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Activation of the IL-1β/CXCL1/MMP-10 axis in chorioamnionitis induced by inactivated Group B *Streptococcus*

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ABSTRACT

Infection or inflammation during pregnancy is known to lead to maternal immune activation triggering a fetal inflammatory response syndrome associated with deleterious effects, such as brain injury and neurodevelopmental disabilities. Group B *Streptococcus* (GBS) - one of the most common bacterium colonizing pregnant women - can be responsible for chorioamnionitis. Given that interleukin (IL)-1β has a major role in anti-GBS host defense, we hypothesized that IL-1β-driven innate immune response is implicated in GBS-induced chorioamnionitis. Using a rat model of GBS-induced chorioamnionitis, this study showed that inflammatory response to this pathogen was associated with maternal and placental IL-1β hyper expression. Following placental chemokine (C-X-C motif) ligand 1 (CXCL1) production, polymorphonuclear leukocytes (PMN) placental infiltration started at 24 h post-GBS exposure, and MMP-10 was released within these placentas. At 72 h, PMN infiltration extended to membranes and to membranes' arteries. This was associated with IL-1β release within the fetus blood at 72 h. Such a GBS-associated inflammatory cascade might be deleterious for fetal organs. These results pave the way toward targeted placento-protective anti-inflammatory strategies against GBS-induced chorioamnionitis.

1. Introduction

Chorioamnionitis is an infectious/inflammatory condition affecting the maternofetal compartments of the placenta [1]; it occurs in more than 50% of preterm birth and in 5–10% of term pregnancy [2]. Chorioamnionitis increases the risk of the newborn to present long term disabilities such as those arising from retinopathy, bronchopulmonary dysplasia, necrotizing enterocolitis, and cerebral injury [3–8]. Group B *Streptococcus* (GBS) is one of the most common pathogenic microorganisms colonizing pregnant women (10–30%), infecting the placenta, and causing perinatal morbidity and mortality, in which inflammatory determinants likely play a pivotal role [9,10].

Up to now, most experimental paradigms of pathogen-induced maternal immune activation (MIA) have used toll like receptor (TLR)3 or TLR4 agonists to mimic inflammation exerted by either viral or Gram-negative pathogens [11–13]. Gram-positive GBS-induced inflammation acts in part through TLR2 and other pathogen recognition receptors (PRR), which remain largely undefined [9]. Despite its clinical relevance, the impact of GBS-induced MIA on the placenta started only recently to be investigated - at the histopathological level - using a pre-clinical model of inactivated GBS-induced chorioamnionitis. In this model, maternal exposure to inactivated-GBS elicits chorioamnionitis and fetal brain injuries through inflammatory cascades, which remain unsettled [14]. This prompted us to further characterize the inflammatory process involved in inactivated GBS-induced chorioamnionitis.

Given that interleukin (IL)-1β and polymorphonuclear cells (PMN) have a major role in the host defense against GBS, their

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implication in GBS-induced chorioamnionitis was tested in our model, as well as the expression of the chemokine (C-X-C motif) ligand 1 (CXCL1), an IL-1-induced chemoattractant of PMN. PMN infiltration, which is one of the hallmarks of human chorioamnionitis, has already been documented in our model of inactivated GBS-induced chorioamnionitis [14]. In regard to the human context, CXCL1 is a relevant molecule to investigate as IL-8 (human equivalent of CXCL1) is overexpressed in human chorioamnionitis, which is also characterized by PMN infiltration of the placenta [15]. Metalloproteases (MMP) and other IL-1β-driven inflammatory molecules produced by PMN might be involved in inactivated GBS-induced chorioamnionitis, as documented in human [16–21]. This putative IL-1β-driven pathway is an interesting translational target for many reasons: *(i)* IL-1β is known to be associated with human chorioamnionitis and preterm birth [22–25]; *(ii)* the causal role of the IL-1 system in TLR4-induced chorioamnionitis was pre-clinically established [25–28]; *(iii)* mounting preclinical evidence points to IL-1 blockade as a protective drug against maternofetal inflammatory responses [28].

We crossed two approaches to test these hypotheses: *(1)* a large approach using microarray to identify the modifications of inflammatory gene expression following GBS-induced inflammation and *(2)* specific protein analyses targeting the above-mentioned molecules of interest.

2. Methods

2.1. Animals and study design

Dams obtained from Charles River Laboratories (Saint-Constant, QC) at gestational day (G) 14 were allowed to acclimatize to our animal facility prior to experimental manipulations. Rats were reared at 20–23 °C with a 12 h light/dark cycle and access to food and water *ad libitum*. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the *Université de Sherbrooke* in accordance with the Canadian Council on Animal Care guidelines. Dams were injected intraperitoneally every 12 h from G19 to G22 either with GBS (1×10^9 CFU of inactivated GBS suspended in 100 μl of sterile saline) - GBS-exposed group - or with 100 μl of sterile saline - GBS -unexposed group (control group). Dams were exposed to GBS from G19 to G22; we chose this time frame based on a level of fetal development in rats equivalent to the early third trimester of human gestation [29], and on epidemiological studies showing that placental infections peak at the end of the third trimester of gestation [14]. As the diagnosis of chorioamnionitis is most often delayed, *i.e.* during labor (clinical chorioamnionitis) or after delivery (histological chorioamnionitis), meaning that the exposure to bacterial components and inflammation last often for days or weeks, we decided to administer inactivated GBS every 12 h from G19 to G22 in order to mimic the time course of human placental inflammation. Cesarean section (C-section) were performed as previously described [26] at 1, 3, 6, 24, 48, and 72 h post-injection to collect maternal blood, placentas, and fetal tails. Due to sampling limitations, fetal blood could not be harvested prior to 48 h post-GBS exposure. Sex of fetuses was determined by amplification of a sequence within the SRY gene specific to the Y chromosome using polymerase chain reaction (PCR) as previously described [30]. We focused our study on placenta associated with male fetuses, and on male fetal tissues, given the GBS-induced sex dichotomous placental and cerebral effects we previously observed [14,31].

2.2. Microarray and qPCR

Placentas from GBS-exposed *versus* unexposed dams were collected at 6 h post-GBS. Total RNA was isolated using an RNeasy Plus Universal Mini kit (Qiagen, Germany) in accordance with the manufacturer's instructions. The concentration of the RNA samples was quantified using a Nanodrop ND-1000 (Nanodrop Technologies, DE). RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, CA). For microarray experiments, the *AffymetrixGeneChip® Rat Genome 2.0 array* was used to examine gene expression profile shifts in the placenta of male fetuses from GBS-exposed *versus* unexposed dams. Each gene chip identified up to 30,000 different probe sets. Data normalization was performed using Expression Console (EC) software (Affymetrix®, www.affymetrix.com) and statistical analyses were performed using Transcriptome Analysis Console (TAC) 3.0 software (Affymetrix®, www.affymetrix.com). Data were analyzed further using Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com) to define the potentially altered pathways. For qPCR experiments, RT was performed on 2.2 μg total RNA with Transcriptor reverse transcriptase, random hexamers, dNTPs (Roche Diagnostics, France), and 10 units of RNAseOUT (*Invitrogen*) following the manufacturer's protocol in a total volume of 20 μl. Quantitative PCR (qPCR) reactions were performed in 10 μl in 96 well plates on a CFX-96 thermocycler (BioRad, CA) with 5 μL of 2X iTaq Universal SYBR Green Supermix (BioRad, CA), 10 ng (3 μl) cDNA, and 200 nM final (2 μl) primer pair solutions. The following cycling conditions were used: 3 min at 95 °C; 50 cycles: 15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C. Relative expression levels were calculated using the qBASE framework [32] and the housekeeping genes Tubb5, Rpl19 and Pum1 for rat cDNA. Primer design and validation was evaluated as described before [33]. All primer sequences are available in Supplemental Table 1.

2.3. ELISA

Plasma and placenta were sampled from GBS-exposed, and unexposed, dams and fetuses. Proteins were extracted from placentas and concentrations were determined as previously described [34]. IL-1β and CXCL1 (R&D System, MN), as well as MMP-10 and S100A9 (NeoScientific, MA) and MMP-8 (Raybiotech, GA) ELISA were used according to manufacturer's instructions.

2.4. Tissue preparation and immunohistochemistry (IHC)

Placentas were fixed (4% formaldehyde, 0.1% glutaraldehyde) and paraffin-embedded, as previously described [26]. Median coronal sections of placentas were performed to allow the study of decidua, junctional zone, labyrinth and membranes on the same section. Briefly, 5-μm-thick sections were mounted on silanized slides (VWR, ON) [35]. Primary antibodies against: ionized calcium-binding adapter protein 1 (Iba-1) (macrophage marker, 1:250, Wako Chemicals, VA), IL-1β (1:100, AbD Serotec, NC) and MMP-10 (1:100, Millipore, ON) were used. Polyclonal antibodies against CXCL1 (1:50, LifeSpan Biosciences, WA) and PMN (PMN; 1: 500; Cederlane, ON) were used. The secondary antibodies were coupled to horseradish peroxidase. The substrate for the colorimetric reaction was 3,3'-diaminobenzidine. Negative IHC controls consisted of additional sets of placental sections treated in a similar way but without the primary antibody. For PMN counts, we counted labeled cells in five predetermined fields equally distributed within the placenta (Supplemental Fig. 1), respectively within the decidua, junctional zone, labyrinth and membranes to investigate the propagation of inflammation beyond the maternal/decidual compartment from each placenta at 400× magnification using ImageJ software application cell counter, as described [35]. We defined diffuse PMN infiltration as scattered PMN, and clusters of PMN infiltration as nodules of at least 25 adjacent PMN.