al. 2009). TCA and DCVC have been detected in serum of exposed rodents (Lash, Putt et al. 2006, Kim, Collins et al. 2009, Bradford, Lock et al. 2011) and humans (Lash, Putt et al. 1999). The placenta is a highly perfused organ that would readily be exposed to circulating TCE and metabolites. In addition, the human placenta expresses key enzymes necessary to initiate TCE metabolism to bioactive forms, including CYP2E1, which is important for TCA formation (Hakkola, Raunio et al. 1996, Collier, Tingle et al. 2002) and glutathione-*S*-transferase (GST) (Nogutii, Barbisan et al. 2012), needed to generate DCVC.

Here we utilized human extraplacental membranes cocultured with LTA, LPS, and GBS to test our hypothesis that trichloroethylene, through its metabolites, decreases the innate immune response of extraplacental membranes.

Materials and Methods

Reagents and Materials

TCA was from Sigma-Aldrich (St. Lewis, MO). DCVC was synthesized at the University of Michigan Medicinal Chemistry Core Synthesis Lab. The GBS strain used in this study (A909, construct RS020, a gift from Amanda Jones, University of Washington), was initially isolated

from a septic newborn (Lancefield, McCarty et al. 1975). GBS was grown at 37 ºC in culture using Todd Hewitt Broth (THB, Becton-Dickinson, Franklin Lakes, NJ) or on sheep's blood agar plates (Blood Agar Base #2, Remel, Lenexa, KS and BBL defibrinated sheep blood, Franklin Lakes, NJ) with 5 μg/mL erythromycin (Hemostat Labs, Dixon, CA). Media, buffers, fetal bovine serum (FBS; catalog #10438), 0.25% trypsin-EDTA (catalog #25200), and penicillin/streptomycin (pen/strep; catalog #15140) were from GIBCO (Grand Island, NY). Lipoteichoic acid (LTA) from *Staphylococcus aureus* was from Invivogen (San Diego, CA). Lipopolysaccaride (LPS) from *Salmonella typhimurium* was from List Biological Laboratories (Campbell, CA).

Culture of Extraplacental Choriodecidual Membranes

Human extraplacental membranes were collected from healthy, non-smoking, singleton pregnancies undergoing scheduled cesarean delivery prior to onset of labor at the University of Michigan Birth Center as previously described (Boldenow, Jones et al. 2013). The University of Michigan Institutional Review Board approved this research (IRBMED#HUM0013915).

Immediately following delivery, the membranes were transported to the research laboratory in Dulbecco's phosphate-buffered saline (DPBS). Membranes were rinsed with medium and blood clots removed. Membranes were then punched using a 12-mm biopsy punch and placed in 12-well plates with 1 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% FBS and pen/strep. Cultures were incubated at 37 ˚C and 5% CO_2. After 4 h, the medium was changed, and cultures were incubated and additional 24 h. LTA and LPS treatments were initially dissolved in water and diluted in DMEM/1% FBS. Following acclimation, the medium

of the extraplacental membranes punches was replaced with exposure medium of DMEM/1%FBS with LTA (1 µg/mL), LPS (100 ng/mL), or no LTA or LPS (controls). Tissue punch experiments were conducted in triplicate using extraplacental membranes from seven or eight women.

Next, to better simulate the ascending uterine infection model, extraplacental membranes were cultured in a two-compartment transwell system as described previously published (Zaga, Estrada-Gutierrez et al. 2004, Thiex, Chames et al. 2009). The membranes were then mounted on sterile transwell frames that had no synthetic membrane (gift from Corning, NY) and held in place with sterile latex elastic bands (ORMCO, Orange, CA). The membranes were affixed with the choriodecidua facing the inner chamber of the transwell and the amnion facing the outer chamber. The transwell inserts with extraplacental membranes were placed in wells of 12-well culture plates containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% FBS and pen/strep. The smaller inner chamber had 0.5 mL medium and the larger outer chamber had 1.5 mL medium. Cultures were incubated at 37 ˚C and 5% CO_2. After 4 h, the medium was exchanged for DMEM/1% FBS without antibiotics. GBS in early exponential growth phase was diluted with DMEM/1% FBS to $1x10^6$ colony forming units/mL (CFU/mL). Inoculant concentrations were validated by overnight growth on 5% sheep blood agar with erythromycin. Following a 24-h acclimation, the medium in the inner choriodecidua-facing chamber was replaced with fresh DMEM/1%FBS medium containing 0.5 mL GBS inoculant ($1x10^6$ CFU/mL), DCVC (1, 5, or 10 µM), DCVC (1, 5, or 10 µM) + GBS ($1x10^6$ CFU/mL). Medium in the outer amnion compartment was replaced with fresh DMEM/1%FBS containing DCVC (1, 5, or 10 µM). Control cultures had fresh medium without DCVC or GBS. Transwell experiments

were conducted in duplicate or triplicate using extraplacental membranes from four women.

Cytokine ELISAs

Cytokine release from the extraplacental membranes punches into medium was measured by the University of Michigan Immunology Core using commercially available enzyme linked immunosorbant assays (ELISA; R&D Systems). The ELISA detection ranged from 2.91-2500 pg/mL for IL-1β,9.38-125,000 pg/mL for IL-6, 31.2-2000 pg/mL for IL-8, and 15.6-5000 pg/mL for TNF-α. Samples were diluted as necessary. Values are reported as pg or ng protein/mL medium.

RNA Extraction and PCR

Total RNA from tissue punches was extracted using RNeasy Plus Mini kits (Quigen, Valencia, CA). The cDNA was synthesized with 1 µg total RNA using the iScript cDNA Synthesis Kit (BioRad, Hercules, CA) according to manufacturer's instructions. Replicates within each woman were pooled. Forward and reverse primers used were: (TNF-α) 5'-GGAGGACGAACATCCAACCTT-3' and 5'-GGTTGAGGGTGTCTGAAGGAG; beta-2-microglobulin (B2M) 5'-TGGAGGCTATCCAGCGTACT-3' and 5'-CGGATGGATGAAACCCAGACA-3'. Quantitative RT-PCR reactions were performed on a Bio-Rad CFX Connect Real-Time System according to SsoAdvanced SYbR Green Supermix directions (BioRad, Hercules, CA). The following conditions were used for PCR: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 15 sec at 95 °C, 5 sec at 60 °C. Signal intensities of target genes were quantified using standard curves and normalized to the signal of

β-microglobulin using Bio-Rad CFX manager software. All samples were run in triplicate. Data were analyzed using CFX software (BioRad, Hercules, CA).

GBS Viability

GBS viability in the presence of DCVC and TCA was assessed using the AlamarBlue assay (Serotec) according to manufacture directions and previously published methods (Chen, et al). Briefly, GBS was grown to exponential log phase at 37 °C with shaking in THB and diluted to $1x10^6$ CFU/mL in DMEM medium supplemented with 1% FBS. Bacterial suspension, DCVC or TCA, and AlamarBlue dye (10%) were incubated in 96-well plates at 37 °C. Fluorescence was measured after 8 h on a SpectraMax $M2^e$ plate reader (excitation 560nm, emission 590nm) and percent cell viability was determined. Three independent experiments were performed with five replicates per treatment.

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). ANOVAs with Tukey's post hoc test were performed. Data were considered significant if the p-value was < 0.05.

Results

DCVC Effects on LTA-Stimulated and LPS-Stimulated TNF-α Release from Tissue Punch Cultures of Extraplacental Membranes

To investigate TCE metabolite-pathogen interactions on cytokine release, human extraplacental membranes were cocultured with the TCE metabolites TCA or DCVC in the absence or presence

of LTA or LPS for 24 h. Exposure to TCA or DCVC alone did not significantly increase cytokine release from extraplacental membranes, whereas both LTA and LPS significantly increased TNF-α release from approximately 20 pg/mL to 830 pg/mL and 4300 pg/mL respectively (Figure 5.1; $P < 0.05$). Nonetheless, 5 μM DCVC significantly inhibited LTA-stimulated TNF-α release by 5 fold. In addition, 10 μM DCVC (the lowest concentration tested) significantly inhibited LPS-stimulated TNF-α release with a 21-fold reduction. To investigate the toxicodynamics of DCVC inhibition of pathogen-stimulated TNF-α release, extraplacental membranes were cocultured with DCVC or LTA for 4, 8, and 24 h. LTA stimulated a robust increase in TNF-α by 4 h, and 10 μM DCVC inhibited the LTA-stimulated response at 4 h (Figure 5.2; $P < 0.05$). To further probe the mechanism by with DCVC inhibited pathogen-stimulated TNF-α release, RNA was extracted from tissue punches treated for 4 h with 10 μM DCVC, LTA, LTA + 10 μM DCVC, or no treatment (control). Similar to the protein results, LTA-stimulated TNF-α mRNA expression was inhibited by 4 h of exposure to DCVC by approximately 8 fold (Figure 5.3; $P < 0.05$). In contrast, the TCE metabolite TCA did not inhibit LTA-stimulated cytokines (Figure 5.4) at up to 500 μM.

DCVC Effects on GBS-Stimulated Release of Pro-Inflammatory Cytokines from Extraplacental Membranes in Transwell Cultures

Extraplacental membranes in transwell culture released increased amounts of IL-1β (66 fold), IL-8 (4.7 fold), and TNF-α (54 fold) into the medium of the choriodecidual compartment after 24 h of exposure to GBS (Figure 5.5; $P < 0.05$). Although, exposure to GBS appeared to increase IL-6 slightly, the data were not statistically different. In addition GBS-stimulated IL-1β and TNF-α release was completely inhibited by coculture with 5 μM and 10 μM DCVC. GBS-stimulated IL-

8 release was inhibited 3.3 fold with 5 µM DCVC and 3.5 fold with 10 µM DCVC. Membranes exposed to GBS with 1 µM DCVC increased IL-6 secretion in the choriodecidual compartment compared to medium only control. However, at higher concentrations, DCVC (5 µM and 10 µM) and GBS co treatment do not increase IL-6. To determine if decreased cytokines were a result of DCVC or TCA killing the bacteria directly, GBS was incubated with DCVC or TCA for 8 h and viability was assessed. DCVC only decreased cell viability at 50 µM, a higher concentration than we used for the punch and transwell tissue culture experiments (Figure 5.6; $P < 0.05$).

Discussion

Despite the recent attention to toxicant-pathogen interactions in the literature, few studies have been published examining the role of toxicant-pathogens interactions during pregnancy. Cytokines are essential for recruitment and function of immune cells during infection. An inhibition of cytokine secretion during infection could result in loss of immune cell recruitment and increased severity of infection. Here, we show for the first time that the TCE metabolite DCVC is capable of inhibiting pathogen-stimulated cytokine (IL-1β, IL-8, and TNF-α) release in a concentration-dependent manner. In addition, we show that this happens both at the protein and mRNA expression level as early as 4 h.

TCE is a common drinking water contaminant (ATSDR 2011) implicated in developmental, reproductive and immune system toxicity (U.S. Environmental Protection Agency 2011, Chiu, Jinot et al. 2013). Drinking water contaminated with TCE has been associated with increased neural tube defects, oral clefts, and cardiac defects in humans (Bove, Shim et al. 2002). TCE exposure during pregnancy is associated with intrauterine growth restriction (IUGR) (Windham, Shusterman et al. 1991). In addition to environmental exposures, infection is associated with

adverse birth outcomes. Given the likelihood that pregnant women are exposed to both environmental contaminants such as TCE and infectious agents, few studies have elucidated toxicant-pathogen interactions during pregnancy. Our study is among the first to explore toxicant-pathogen interactions in a relevant target tissue: the extraplacental membranes. Although the extraplacental membranes model is useful for exploring mechanisms of toxicity *in vitro*, our findings of DCVC suppression of pathogen-stimulated cytokines, warrant further validation in additional pregnant animal models.

Studies in humans and mice implicate a role for TCE in some autoimmune diseases (Cooper, Makris et al. 2009). Workers exposed to TCE have altered cytokine levels including increased IL-2, increased IFN-γ, and reduced IL-4 cytokine levels compared to non-exposed workers (Iavicoli, Marinaccio et al. 2005). Mice exposed to TCE had increased IL-2, TNF-α, and IFN-γ secretion from CD4+ T-cells (Blossom, Doss et al. 2008). TCE also affects the developing immune system in mice with increased delayed type hypersensitivity, suppressed SRBC-specific IgM production, decreased B220 cells in the spleen, and increased thymic T cell subpopulations (Peden-Adams, Eudaly et al. 2006). Despite evidence linking TCE to reproductive and immune outcomes, few if any studies have examined TCE and immune outcomes (such as cytokines) in pregnant women. Our study is one of the first studies to use a gestational tissue model to examine effects of TCE metabolites on cytokine secretion. In our study, neither DCVC nor TCA affected proinflammatory cytokine secretion directly; instead we saw pathogen stimulated suppression. Although the aforementioned studies did not report cytokine suppression, the response could have been overlooked. Without stimulating with an infectious agent we would not have observed suppression in our experiments.

TCE exposure increases susceptibility to bacterial lung infection in mice (Aranyi, O'Shea et al. 1986, Selgrade and Gilmour 2010). Mice co-treated with TCE and *Streptococcus zooepidemicus* show increased mortality, decreased bacterial clearance in the lung and decreased alveolar phagocytosis. Although the mechanisms for this immunosuppression during TCE and *Streptococcus zoopidemicus* infection have not yet been elucidated, this study, along with our data provide the first evidence of toxicant-pathogen interactions with trichloroethylene.

TNF-α has a diversity of functions including cellular growth and differentiation (Semenzato 1990) and proper regulation throughout gestation is essential (Bowen, Chamley et al. 2002, Bowen, Chamley et al. 2002). TNF-α is found to be expressed in placental villi during normal pregnancy and regulates apoptotic death in villous cytotrophoblasts (Yui, Garcia-Lloret et al. 1994). Moreover, TNF-α is involved in both neutrophil recruitment and macrophage phagocytosis (Lukacs, Strieter et al. 1995, Arcuri, Toti et al. 2009). Suppression of TNF-α inhibits nitric oxide (NO) production and microbial properties in macrophages (Oswald, Wynn et al. 1992, Leenen, Canono et al. 1994, Kolls, Xie et al. 1995, Xie, Kolls et al. 1995). Toxicant inhibition of TNF-α may have implications for pathways necessary for fighting infection especially in macrophages.

IL-1β has been implicated in antimicrobial peptide production (Singh, Jia et al. 1998, Moon, Lee et al. 2002, McDermott, Redfern et al. 2003, Pioli, Weaver et al. 2006). Antimicrobial peptides are critical components of the innate immune response to pathogens responsible for killing pathogens and recruiting immune cells (Zasloff 2002, Chen, Niyonsaba et al. 2005, Peschel and Sahl 2006). Recently, we found that GBS stimulation of extraplacental membranes increases IL-

1 (Chapter 3) which is responsible for increasing human β-defensin-2 (HBD-2) in amniotic epithelium (Boldenow, Jones et al. 2013). Here, we found that DCVC inhibits GBS stimulated IL-1β in the choriodecidual compartment. Although, not studied here, inhibition of IL-1β could be implicated in inhibiting antimicrobial peptide production and possibly increased susceptibility to infection.

Although TCE can be detected in rat fetuses following maternal exposure to TCE (Withey and Karpinski 1985), metabolites were utilized in the present study because most TCE is rapidly metabolized after absorption into the body (Chiu, Okino et al. 2006, U.S. Environmental Protection Agency 2011) Furthermore, TCE metabolites are significantly more biologically active than the parent compound (Chiu, Okino et al. 2006), and metabolism appears to be necessary for at least some of TCE's immunomodulatory activity (Griffin, Gilbert et al. 2000). DCVC has been detected in serum of exposed rodents (Lash, Putt et al. 2006, Kim, Collins et al. 2009, Bradford, Lock et al. 2011) and humans (Lash, Putt et al. 1999), and the human placenta expresses key enzymes necessary to initiate metabolism of TCE to this bioactive metabolite (Hakkola, Raunio et al. 1996, Collier, Tingle et al. 2002, Nogutii, Barbisan et al. 2012). LTA, LPS, and GBS stimulation of TNF-α release was inhibited at 5 μM DCVC. The DCVC concentrations used were at or near the lowest bioactive concentrations of DCVC reported in kidney cells (Xu, Papanayotou et al. 2008). Estimates for concentrations of DCVC relevant to human exposure are difficult to make due to insufficient data (Chiu, Okino et al. 2006). Humans exposed to levels as low as 100 ppm TCE in air transiently exhibit levels as high as 50 μM of the DCVC precursor DCVG in serum (Lash, Putt et al. 1999), although typical environmental exposures to TCE are <5 ppb in air (U.S. Environmental Protection Agency 2011). Although the concentrations we used

are higher than the average person in the United States would encounter through an ambient environmental exposure, they are within occupational exposure levels.

Human extraplacental membranes were incubated *ex vivo* with lipoteichoic acid (LTA), lipopolysaccharide (LPS), and live Group B Streptococcus (GBS; *S. agalactiae*) as model infectious agents. LTA and LPS are cell wall or membrane components of gram positive and gram negative bacteria, respectively, that are highly immunogenic in tissue cultures of human extraplacental membranes (Zaga, Estrada-Gutierrez et al. 2004, Thiex, Chames et al. 2009) and induce preterm birth in rodent models (Kajikawa, Kaga et al. 1998, Elovitz and Mrinalini 2004). Moreover, GBS is a gram positive bacterium associated with adverse pregnancy outcomes (Anderson, Simhan et al. 2007, Winn 2007, Verani, McGee et al. 2010) in women and able to induce preterm labor in subhuman primates (Gravett, Haluska et al. 1996). Despite interventions aimed at reducing GBS infections during pregnancy, GBS remains the leading cause of infection-related neonatal death and disease in the United States (Verani, McGee et al. 2010). We previously reported that human extraplacental membranes mount a robust defense to live GBS in tissue culture (Boldenow, Jones et al. 2013). In this study we also show that live GBS mounts a robust cytokine release in extraplacental membranes. Furthermore, we show that LTA and LPS, cell wall or membrane components from different microbial species mount similar cytokine responses compared to GBS. Despite the differences in molecular structure of the infectious agents used in this study, DCVC inhibition of pathogen-stimulated cytokines was similar between all three infectious agents (LTA, LPS, and GBS), suggesting the observed cytokine inhibition is not pathogen specific.

In summary, we have shown that the TCE metabolite DCVC inhibits pathogen-stimulated TNF-α mRNA expression and release in the extraplacental membranes. These findings have implications for mechanisms involved in innate immune dysregulation during pregnancy, potentially leading to increased susceptibility to infection. Future studies will focus on additional aspects of immune function such as macrophage function and antimicrobial peptide response. Current limited knowledge of mechanisms by which GBS acts on the host immune system during pregnancy and the potential toxicant interactions on host defense are critical barriers to the development of targeted preventive and therapeutic approaches to reduce adverse pregnancy outcomes. Findings from this project have the potential to expand current paradigms about GBS infections and environmental contaminant exposures that may put pregnant women at increased risk for intrauterine infection.

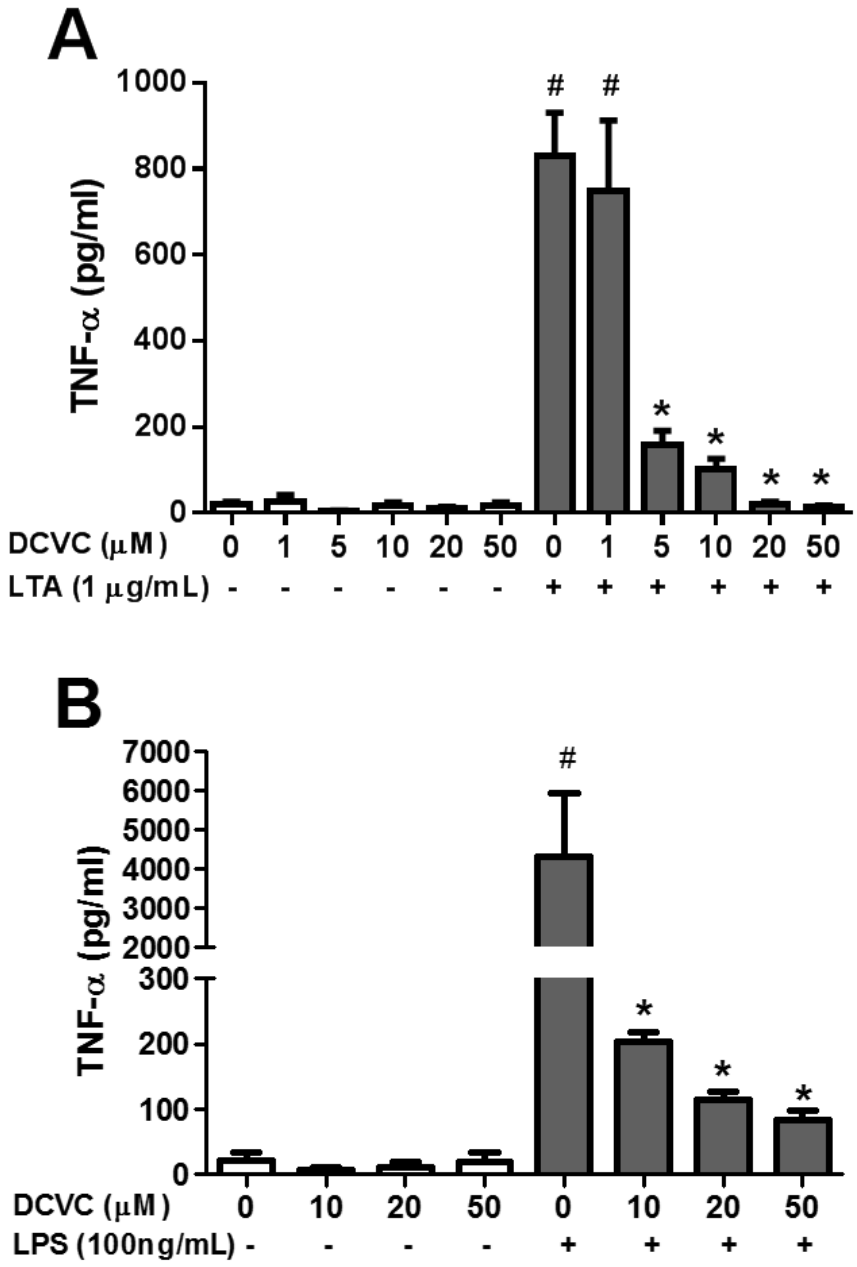


Figure 5.1. DCVC effects on LTA-stimulated and LPS-stimulated cytokines.
Punches of full thickness human extraplacental membranes were floated in culture with and without DCVC and LTA (A; 1 μg/mL) or LPS (B; 100 ng/mL) for 24 h, and then the medium was assayed by ELISA for TNF-α. Columns represent mean ± SEM; N=3-7 women. #, Significant differences compared to control (medium only). *, Significant compared to LTA or LPS alone by ANOVA with Tukey's post-hoc test ($p \leq 0.05$).

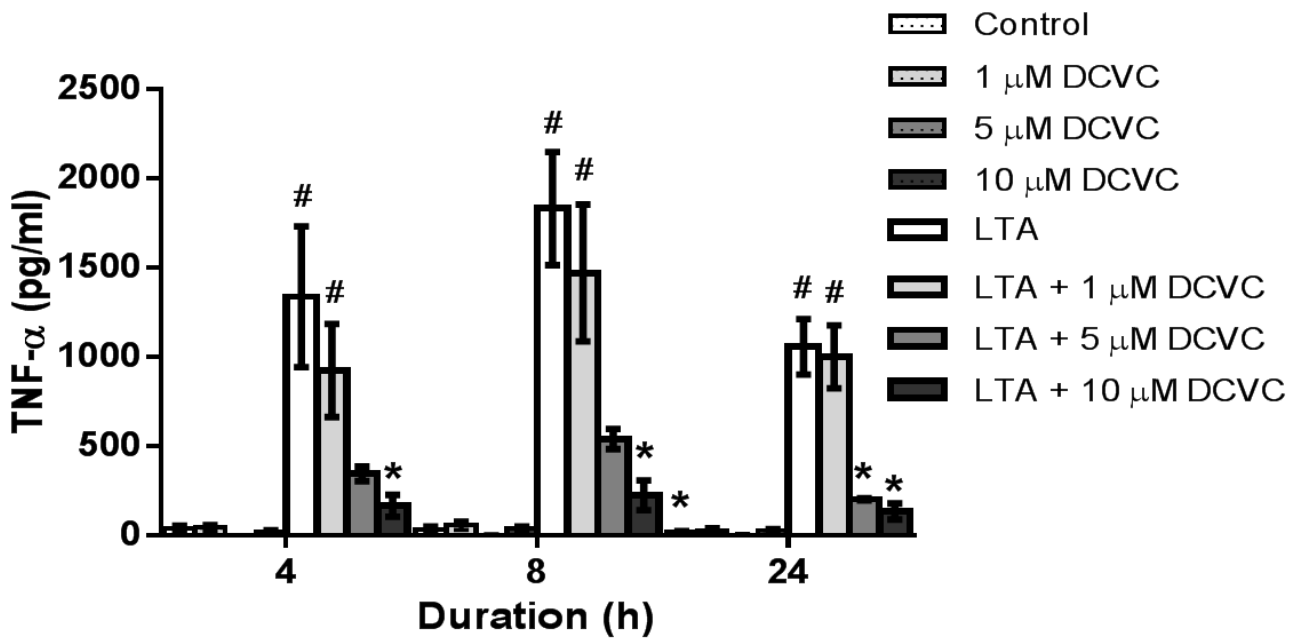


Figure 5.2. DCVC effects on LTA-stimulated TNF-α over time.
Punches of full thickness human extraplacental membranes were floated in culture with and without DCVC and LTA (1 μg/mL) for 4, 8, and 24 h, and then the medium was assayed by ELISA for TNF-α. Columns represent mean ± SEM; N=5 women. #, Significant differences compared to control (medium only). *, Significant compared to LTA alone by ANOVA with Tukey's post-hoc test ($p \leq 0.05$).

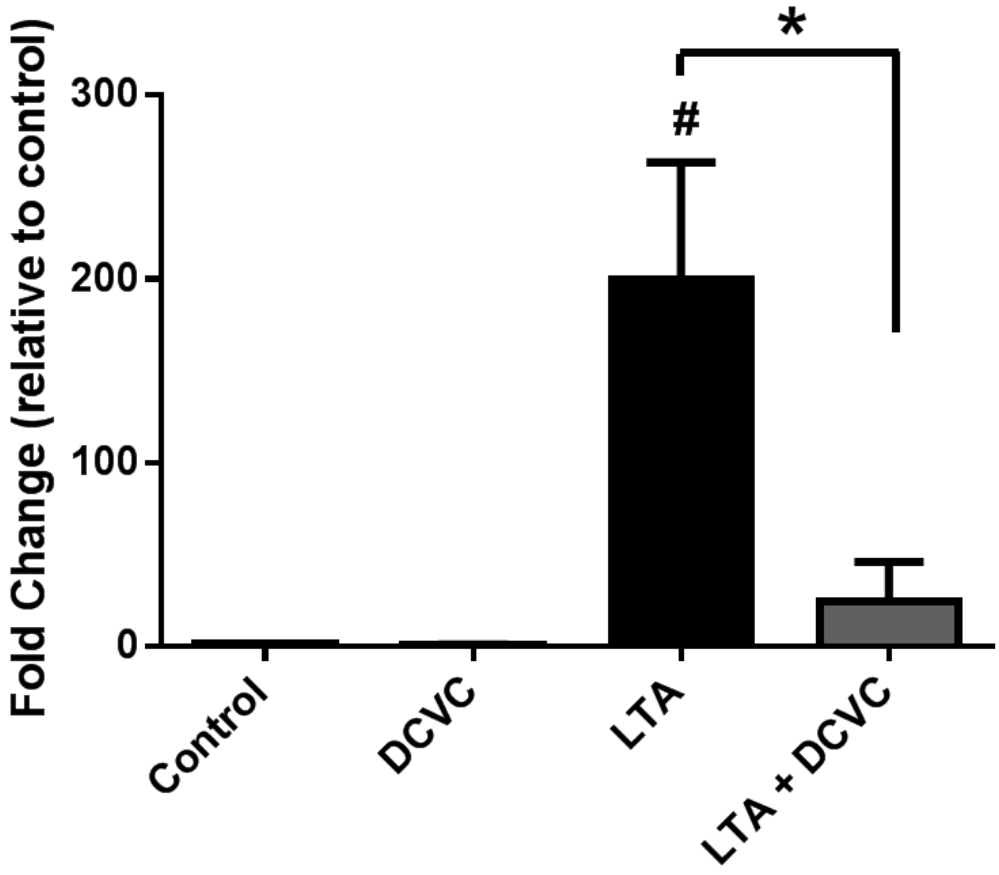


Figure 5.3. DCVC effects on LTA-stimulated TNF-α mRNA expression.
Punches of full thickness human extraplacental membranes were floated in culture with and without DCVC (10 µM) and LTA (1 µg/mL) for 4 h, and then the RNA was extracted and assayed by PCR for TNF-α expression and the data presented as fold change in mRNA expression compared to control. Columns represent mean ± SEM; N=3 women. #, Significant differences compared to control (medium only). *, Significant compared to LTA alone by ANOVA with Tukey's post-hoc test ($p \leq 0.05$).

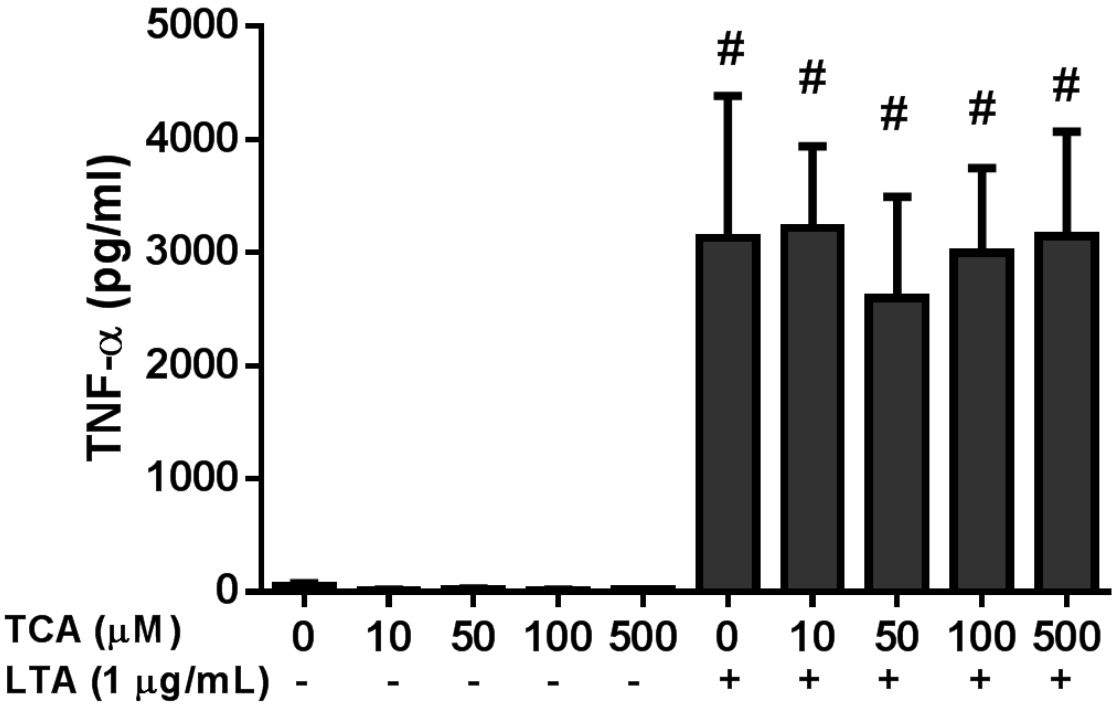
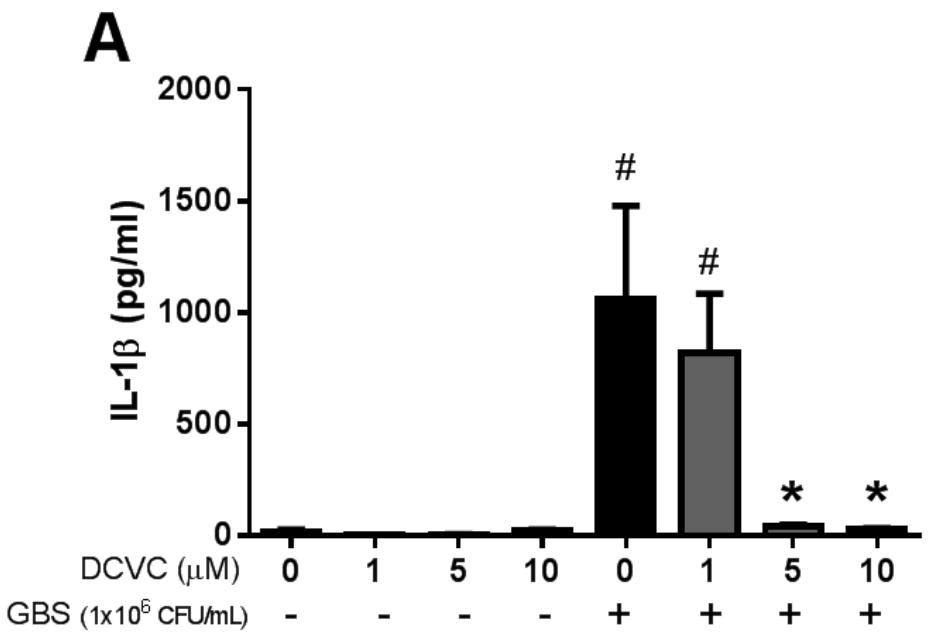


Figure 5.4. TCA effects on LTA-stimulated release of TNF-α.
Punches of full thickness human extraplacental membranes were floated in culture with and without TCA and LTA (1 μg/mL) for 24 h, and then the medium was assayed by ELISA for TNF-α. Columns represent mean ± SEM; N=3 women. #, Significant differences compared to control (medium only) by ANOVA with Tukey's post-hoc test ($p \leq 0.05$).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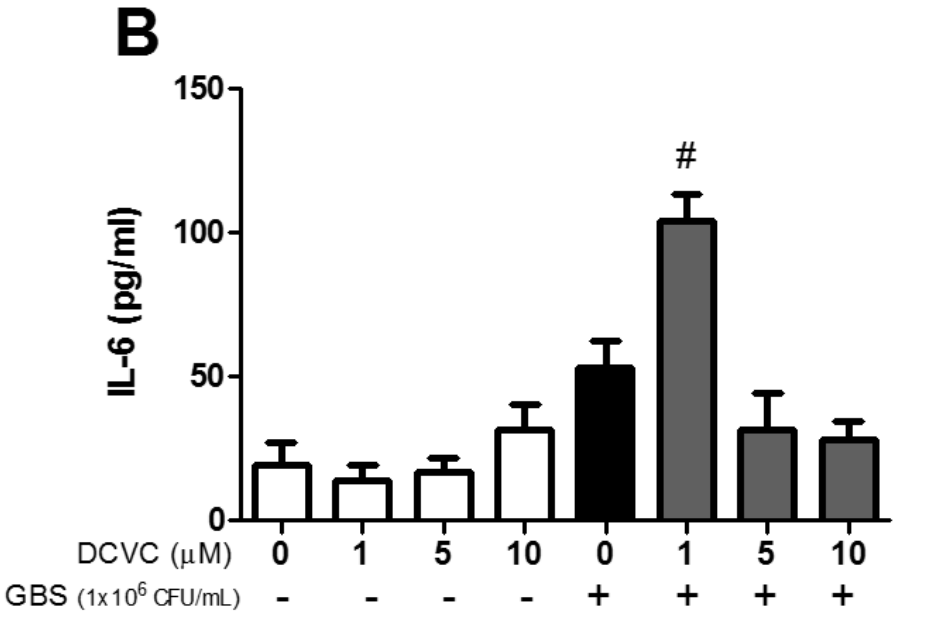
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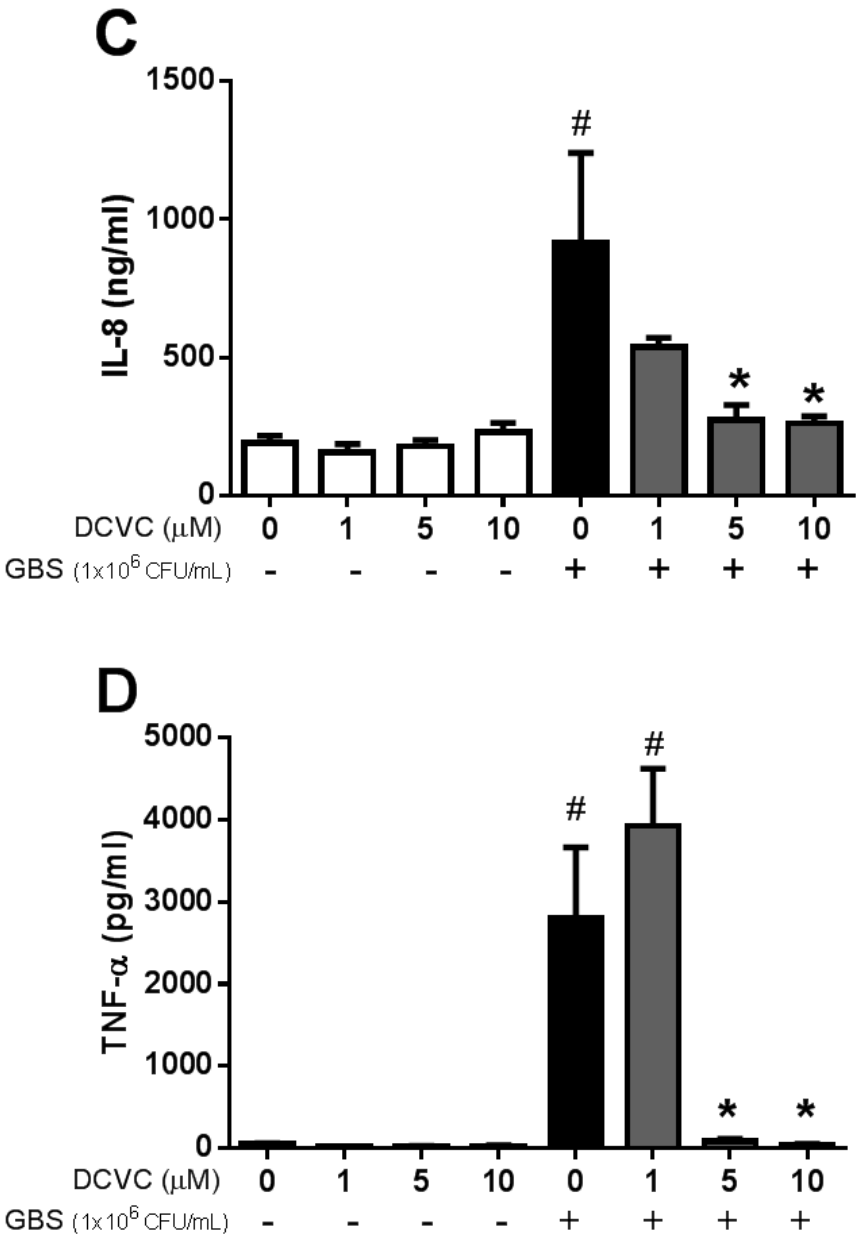

Figure 5.5. DCVC effects on GBS-stimulated cytokines.
DCVC effects on GBS-stimulated release of pro-inflammatory cytokines from extraplacental membranes in transwell cultures. Full thickness human extraplacental membranes were cocultured with live GBS for 24 h, and then the medium from the choriodecidua compartment was assayed by ELISA for IL-1β (A), IL-6 (B), IL-8 (C), and TNF-α (D). Columns represent mean ± SEM; N=5 women (N=2 women for 1 μM DCVC and N=3 for 1 μM DCVC + GBS). #, Significant differences compared to control (medium only). *, Significant compared to GBS alone by ANOVA with Tukey's post-hoc test ($p \leq 0.05$).

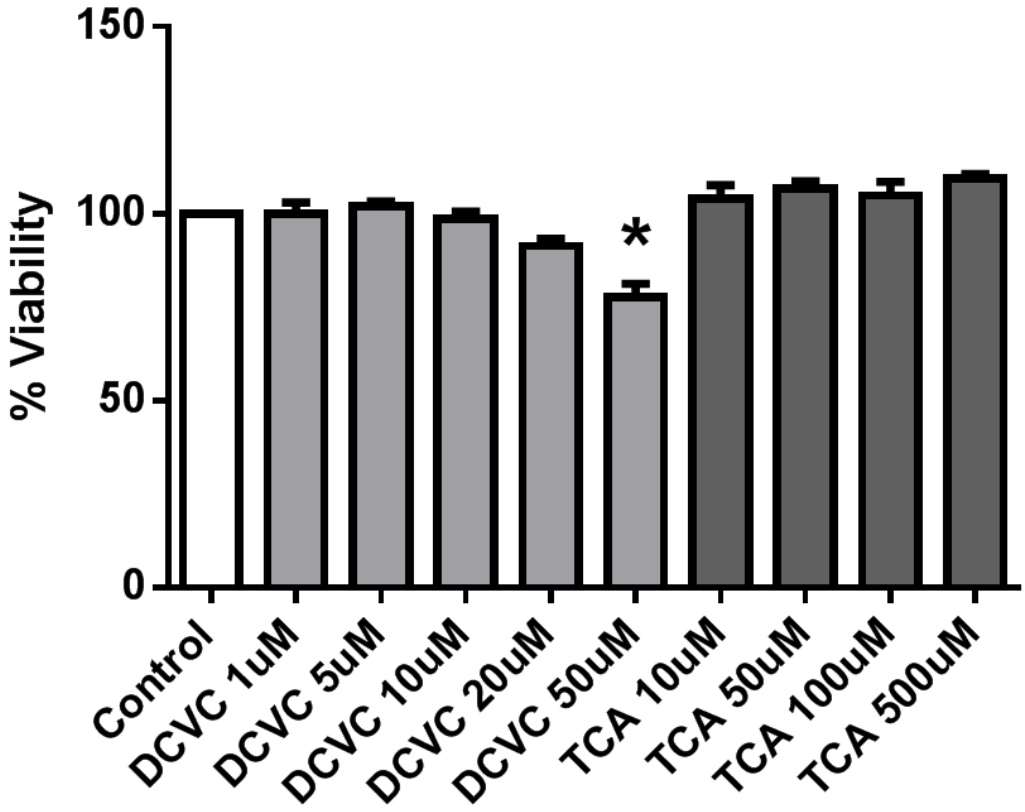

Figure 5.6. DCVC and TCA effects on GBS viability.
GBS was cultured with DCVC or TCA for 8 h and bacterial viability was assessed by AlamarBlue. Columns represent mean ± SEM; N=3 experiments, five replicates per experiment. *, Significantly decreased compared with nontreated control and other DCVC treatment groups using ANOVA with Tukey's post-hoc test ($p \leq 0.05$).

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CHAPTER 6. DISCUSSION

Group B *Streptococcus* (GBS) remains a serious public concern as the leading cause of infectious neonatal morbidity and mortality in the United States (Verani, McGee et al. 2010). Elucidating host-pathogen interactions is essential for understanding why this pathogen persists. This thesis explores mechanisms by which GBS stimulates host responses within the extraplacental membranes (Figure 6.1). GBS stimulates host response in immune cells (Wennekamp and Henneke 2008); however, few studies have examined GBS-host responses in the gestational tissues, which represent one the first tissues to interact with GBS during an ascending infection during pregnancy. Furthermore, this thesis explores toxicant-pathogen interactions using a bioactive metabolite of trichloroethylene as a model toxicant and GBS, LTA, or LPS as model infectious agents.

As the first line of defense, antimicrobial peptides represent an important part of the innate immune response, yet the mechanisms and implications of antimicrobial peptide expression in the extraplacental membranes remain understudied. Consistent with previous reports, we found multiple antimicrobial peptides constitutively expressed throughout the extraplacental membranes (Chapter 2). In addition, human beta defensin (HBD)-2 secretion into the medium increased in the choriodecidual and amnion compartments after GBS treatment on the choriodecidual side of the membranes. Of particular note, amnion epithelial cells appear to be the

major contributor of HBD-2 in the extraplacental membranes (Chapter 3). Although we did not conduct an exhaustive study, our data suggest that HBD-2 is likely the antimicrobial peptide responsible for killing GBS on the choriodecidual side of the membrane (Chapter 2). Immunohistochemical data (Figure 2.3) suggest HBD-2 is present across various cell types in the extraplacental membranes. However, our ELISA data (Appendix 3.5) suggest that amnion epithelial cells, not choriodecidual tissue, are primarily responsible for increases in HBD-2 during GBS infection. Constitutive expression of HBD-2 in the choriodecidual tissue may be enough to kill the GBS or HBD-2 may migrate through the tissue. To our knowledge, no studies have demonstrated HBD-2 migration through the tissue. However, cytokines transfer through extraplacental membranes (Kent, Sullivan et al. 1994) and HBD-2 secreted by the amnion epithelial cells may act similarly to cytokines. Alternatively, additional antimicrobial peptides could be responsible for killing GBS on the choriodecidual side of the membrane.

Though the link between infection and increased cytokine secretion has been previously established in gestational tissues (Gravett, Witkin et al. 1994, Menon, Swan et al. 1995, Griesinger, Saleh et al. 2001, Zaga, Estrada-Gutierrez et al. 2004, Menon, Peltier et al. 2009, Adams Waldorf, Gravett et al. 2011), the link between cytokine increases and antimicrobial peptides during infection has not been fully elucidated in the extraplacental membranes. Using novel culture methods of the extraplacental membranes, the present study is the first to identify a mechanism by which HBD-2 is increased in the amnion epithelial cells (Chapter 3). Our results demonstrate that IL-1α and IL-1β secreted from the choriodecidua are responsible for the increase in HBD-2 in the amnion epithelial cells. In addition, blocking IL-1α or IL-1β with either an IL-1β neutralizing antibody or IL-1Ra (receptor antagonist) inhibits the HBD-2 secretion from

the extraplacental membranes. This represents one potential mechanism by which antimicrobial peptides are regulated and is consistent with cell models from other tissue types such as lung (Tsutsumi-Ishii and Nagaoka 2003). GBS increases IL-1α, IL-1β, and IL-1Ra secretion from the extraplacental membranes (Chapter 4). Because IL-1α and IL-1β seem biologically redundant for increasing HBD-2 and because IL-1Ra inhibits the increase, it may be beneficial to look at ratios of the three secreted factors in the future to help define some of the intra-individual differences we observed. Here, we investigated mechanisms on only one antimicrobial peptide. However, as demonstrated in chapter 2, a host of antimicrobial peptides are present in the tissue and the mechanisms by which they act to help eliminate infection needs to be explored further.

Several literature reviews have suggested that TLRs are involved in antimicrobial peptide production in the extraplacental membranes (King, Paltoo et al. 2007, Horne, Stock et al. 2008), yet few studies have studied this directly. Although we showed that TLR-2 is present in various cells throughout the extraplacental membranes, LTA, a classic TLR-2 ligand, did not increase HBD-2 secretion in full thickness extraplacental membranes or in amnion epithelial cells (Chapter 3). TLR-4 is also present in the extraplacental membranes; however, we found that LPS, a classic TLR-4 ligand, also does not stimulate HBD-2 secretion. Furthermore, heat-killed GBS does not stimulate cytokine or HBD-2 secretion from the membranes. In contrast, live GBS stimulates HBD-2 secretion from whole membranes. Taken together, the data are consistent with a model wherein TLR stimulation alone is not sufficient to stimulate HBD-2 secretion. Additionally, we propose that cellular internalization is critical for activating the immune response, which has been demonstrated during GBS infection of mouse dendritic cells (Costa, Gupta et al. 2012).

Although not directly tested, we propose that data in this dissertation support a role for inflammasome activation during GBS infection. The inflammasome is a multi-protein complex composed in part of caspase-1, which is responsible for cleaving pro-IL-1β to the active form which can then be secreted from the cell (Latz 2010). To activate the inflammasome, a cell or tissue needs be stimulated by both a TLR ligand and an additional component such as ATP and calcium. Our data (Chapter 3) suggest that IL-1β secretion, which is dependent on the inflammasome, plays a particularly important role during GBS infection of the extraplacental membranes. In addition, Mitchell et al. has demonstrated increases in IL-1β in maternal and fetal plasma from GBS-colonized mothers (Mitchell, Brou et al. 2013). Caspase-1 has been shown to be significantly elevated in amniotic fluid from women who delivered preterm with an infection (Gotsch, Romero et al. 2008) compared to women who delivered preterm without an infection. Furthermore, the inflammasome has been shown to be important in IL-1β, but not TNF-α secretion during GBS infection of dendritic cells (Costa, Gupta et al. 2012). These studies highlight the complexity of the pathways involved in GBS-stimulated host defense and suggest that IL-1β and TNF-α have different secretion pathways. Inflammasome activation is likely needed for GBS-stimulated IL-1β secretion, whereas, TNF-α may rely more simply on TLR-2 binding. Future studies need to further examine the inflammasome during GBS infection of the extraplacental membranes.

GBS strains utilize different virulence mechanisms to infect host cells and tissues. Although, the virulence mechanisms by which GBS act on host cells remain to be elucidated, this is the first study to link the magnitude of different cytokine secretion from the extraplacental membranes to

GBS strain differences (Chapter 4). One particular virulence mechanism for future work may be biofilm formation. Biofilm formation is thought to be present in human infections and represents a significant problem in treatment because biofilms are physiologically distinct from planktonic (or free floating) cells and more difficult for antibiotic treatment (Costerton, Stewart et al. 1999). GBS forms biofilms (Kaur, Kumar et al. 2009, Borges, Silva et al. 2012, Ho, Li et al. 2013), and previous studies have identified biofilm formation in the amniotic compartment (Kusanovic, Espinoza et al. 2007, Romero, Kusanovic et al. 2007, Romero, Schaudinn et al. 2008). Our initial finding that extraplacental membranes were capable of killing GBS (strain A909; Chapter 2) appears to be GBS strain specific (Chapter 4). We identified clusters of GBS strain GB112 on the surface of the extraplacental membranes. This latter strain also promoted a less robust cytokine response compared to the invasive GBS strains, suggesting a possible mechanism by which GB112 evades host responses. Although compelling, these experiments were only collected in a small number of women and need to be validated in a larger sample size. Exploration into additional GBS strains and the mechanisms by which the GBS strains tested in this dissertation colonize and invade gestational tissues is critical to gain a more complete understanding of host-pathogen interactions.

The lack of knowledge of toxicant actions on host defense to infection of extraplacental membranes is a critical barrier to the development of targeted preventive and therapeutic approaches to reduce adverse pregnancy outcomes. Previous studies from our lab have demonstrated the potential role of environmental toxicants in adverse birth outcomes. However, this is the first study to explore toxicant-pathogen interactions in the extraplacental membranes which form a critical barrier to infection of the gestational compartment. We found a decrease in

pathogen-stimulated inflammatory cytokines (IL-1β and TNF-α) with the trichloroethylene metabolite DCVC treatment (Chapter 5). These findings of alterations of the normal host response have implications for increased risk and severity of intrauterine infection during environmental exposures. As demonstrated in Chapter 3, IL-1β is critical for HBD-2 secretion in the amnion epithelial cells and is likely involved in bacterial clearance. These findings suggest that DCVC inhibition of pathogen-stimulated TNF-α could also impact monocyte recruitment and macrophage killing of the bacteria, which are both reliant on TNF-α (Esparza, Mannel et al. 1987, Ming, Bersani et al. 1987, Bermudez and Young 1988). We recognize that these data are preliminary and could represent the opposite of expected – intrauterine inflammation is associated with neonatal brain damage, consequently the inhibition of pathogen stimulated inflammatory cytokines could actually benefit the fetus during infection. Complete inhibition of stimulated TNF-α could have different implications at different times during pregnancy such as placentation, which is regulated in part by cytokines. Regardless, the data generated from our experiments expand current paradigms of risk for intrauterine infection to include exposure to environmental contaminants. Furthermore, these data could have implications for immune inhibition during infection in more general populations and should be explored further in additional models.

DCVC and TCA are the critically bioactive metabolites responsible for TCE toxicity in the kidney and liver, respectively. Both metabolites circulate in the blood and can reach the placenta and membranes through the maternal blood supply (Lash, Putt et al. 1999, Lash, Putt et al. 2006, Kim, Collins et al. 2009, Bradford, Lock et al. 2011). Furthermore, the placenta has the necessary enzymes to metabolize TCE to DCVC and TCA (Hakkola, Raunio et al. 1996, Collier,

Tingle et al. 2002, Noguti, Barbisan et al. 2012). Studies in our lab are currently underway to identify TCE metabolites in rat amniotic fluid and placenta. The concentrations of DCVC (1 – 50 μM) used in the present study are in the lower effective concentration range of DCVC for cultured renal proximal tubular cells, known targets for DCVC toxicity (Lash, Qian et al. 2001, Lash, Putt et al. 2007, Xu, Papanayotou et al. 2008). Estimates for concentrations of DCVC relevant to human exposure are difficult to make due to insufficient data; however, humans exposed to levels as low as 100 ppm TCE in air transiently exhibit levels as high as 50 μM of the DCVC precursor DCVG in serum (Lash, Putt et al. 1999). Although, no changes in cytokine secretion were noted for TCA, the concentrations used in the present study (10-500 μM) were also comparable to occupational exposures: TCA concentrations are approximately 40 μM in blood from humans exposed for 4 h to 50 ppm TCE (the 8-h timeweighted average threshold limit recommended by the American Conference of Governmental Industrial Hygienists) (Fisher, Mahle et al. 1998).

This study employed new applications of tissue culture models to examine toxicant actions in the extraplacental membranes. The benefits of using *ex vivo* human tissues include availability, relevant target tissue, and ability to capture cell-cell interactions. We recognize that this model has limitations, which include heterogeneity of cell populations, and inter- and intra-individual differences. We did not control for environmental factors when collecting the tissue. Although we have specific inclusion criteria for tissue collection we did not control for fetal sex, fetal age, maternal BMI, etc. In most of our studies, we noted high responders and low responders. Therefore, future studies should be conducted in whole animal models (especially for toxicant-pathogen interaction) to validate findings. In addition, more studies are needed linking the

immune markers identified in this study (such as increases in IL-1α, IL-1β and HBD-2) to adverse birth outcomes and neonatal infection from GBS-positive pregnant women.

In summary, the findings from this compendium of related studies demonstrate: 1) Antimicrobial peptides, especially HBD-2, appear to be an important immune response during GBS infection in the extraplacental membranes; 2) cytokines produced by the choriodecidua, namely IL-1α and IL-1β, are responsible for increasing HBD-2 in the amnion epithelial cells during GBS infection of the extraplacental membranes; 3) host response in the extraplacental membranes is GBS strain dependent; and 4) exposure to environmental toxicants such as trichloroethylene can alter host response mechanisms during infection (Figure 6.1). These studies provide new insight into host response of the extraplacental membranes during GBS infection with special emphasis placed on the necessity of IL-1 secretion for HBD-2 increases. Furthermore, this dissertation provides data that expands our current thinking of infection during pregnancy to include toxicant-pathogen interactions as a potential concern.

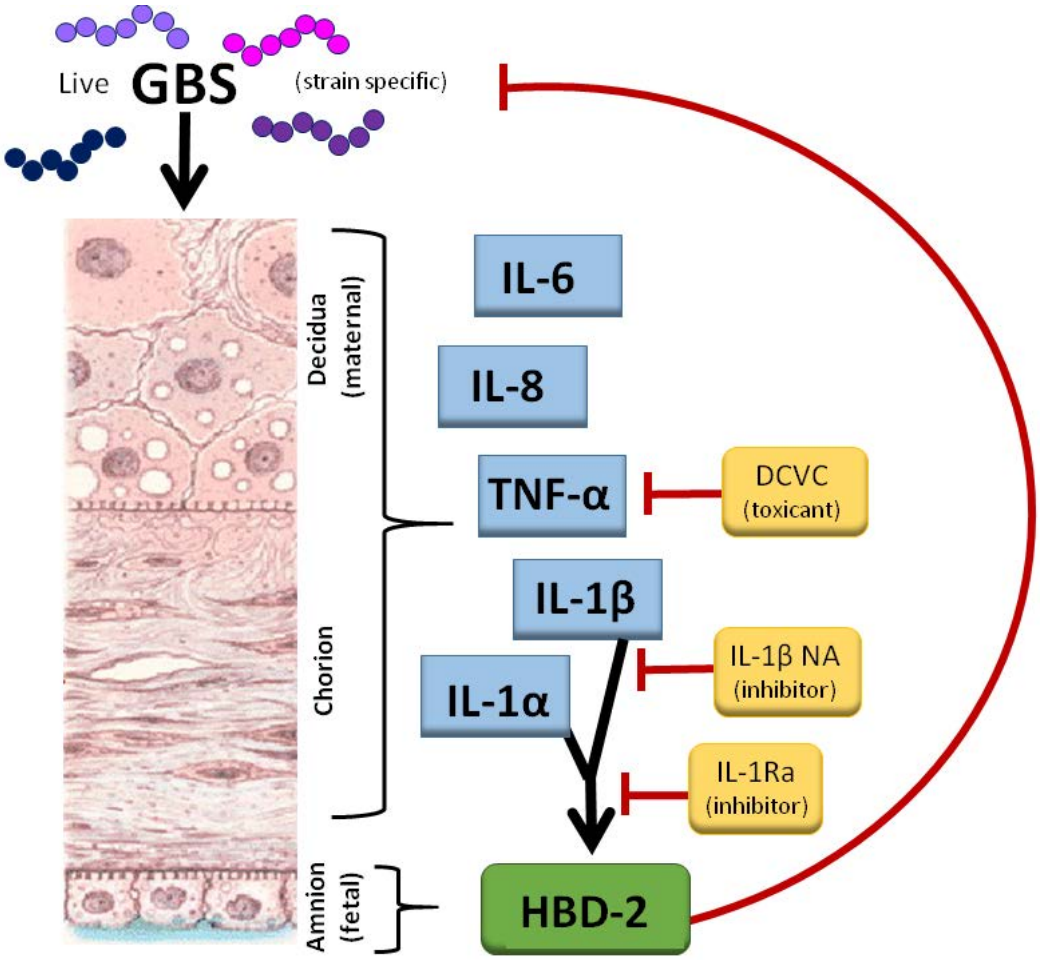

Figure 6.1. Conceptual model of GBS infection in the extraplacental membranes.
Live GBS increases secretion of cytokines IL-1α, IL-1β, IL-6, IL-8, and TNF-α in a strain-dependent manner in the choriodecidual tissue. Heat-killed GBS fails to elicit an increased cytokine response. IL-1α and IL-1β from the choriodecidual tissue increase HBD-2 in the amnion epithelial cells. HBD-2 then kills GBS. IL-1β NA (neutralizing antibody) and IL-1Ra (receptor antagonist) inhibit IL-1 stimulated increases in HBD-2. Finally, *S*-(1,2)-dichlorovinyl-L-cysteine (DCVC) inhibits GBS-stimulated TNF-α.

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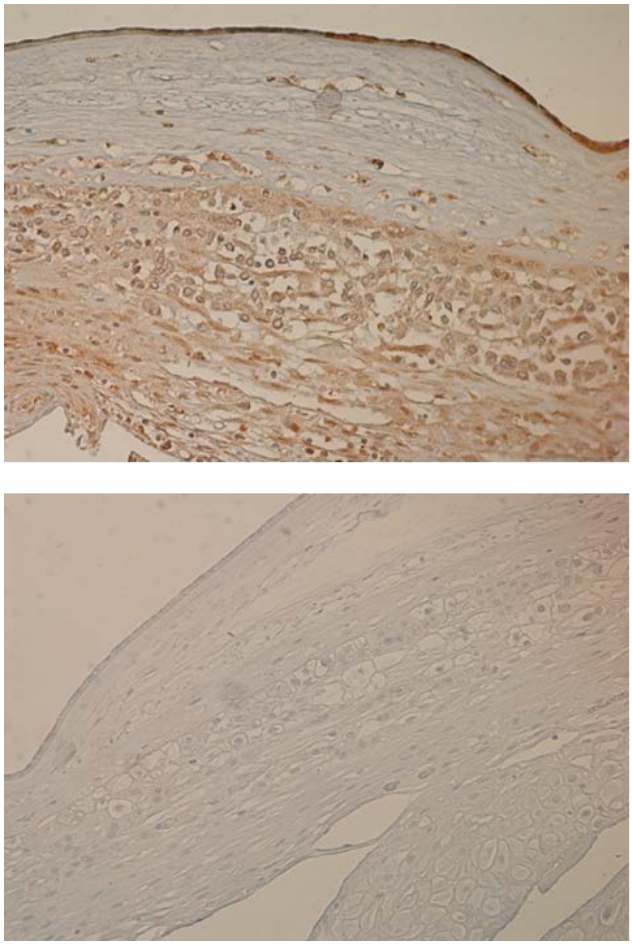
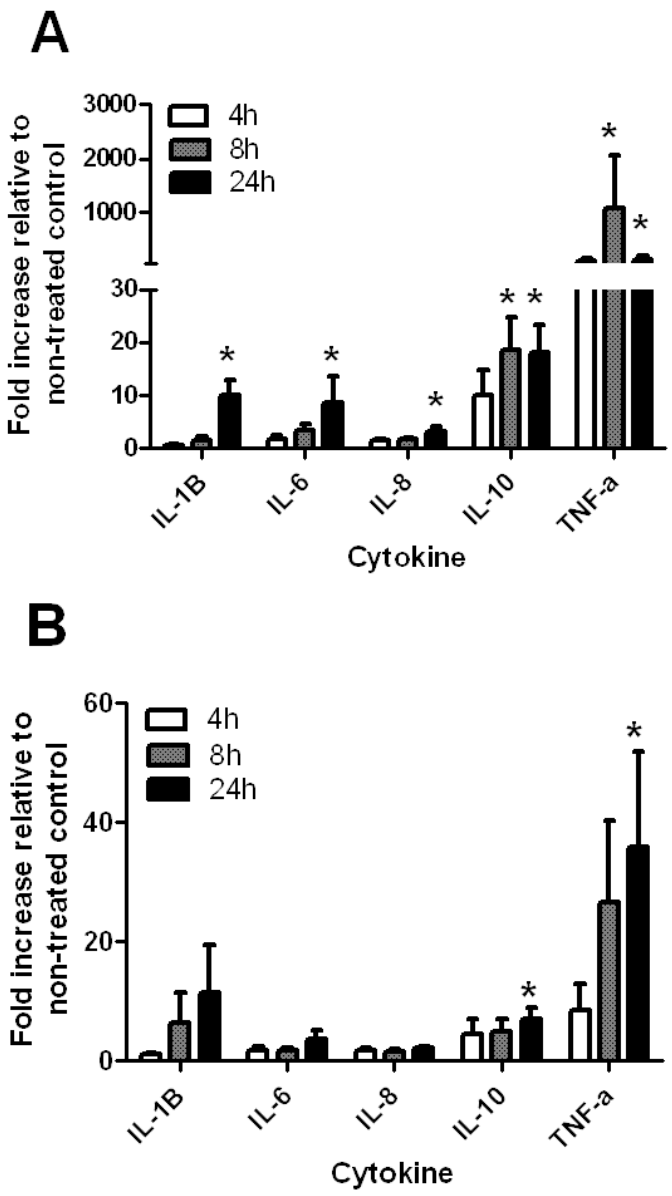
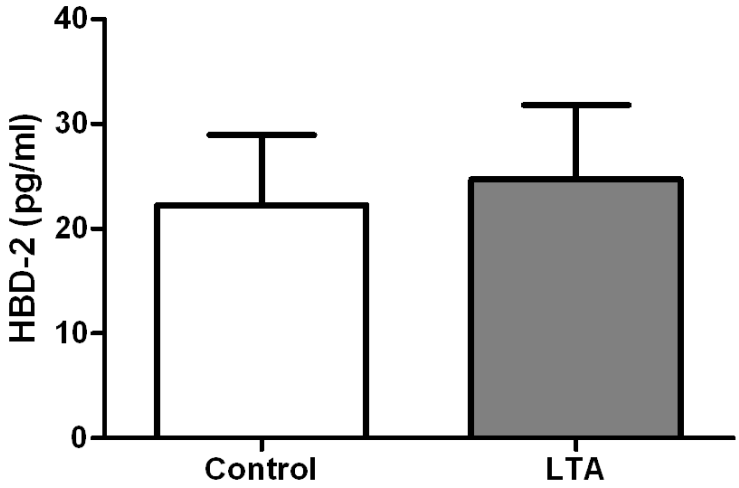


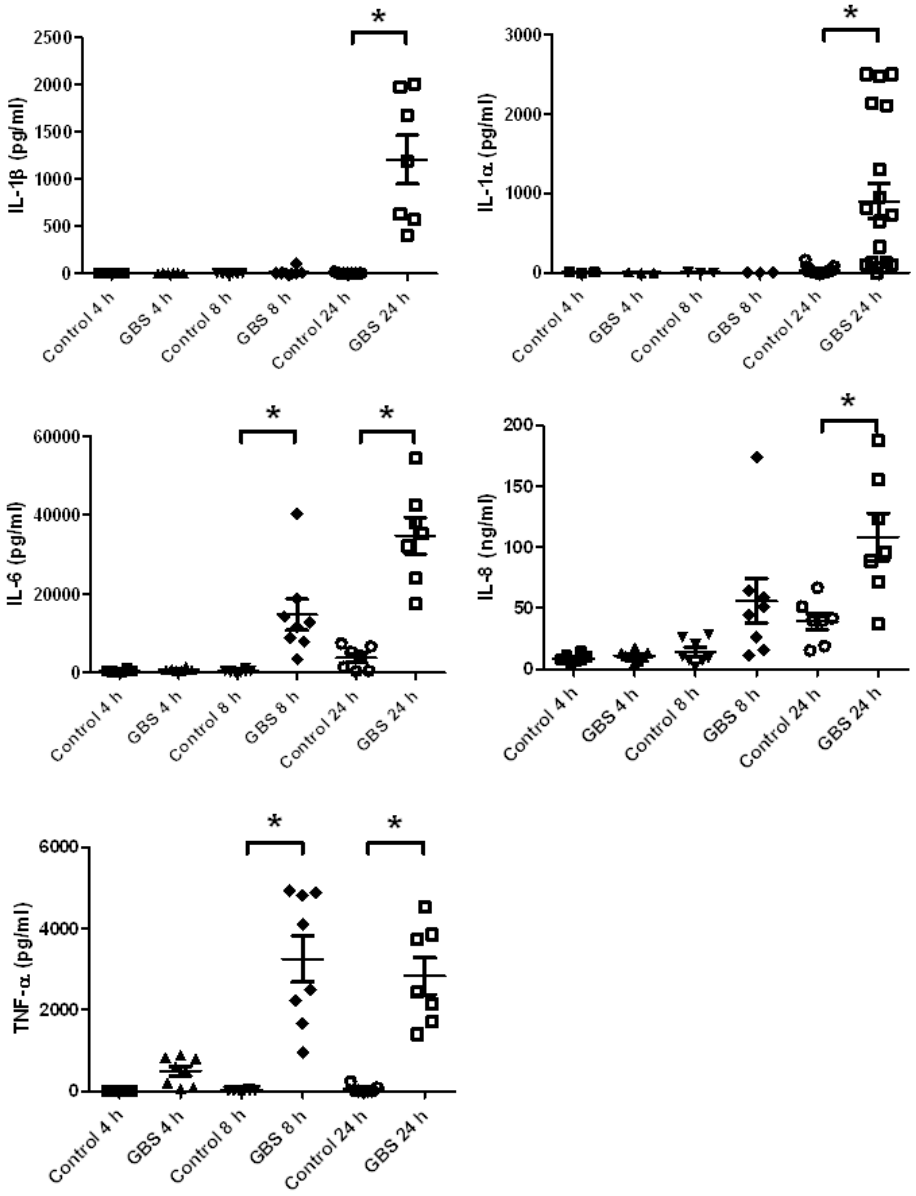
Appendix 1. Immunohistochemical staining for TLR-2 in extraplacental membranes.
The top shows a representative image for TLR-2 staining in human extraplacental membranes. The bottom shows a representative image of negative control sections incubated with secondary antibody only. No differences were noted between no treatment controls and GBS treated tissues (data not shown).



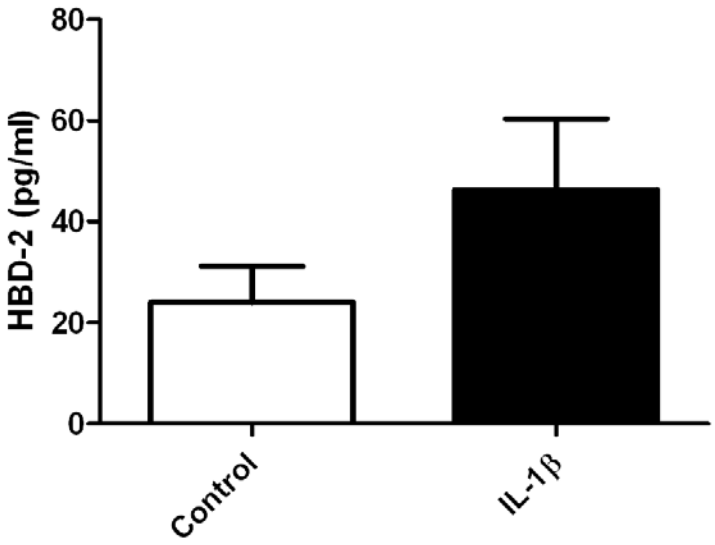
Appendix 2. Cytokine release by extraplacental membranes treated with LTA over time. Cytokine release into medium by extraplacental transwell cultures treated with medium alone (control) or LTA (1 μg/mL) in the choriodecidua compartment. Cytokines in the medium were measured by ELISA in the choriodecidual compartment (A) and amnion compartment (B). Columns are mean ± SEM (N=6 women). Asterisks (*) represent significant differences between treatment and control when compared by Tukey's post hoc test following ANOVA ($P < 0.05$) on non transformed data. These results show that the choriodecidua (side of treatment) produces a more robust cytokine response. In addition, TNF-α is secreted early compared to IL-1β, IL-6, and IL-8 which are produced later.



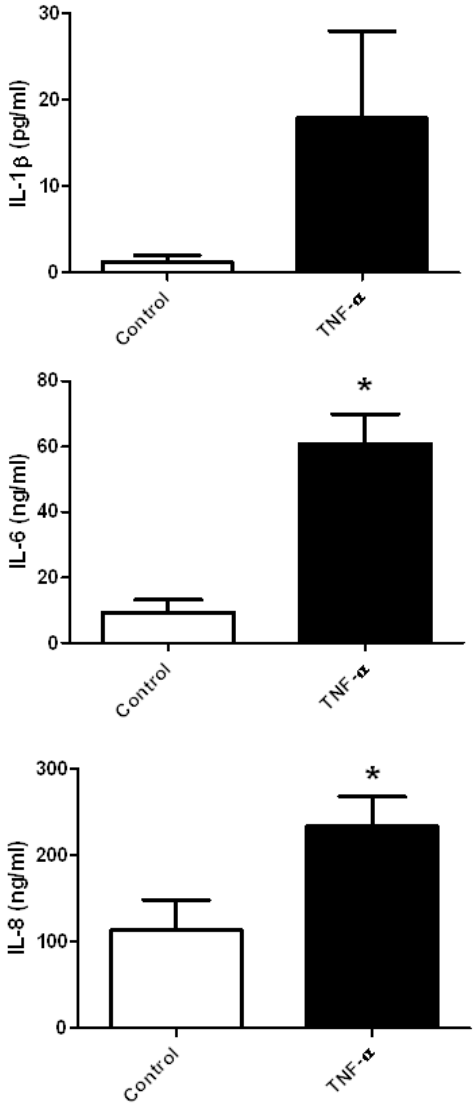
Appendix 3. HBD-2 release into medium by extraplacental membranes treated with LTA. HBD-2 release into medium by extraplacental transwell cultures treated with medium alone (control) or LTA (1 μg/mL) on the choriodecidua for 24 h. HBD-2 in the medium of the amnion compartment was measured by ELISA. Columns are mean ± SEM (N=6 women). No significant differences were observed between treatment and control when compared by Tukey's post hoc test following ANOVA. These data suggest that LTA does not stimulated HBD-2 in the extraplacental transwell model.



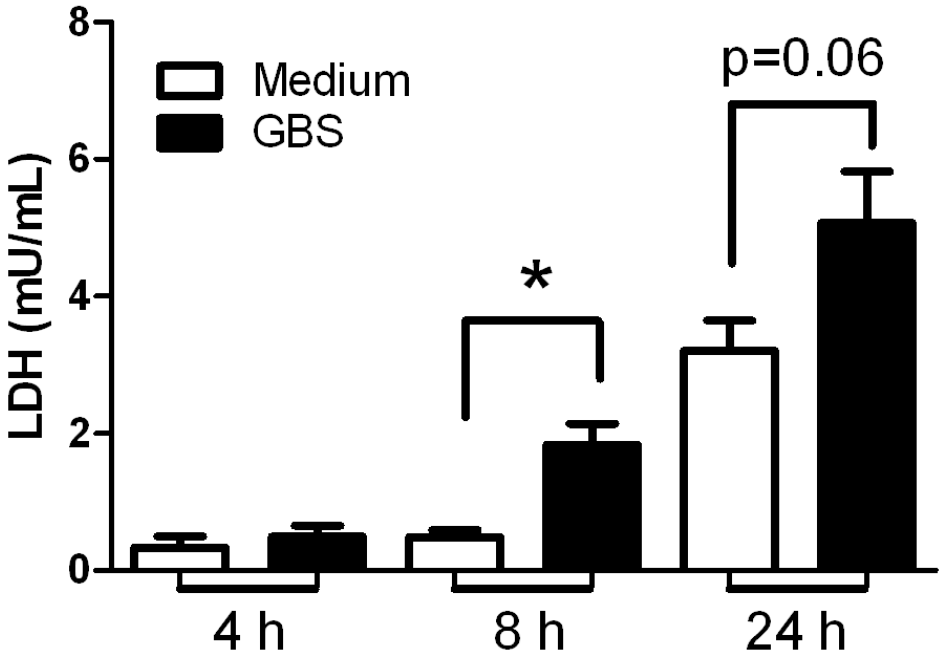
Appendix 4. Cytokine release by extraplacental membranes treated with GBS over time. Cytokine release into medium by extraplacental membranes choriodecidua punch cultures treated with medium alone (control) or GBS ($1x10^6$ CFU/mL) for 4, 8, or 24 h. Cytokines in the medium were measured by ELISA. Columns are mean ± SEM (for IL-1β, IL-6, IL-8 and TNF-α N=7-8 women, for IL-1α N=3-19 women). Asterisks (*) represent significant differences between treatment and control when compared by Tukey's post hoc test following ANOVA ($P < 0.05$). These results show that TNF-α is secreted early compared to IL-1α and IL-1β which are produced later.



Appendix 5. HBD-2 release by extraplacental choriodecidua punches treated with IL-1β. HBD-2 release into medium by extraplacental choriodecidua punch cultures treated with medium alone (control) or IL-1β (10 ng/mL). HBD-2 in the medium was measured by ELISA. Columns are mean ± SEM (N=5 women). No significant differences were observed between treatment and control when compared by student's t-test. This result suggest that choriodecidua is not the primary producer of HBD-2 in the extraplacental membranes.



Appendix 6. Cytokine release by extraplacental membranes treated with TNF-α.
Cytokine release into medium by extraplacental membranes punch cultures treated with medium alone (control) or TNF-α (100 ng/mL) for 24 h. Cytokines in the medium were measured by ELISA. Columns are mean ± SEM (N=4 women). Asterisks (*) represent significant differences between treatment and control when compared by student's t-test ($P < 0.05$).The results suggest that TNF-α is the not the driver of IL-1β expression in the extraplacental membranes.



Appendix 7. LDH release into medium by extraplacental choriodecidua punch cultures. LDH release into medium by extraplacental choriodecidua punch cultures treated with medium alone (control) or GBS ($1x10^6$ CFU/mL) for 4, 8, or 24 h. LDH in the medium was measured by LDH cytotoxicity assay kit. Columns are mean ± SEM (N=4-5 women). Asterisks (*) represent significant differences between treatment and control when compared by Tukey's post hoc test following ANOVA ($P < 0.05$).