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Research Article

Group B streptococcus induces cellular senescence in human amnion epithelial cells through a partial interleukin-1-mediated mechanism

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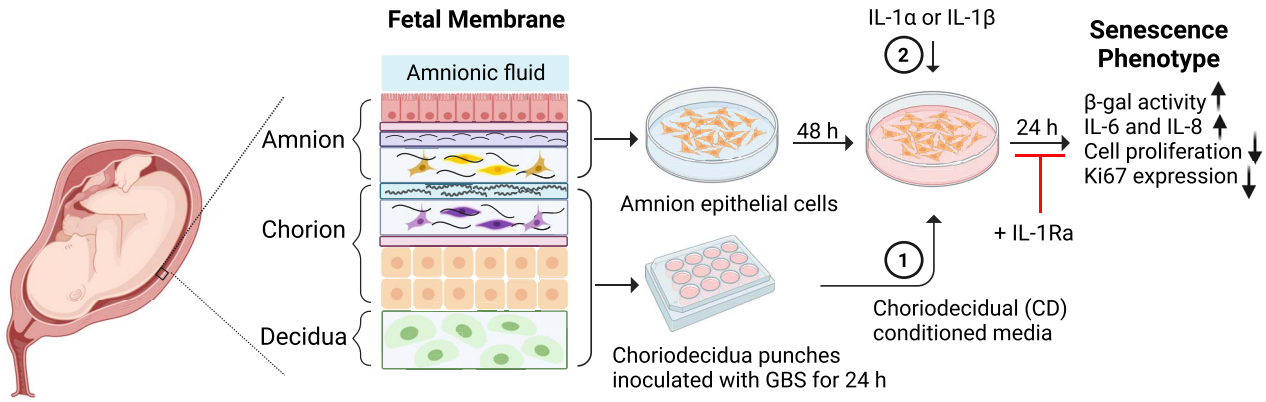
Abstract

Group B streptococcus (GBS) infection is a significant public health concern associated with adverse pregnancy complications and increased neonatal mortality and morbidity. However, the mechanisms underlying the impact of GBS on the fetal membrane, the first line of defense against pathogens, are not fully understood. Here, we propose that GBS induces senescence and inflammatory factors (IL-6 and IL-8) in the fetal membrane through interleukin-1 (IL-1). Utilizing the existing transcriptomic data on GBS-exposed human fetal membrane, we showed that GBS affects senescence-related pathways and genes. Next, we treated primary amnion epithelial cells with conditioned medium from the choriodecidual layer of human fetal membrane exposed to GBS (GBS collected choriodecidual [CD] conditioned medium) in the absence or presence of an IL-1 receptor antagonist (IL-1Ra). GBS CD conditioned medium significantly increased β-galactosidase activity, IL-6 and IL-8 release from the amnion epithelial cells. Cotreatment with IL1Ra reduced GBS-induced β-galactosidase activity and IL-6 and IL-8 secretion. Direct treatment with IL-1α or IL-1β confirmed the role of IL-1 signaling in the regulation of senescence in the fetal membrane. We further showed that GBS CD conditioned medium and IL-1 decreased cell proliferation in amnion epithelial cells. In summary, for the first time, we demonstrate GBS-induced senescence in the fetal membrane and present evidence of IL-1 pathway signaling between the choriodecidua and amnion layer of fetal membrane in a paracrine manner. Further studies will be warranted to understand the pathogenesis of adverse pregnancy outcomes associated with GBS infection and develop therapeutic interventions to mitigate these complications.

Summary Sentence

Role of IL-1 in GBS-induced senescence of human primary amnion epithelial cells.

Graphical Abstract



Key words: fetal membrane, GBS, amnion epithelial cells, senescence, inflammation, senescence-associated secretary phenotype, β-gal, IL-6, IL-8

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Introduction

Intrauterine bacterial infections during pregnancy pose a significant risk for adverse birth outcomes and contribute to neonatal morbidity and mortality [1, 2]. Infections result when bacterial species including *Ureaplasma* spp., *Mycoplasma* spp., *Fusobacterium* spp., *Escherichia coli* and *Streptococcus* spp. colonize the vagina and cervix, migrate to the fetal membranes, and invade the amniotic cavity and fetus [2–5]. *Streptococcus agalactiae*, or group B *Streptococcus* infection in pregnant women is a leading cause of infectious neonatal morbidity and mortality worldwide [6]. Intrauterine GBS infection is associated with neonatal sepsis [7], miscarriage [8], preterm birth [9], and chorioamnionitis [5]. Human fetal membranes serve as a barrier between the feto-placental and maternal compartments, and consist of the amnion (innermost layer of the intraamniotic cavity) and the choriodecidua (fetal tissue juxtoposed to maternal decidua) [10] (Figure 1). During the course of ascending microbial infection in the gestational compartment, GBS initially encounters the choriodecidua layer of the fetal membranes, which serves as a crucial line of defense against intrauterine pathogens [11–13].

How gestational tissues respond to pathogens is an area under active investigation. Fewer studies have characterized the role of senescence, which is a state of cellular aging characterized by irreversible growth arrest and a pro-inflammatory secretory phenotype, termed the senescence-associated secretory phenotype (SASP). SASP exacerbates local inflammation and disrupts the intrauterine microenvironment [14, 15]. Emerging evidence suggests that inflammation and senescence in fetal membranes play critical roles in adverse pregnancy outcomes including preterm labor or preterm premature rupture of membranes (PPROM) [10, 15–17]. Increased senescence markers including senescence-associated (SA)-β-galactosidase (gal) staining, IL-6 and IL-8, were associated with the fetal membranes from women in labor at term, when compared with nonlaboring women at term [16]. In addition, fetal membranes in women with PPROM or preterm birth showed increased markers of senescence phenotypes such as p53, p21, and phospho (p)-p38 mitogen-activated protein kinase (MAPK) [17]. Senescence-associated inflammation in the fetal membrane can impact adjacent tissues including the decidua, myometrium, and cervix and initiate parturition [10, 18].

Although specific studies on GBS-induced senescence in the fetal membrane are limited [19], GBS has been shown to activate inflammatory pathways and stimulate release of proinflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8), which are part of SASP from human fetal membranes [12, 19, 20]. Among SASP factors, IL-1 is a key proinflammatory cytokine that plays a crucial role in various cellular processes, which has also been implicated in regulation of senescence. For example, studies in human fibroblasts demonstrate that IL-1 induces cellular senescence and SASP through activation of the NF-κB pathway [21, 22]. In addition, IL-1β increases SA-β-gal staining and IL-6 and IL-8 release in rat astrocytes [23]. Furthermore, overexpression of IL-1α leads to endothelial senescence [24]. Although these studies implicate IL-1 in the regulation of cellular senescence and SASP, the precise mechanisms underlying GBS-induced senescence and inflammation in the fetal membrane remain poorly understood.

To address these research gaps, this study aims to investigate the relationship between GBS infection, inflammatory cytokines, and senescence in fetal membranes. Specifically, we hypothesize that GBS-induced senescence is mediated by IL-1 signaling, which results in SASP factor production in the fetal membrane. By unraveling these mechanisms, we hope to shed light on the pathogenesis of adverse pregnancy outcomes associated with GBS infection and provide potential targets for therapeutic intervention.

Materials and methods

Ethics statement

The University of Michigan Institutional Review Board approved this research (IRBMED#HUM0013915).

Pathway analysis of transcriptomics dataset

To identify whether GBS activates pathways involved in senescence, we reanalyzed an available transcriptomic dataset from a previous study [20]. We downloaded the publicly available transcriptomic profiles and lists of differentially expressed genes at each time point from Gene Expression Omnibus (accession ID GSE96557). Lists of significantly changed genes for each timepoint were used as input into DAVID online pathway analysis software. We then used the functional annotation tool to identify significantly enriched Gene Ontology terms for each timepoint (Padj < 0.05). P-values were corrected for multiple testing using the Benjamini and Hochberg False Discovery Rate approach.

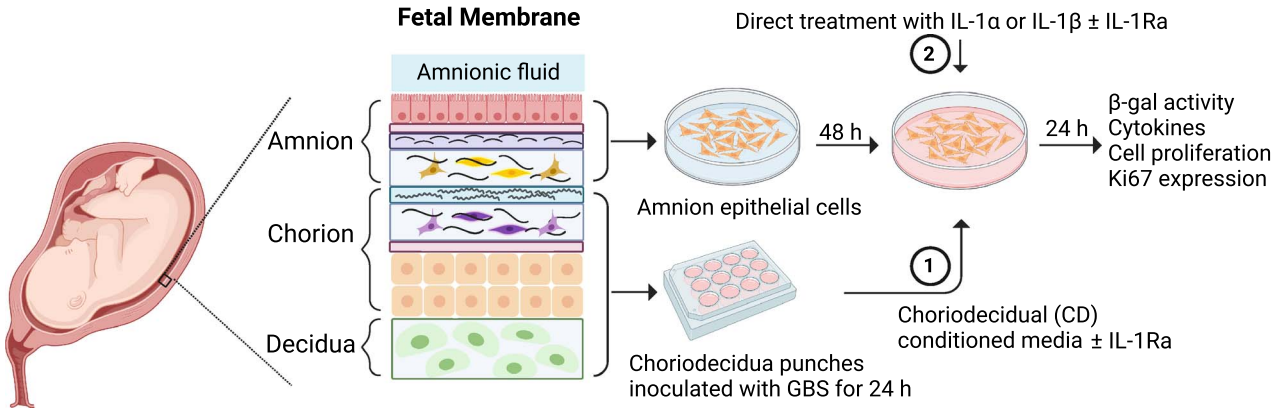

Figure 1. Schematic workflow of the study. Created using bioRender.

Reagents and materials

The GBS used in this study was strain A909, initially isolated from a septic newborn [25], 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°C in planktonic culture using Todd Hewitt Broth (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 {Dulbecco's Modified Eagle Medium (DMEM) catalog # 21063 and DMEM:F12 catalog #11039}, buffers, fetal bovine serum (FBS; catalog #10438), and penicillin/streptomycin (pen/strep; catalog #15140) were from GIBCO (Grand Island, NY). Recombinant cytokines (IL-1α and IL-1β) were from Peprotech (Rocky Hill, NJ). IL-1Ra was from Sigma-Aldrich (Saint Louis, MO).

Tissue collection

Human extraplacental membranes were collected from healthy pregnancies undergoing scheduled cesarean delivery at term or near term (36–40 weeks) prior to onset of active labor at the University of Michigan Birth Von Voigtlander Women's Hospital Birth Center. Only healthy, nonsmoking, singleton mothers were included. Women were excluded if they had spontaneous rupture of membranes, more than 6 contractions per hour, significant obstetrical complications such as preterm labor or cervical cerclage, third trimester bleeding, multifetal pregnancy, suspicion for active vaginal or intrauterine infection, immunocompromised conditions, or major medical conditions (e.g. diabetes, hypertension, collagen vascular disease, chronic renal disease, sarcoidosis, hepatitis, HIV). Patients were also excluded 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 2 weeks preceding delivery.

Choriodecidual membrane explant culture and treatment

Full thickness gestational membranes were transported to the laboratory in Dulbecco's phosphate-buffered saline and washed in warm media. The amnion and choriodecidua were separated, and then, explants were created from choriodecidua using a 12-mm biopsy punch. The explants were floated in three separate wells per treatment group in a 12-well plate in 1-mL Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% FBS and 10 000 U/mL penicillin/streptomycin antibiotic (Gibco). Following acclimation of membranes in culture for 24 h, medium was replaced with DMEM +1% FBS containing 1×10^6 colony-forming units/mL (CFU/mL) of GBS according to Boldenow et al. [12]. Following incubation of choriodecidual membranes with GBS for 24 h, conditioned medium was collected, filtered through a 0.2-μm membrane to remove GBS, and stored at −80°C until human amnion epithelial cells were ready to be treated with the conditioned medium. Non-treated collected choriodecidual (NT CD) conditioned medium from choriodecidual membranes without GBS exposure was included as a control. We observed no microbial growth in agar, and the cytokine levels in the medium remained at low levels in the no-treatment controls.

Isolation and treatment of amnion epithelial cells

Amnion isolation was performed according to Boldenow et al. [12]. Briefly, dissected amnion was incubated with 0.25% trypsin–EDTA for 30 min at 37°C and transferred to fresh trypsin–EDTA for an additional 30 min incubation at the same temperature. Trypsin was neutralized with DMEM:F12 medium containing 10% FBS, pen/strep, and 10 ng/mL EGF. We confirmed the expression of epithelial cell markers, including E-cadherin and pan-keratin, through western blot analysis (Supplementary Figure S1). Amnion epithelial cells were seeded in triplicate for each treatment group in 12-well plates at 500 000 cells/well in 1 mL of medium. Cells were permitted to adhere and equilibrate for 48 h, and on day 3, the medium was changed. Cells grown to 70–80% confluence were treated with medium (NT), Non-treated CD (NT CD) conditioned medium, GBS-treated choriodecidual (GBS CD) conditioned medium or with either 0.1–10 ng/mL IL-1α or IL-1β. Treatment groups included co-treatment with GBS CD conditioned medium and 10–100 ng/mL IL-1 receptor antagonist (IL-1Ra) according to Boldenow et al. [12]. Physiological and pathophysiological levels of IL-1 in human pregnancy are reported to be 52.21 and 185.8 pg/mg protein, respectively [26]. Although our units differ because our samples were not normalized to proteins, these values fall within the range of doses used in our studies.

SA-β-galactosidase (SA-β-gal) activity

Using a 96-well Cellular Senescence Assay Kit (Cell Biolabs, San Diego, CA), cell lysates were collected in triplicate for each treatment group. Triplicates were pooled and centrifuged to obtain supernatant. Protein content in supernatants was quantified by BCA Protein Assay Kit (Pierce, Grand Island, NY) and incubated in reaction buffer containing SA-β-gal substrate at 37°C for 2 h according to manufacturer's protocol. Reaction mixtures were transferred in triplicate in a 96-well plate, read at 360 nm (excitation)/465 nm (emission) (RFUs), blank subtracted, and normalized to respective protein levels.

Measurement of pro-inflammatory cytokines

Concentrations of IL-6 and IL-8 levels were measured in conditioned medium collected from amnion epithelial cells 24 h posttreatment the University of Michigan Immunologic Monitoring Core using an enzyme linked immunosorbent assay (ELISA) kit according to manufacturer's directions (R&D Systems, Minneapolis, MN). Samples were diluted as necessary for detection and limit of quantitation (LOQ) was determined by the lowest level detectable on the standard curves [27]. In these studies, LOQ was 0.005 pg/mL. The 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; and 31.2–2000 pg/mL for IL-8.

Cell proliferation assay

Amnion epithelial cells were plated in black wall, clear bottomed 96-well plates at a density of 5000 cells/well, and incubated overnight. Then, the cells were treated with IL-1α (10 ng/ml), IL-1β (10 ng/ml), or GBS CD conditioned medium in the absence or presence of IL-1Ra (100 ng/ml) for 48 h. Cell proliferation was measured using CyQUANT Cell Proliferation Assay (Invitrogen, C7026) following the manufacturer's protocol.

Western blot

Amnion epithelial cells were plated in 12-well plates at a density of 100 000 cells/well and incubated overnight. Then, the cells were treated with IL-1α (50 ng/ml) or IL-1β (50 ng/ml) in the absence or presence of IL-1Ra (100 ng/ml) for 48 h. Whole-cell lysates were prepared in NP40 buffer supplemented with 1× Halt Protease Inhibitor Cocktail (Thermo) and 1-mM PMSF. Cleared lysates were denatured with 1× Laemmli Sample Buffer (Bio-Rad) and beta-mercaptoethanol as a reducing agent, and heated at 95°C for 10 min before being run in SDS-PAGE and transferred to a PVDF blotting membrane. Primary antibodies used in this study include anti-Ki67 (Abcam), anti-E-cadherin (Cell Signaling), anti-pan-keratin (Cell Signaling), and anti-GAPDH (Biolegend). The band densities were quantified using ImageJ and normalized to GAPDH.

Statistical analysis

Treatment groups were compared using ANOVA with Tukey's Multiple Comparison. Analyses were performed using the Prism statistical package (San Diego, CA) and $P \leq 0.05$ was used to determine statistically significant effects. To account for variability among donated human gestational membranes demonstrating a similar pattern of response to GBS CD conditioned medium and accommodate analysis of data with missing values, a linear mixed model analysis was performed using the SPSS statistical package (Armonk, NY) and $P \leq 0.05$ was used to determine statistically significant effects. In this model, the average of technical replicates from measures of cytokine or beta galactosidase concentrations was considered dependent variables, treatment was considered a fixed variable, and donated tissues from individual women were considered a random variable. Data were expressed as means ± SEM.

Results

GBS impacts on senescence pathways/genes in fetal membrane explants

We previously reported GBS-activated pathways related to inflammation and preterm birth in human fetal membranes [20]. To further interrogate GBS impacts on fetal membranes, we reanalyzed existing datasets focusing on senescence-related pathways/genes. DAVID pathway analysis of significantly regulated genes at each time point revealed enrichment of cellular senescence, cell cycle regulation, and p53 signaling pathways in the choriodecidual membranes (Table 1). Expression of genes encoding senescence-associated proinflammatory cytokines including *TNF* (tumor necrosis factor), *IL6* (interleukin 6), *IL1A* (interleukin 1 alpha), and *IL-8* (interleukin 8) was upregulated as early as 4 h indicating critical roles of these genes in early response to GBS infection (Figure 2). In addition, genes involving cell cycle regulation including *PLK3* (polo-like kinase 3), *CDKN1A* (cyclin-dependent kinase inhibitor 1A), *CDK1* (cyclin-dependent kinase 1), *CCNE1* (cyclin E 1), and *CCNA2* (cyclin A2) are differentially regulated by GBS (Figure 2). Altogether, these data suggest that GBS affects pathways/genes involved in cellular senescence characterized by SASP and cell cycle arrest in the fetal membrane.

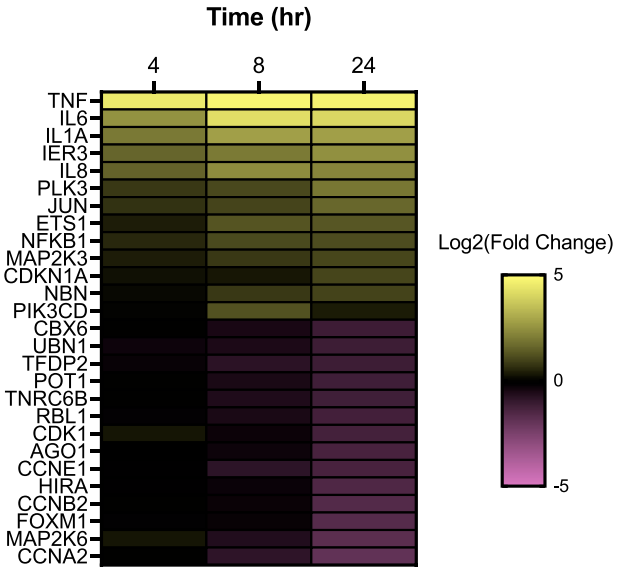

Figure 2. Time-course expression of genes involved in senescence and cell cycle regulation in the fetal membrane explants exposed to GBS. Choriodecidual membrane explants were inoculated with GBS (1×10^6 colony forming units/mL) and incubated for 4, 8, or 24 h. The transcriptome was analyzed by microarray. $n = 4$ subjects.

GBS-induced senescence and cytokine release in primary amnion epithelial cells

GBS infection in fetal membranes stimulates the release of proinflammatory cytokines such as IL-1, IL-6, and IL-8 [12, 19, 20], which is part of SASP [28]. Based on these studies and our findings from the transcriptomics data (Table 1 and Figure 2), we investigated GBS-induced senescence and inflammation in fetal membranes. We first inoculated choriodecidua punches of the fetal membranes; CD conditioned medium; and filtered it through a 0.2-μm membrane to remove GBS. Next, the medium was applied to primary human amnion epithelial cells, which was isolated from the amnion layer of the fetal membranes. As shown in Figure 3A, GBS-treated CD conditioned medium (GBS CD) resulted in about 3.0-fold increase in β-galactosidase activity in amnion epithelial cells, while non-treated CD conditioned medium (NT CD) was not significantly different from NT (medium only). In addition, treatment with GBS CD significantly increased IL-6 and IL-8 release from the cells (Figure 3B and C). NT CD also increased cytokine release likely due to basal levels of cytokines or other biomolecules in the conditioned medium [12]. Co-treatment with IL-1Ra, an IL-1 receptor antagonist, significantly decreased β-galactosidase activity, release of IL-6 and IL-8, implicating the roles of IL-1 signaling in the regulation of cellular senescence and SASP (Figure 3). However, IL-1Ra did not lead to complete reduction of these responses, indicating existence of other mechanisms. IL-1Ra alone did neither affect cellular senescence nor cytokine secretion (Supplementary Figure S2). Altogether, these data suggest that secreted biomolecules released from the choriodecidual membrane in response to GBS play a role in increasing cellular senescence in the amnion cells and that IL-1 signaling, at least in part, regulates cellular senescence of amnion epithelial cells.

Table 1. Enriched terms in the gene list differentially regulated by GBS inoculation on the fetal membrane explants.

Time (hr)	Category	Term	# of genes	*Padj*[*]
8	KEGG_PATHWAY	hsa04218:Cellular senescence	6	**0.033**
	REACTOME_PATHWAY	R-HSA-2559582 ~ Senescence-Associated Secretory Phenotype (SASP)	4	0.240
	REACTOME_PATHWAY	R-HSA-2559583 ~ Cellular Senescence	5	0.265
24	REACTOME_PATHWAY	R-HSA-2559583 ~ Cellular Senescence	19	**0.002**
	KEGG_PATHWAY	hsa04218:Cellular senescence	15	**0.001**
	REACTOME_PATHWAY	R-HSA-2559586 ~ DNA Damage/Telomere Stress Induced Senescence	7	0.465
	REACTOME_PATHWAY	R-HSA-2559585 ~ Oncogene Induced Senescence	4	0.726
	UP_KW_BIOLOGICAL_PROCESS	KW-0131 ~ Cell cycle	46	**8.57E−07**
	GOTERM_BP_DIRECT	GO:0051726 ~ regulation of cell cycle	23	**1.13E−04**
	REACTOME_PATHWAY	R-HSA-1640170 ~ Cell Cycle	41	**0.002**
	REACTOME_PATHWAY	R-HSA-69278 ~ Cell Cycle, Mitotic	34	**0.006**
	REACTOME_PATHWAY	R-HSA-6791312 ~ TP53 Regulates Transcription of Cell Cycle Genes	8	**0.017**
	REACTOME_PATHWAY	R-HSA-3700989 ~ Transcriptional Regulation by TP53	24	**0.016**
	KEGG_PATHWAY	hsa04115:p53 signaling pathway	9	**0.008**

[*] *P*adj: adjusted *P*-values for multiple comparisons by the Benjamini Hochberg correction. *P*adj < 0.05 shown in bold.

IL-1-induced senescence and cytokine release in primary amnion epithelial cells

To further investigate the role of IL-1 signaling in the regulation of senescence and cytokine release, we directly treated primary amnion epithelial cells with either IL-1α or IL-1β in the absence or presence of IL-1Ra. As shown in Figure 4A, treatment with IL-1α significantly increased β-galactosidase activity in the cells, while IL-1Ra decreased β-galactosidase activity to the basal level (NT). However, the fold increase of β-galactosidase activity with IL-1α was modest (1.2-fold) compared with that with GBS CD (Figure 4A; 3.0-fold), indicating additional mechanisms for GBS-induced senescence in the fetal membranes. In addition, IL-1α stimulated IL-6 and IL-8 release, while IL-1Ra suppressed the release (Figure 4B and C). Similarly, treatment with IL-1β significantly increased β-galactosidase activity compared with NT in the amnion epithelial and its increase was suppressed with IL-1Ra treatment (Figure 5A). Effect of IL-RA on IL-1β-induced IL-6 and IL-8 release resulted in marginal, but significant decrease (Figure 5B and C). Altogether, these data suggest the involvement of IL-1α and IL-1β in the regulation of cellular senescence and cytokine release and their differential roles. These data also suggest additional mechanisms for GBS-induced cellular mechanisms.

GBS or IL-1-induced growth inhibition in primary amnion epithelial cells

Next, we examined the impact of IL-1 and GBS on the proliferation of amnion epithelial cells. As shown in Figure 6A, the treatment of amnion epithelial cells with IL-1α or GBS CD-conditioned medium significantly reduced cell numbers, as determined by the CyQuant proliferation assay. Although IL-1β also led to a decrease in cell numbers, this effect did not reach statistical significance. Notably, the reduction observed with GBS CD-conditioned medium was more pronounced compared with IL-1α or IL-1β. We further assessed the expression of Ki-67, a marker of cell proliferation, through western blot analysis. IL-1α and IL-1β both decreased Ki-67 expression, but co-treatment with IL-1Ra reversed this effect (Figure 6B). Collectively, these findings suggest that GBS and IL-1 play regulatory roles in amnion epithelial cell proliferation.

Discussion

Intrauterine infection significantly contributes to various adverse outcomes during pregnancy, including preterm birth, neonatal sepsis, and miscarriage [1]. GBS is the most commonly identified pathogens responsible for intrauterine infections [29]. However, underlying mechanisms for GBS-mediated adverse pregnancy complications are not fully understood. This study proposes GBS-induced senescence as a mechanism for GBS-mediated pregnancy complications including preterm labor and preterm premature rupture of membranes (PPROM). Here, we demonstrate for the first time that soluble factors from GBS-stimulated choriodecidual cultures increase a marker of cellular senescence and release of senescence-associated factors and decrease cell proliferation in primary amnion epithelial cells. We also present evidence that IL-1 signaling partially regulates GBS-mediated senescence in the amnion epithelial cells.

Senescence involves irreversible arrest of cell growth [30] and is characterized by prominent β-galactosidase lysosomal enzymatic activity, the increased production of cell cycle arrest markers p53/p21 and p16-pRB axis, enlarged and flattened cells, DNA damage (yH2AX), ROS production, and telomere shortening [31–36]. Senescence is also associated with changes in a set of biomarkers that are collectively called the SASP and includes cytokines, chemokines, angiogenic and other growth factors, matrix-degrading enzymes, as well as inhibitors, cell adhesion molecules, apoptotic inducers, and their ligands [28, 37]. Consistent with these characteristics, we found that GBS affects genes and pathways involved in senescence and cell cycle regulation in the choriodecidual membranes, increased β-galactosidase activity and SASP (IL-6 and IL-8), and decreased cell proliferation in amnion epithelial cells treated with conditioned medium from the choriodecidual membrane explants exposed to GBS (GBS CD). Levels of IL-6 and IL-8 during normal pregnancy can vary but typically below 100 pg/ml [38, 39]. During bacterial infection, levels may reach several hundred or thousands of picograms per

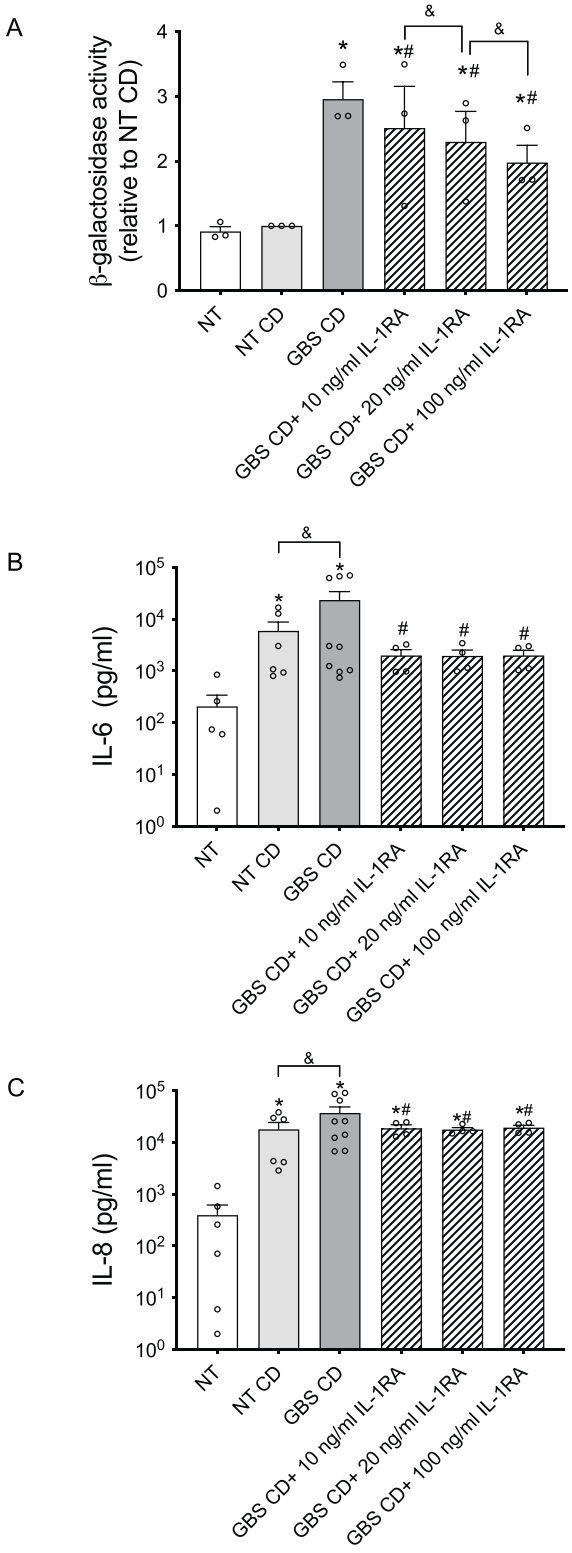

Figure 3. Effect of IL-1 signaling on GBS-mediated senescence in human primary amnion epithelial cells. Human primary amnion epithelial cells were treated with medium (NT), nontreated choriodecidual conditioned medium (NT CD), or GBS-treated choriodecidual conditioned medium in the absence or presence of an IL-1 receptor antagonist (IL-1Ra) for 24 h. β-galactosidase (SA-β-gal) activity (A), secretion of IL-6 (B), and IL-8 (C) were measured. $^{*}P < 0.05$, significant compared to NT, $^{\#}P < 0.05$, significant compared to GBS CD, $^{\&}P < 0.05$, significantly different from each other. $n = 3$ subjects for β-galactosidase activity assay. $n = 4$–9 subjects for cytokine analysis.

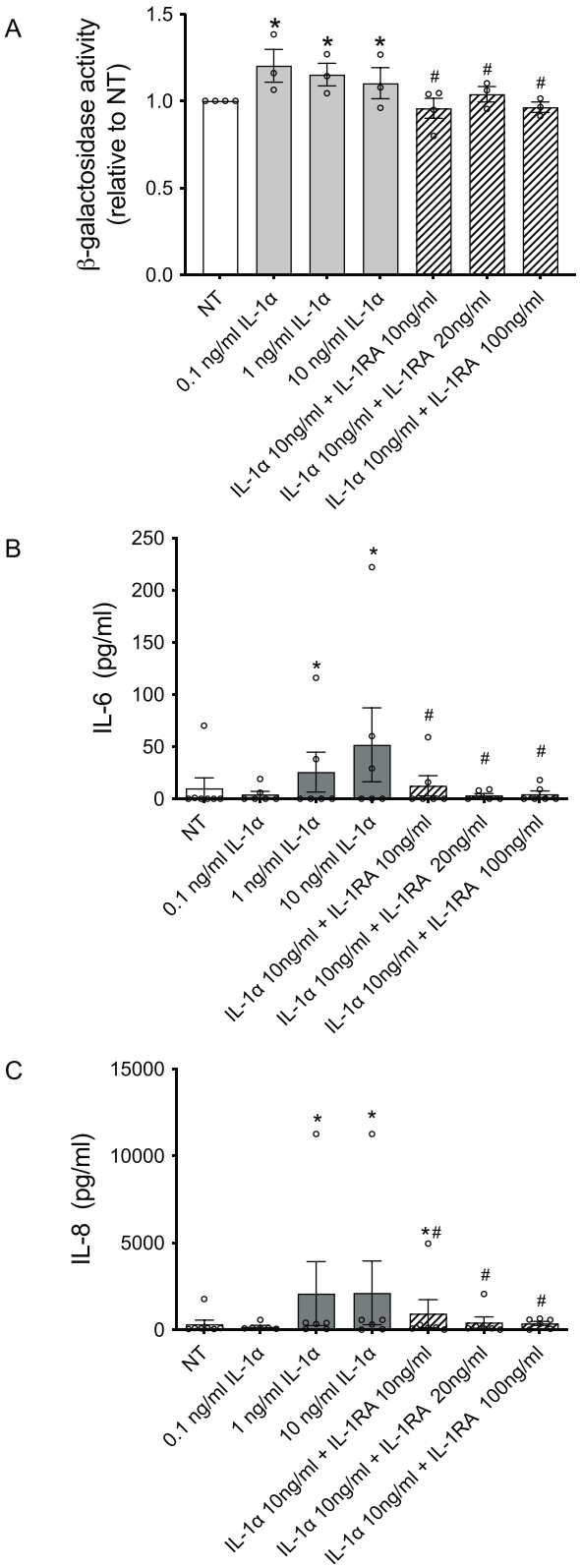

Figure 4. Effect of IL-1α on cellular senescence of human primary amnion epithelial cells. Human primary amnion epithelial cells were treated with IL-1α in the absence or presence of an IL-1 receptor antagonist (IL-1Ra) for 24 h. Then, β-galactosidase (SA-β-gal) activity (A), secretion of IL-6 (B), and IL-8 (C) were measured. $^{*}P < 0.05$, significant compared to NT, $^{\#}P < 0.05$, significant compared to GBS CD, $^{\&}P < 0.05$, significantly different from each other. $n = 3$–4 subjects for β-galactosidase activity assay. $n = 6$ subjects for cytokine analysis.

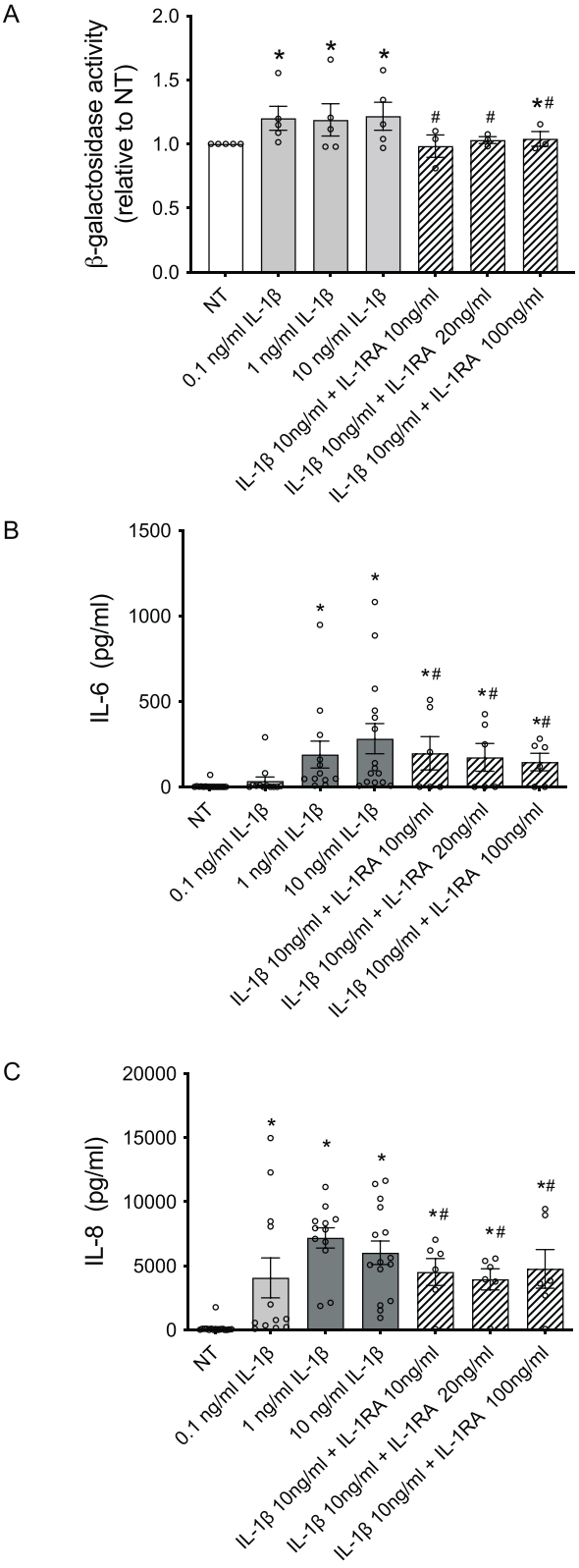

Figure 5. Effect of IL-1β on cellular senescence of human primary amnion epithelial cells. Human primary amnion epithelial cells were treated with IL-1β in the absence or presence of an IL-1 receptor antagonist (IL-1Ra) for 24 h. Then, β-galactosidase (SA-β-gal) activity (A), secretion of IL-6 (B), and IL-8 (C) were measured. $^{*}P < 0.05$, significant compared to NT, $^{\#}P < 0.05$, significant compared to GBS CD, $^{\&}P < 0.05$, significantly different from each other. $n = 3$–5 subjects for β-galactosidase activity assay. $n = 4$–10 subjects for cytokine analysis.

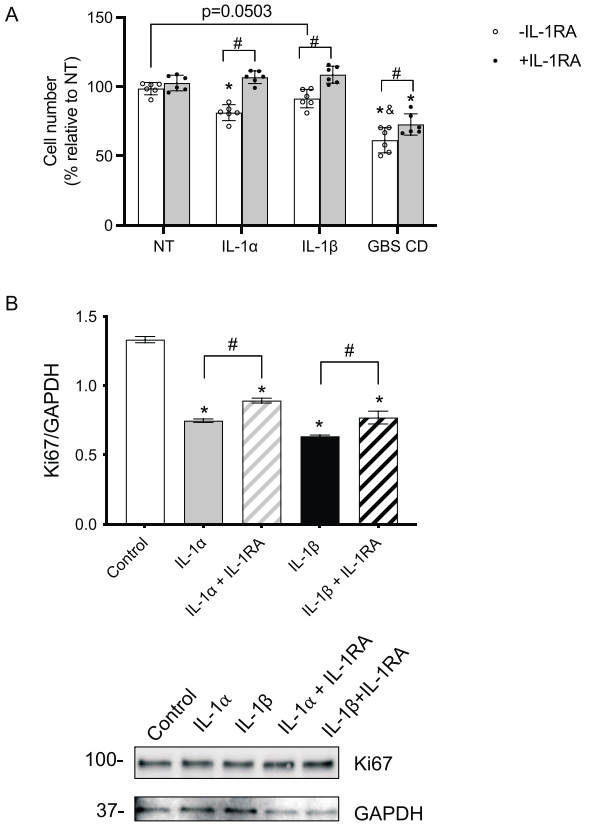

Figure 6. Effect of GBS or IL-1 on cell proliferation of human primary amnion epithelial cells. Human primary amnion epithelial cells were treated with IL-1(A:10 ng/ml or B:50 ng/ml) or GBS CD conditioned medium in the absence or presence of an IL-1 receptor antagonist (IL-1Ra, 100 ng/ml) for 48 h. (A) Cell proliferation was measured by CyQuant assay. $n = 6$. (B) Expression of Ki-67 was measured by western blot. Band densities were quantified by ImageJ. $n = 3$. $^{*}P < 0.05$, significant compared to NT, $^{\&}P < 0.05$, significant compared to GBS CD, $^{\#}P < 0.05$, significantly different from each other.

milliliter (pg/mL) [40, 41]. Therefore, observed IL-6 and IL-8 levels in this study are physiologically relevant.

Accelerated or premature senescence of fetal membranes is a potential mechanism of adverse pregnancy outcomes including preterm labor or PPROM [10, 15, 16]. Although studies of GBS-induced senescence in the fetal membrane are limited, Vanderhoeven *et al.* [19], reported that GBS infection of the choriodecidua increased expression of mRNA for proinflammatory cytokines including IL-1β, IL-6, and IL-8, and downregulated the expression of cytokeratin and cytoskeletal genes critical for membrane integrity and tensile strength, suggesting an increased risk for PPROM by GBS infection. Further study on the role of GBS-induced senescence in the fetal membranes and its implications for pregnancy outcomes is warranted.

In addition to cell cycle regulation pathways, our transcriptomic pathway analysis also showed that GBS activated the p53 signaling pathway in fetal membrane tissue. Interestingly, cell cycle regulation and p53 pathways have been shown to be activated by bacterial species other than GBS (e.g. *Porphyromonas gingivalis*) in placental cell models [42], suggesting that there are some generalizable responses to infection across bacterial species and gestational tissues. However, much more research needs to be done to identify specific and general

responses to different pathogens within the gestational compartment.

We focused on IL-1 signaling, in particular, because IL-1 is a master proinflammatory cytokine known to regulate innate and adaptive immune responses and its dysregulation has been attributed to various infectious and inflammatory diseases [43, 44]. Recent studies suggest roles for IL-1 pathway in the regulation of cellular senescence and SASP in human fibroblasts and rat astrocytes [21–23]. In the present study, we demonstrated IL-1-mediated senescence, SASP (IL-6 and IL-8), and growth inhibition in amnion epithelial cells exposed to GBS CD conditioned medium, indicating paracrine signaling between choriodecidual and amnionic cells [12]. The modest increase in β-galactosidase activity with IL-1α or IL-1β, compared with GBS CD conditioned medium, suggests potential involvement of additional soluble factors or mechanisms in GBS-mediated amnion epithelium senescence. However, accurate comparisons are challenging because GBS or IL-1 was not included in the same experiment because of limited availability of human fetal membranes.

Although the mechanisms by which GBS or IL-1 signaling regulate senescence and SASP in the fetal membrane are not fully understood, however, a few mechanisms have been suggested in previous studies. Binding of IL-1R by either IL-1α or mature IL-1β initiates a signaling cascade that ultimately leads to the activation of the nuclear translocation of the transcription factor NF-κB and subsequent transcriptional activation of numerous inflammatory genes, including IL-6 and IL-8 [43]. NF-κB also controls the transcription of both IL-1α and IL-1β, leading to SASP amplification via a feed-forward loop [21, 45–47]. Additionally, IL-1-induced senescence may involve epidermal growth factor receptor (EGFR) inhibition. For example, EGFR blockage, by tyrosine kinase inhibitors or humanized monoclonal antibodies, promotes senescence by increasing IL-1 cytokine production [48]. Transcriptomic analysis of GBS-inoculated choriodecidual membrane in this study shows upregulation of *NFKB1*(nuclear factor kappa B subunit 1) with concomitant downregulation of *MAP2K3* (Mitogen-Activated Protein Kinase Kinase 3), *MAP2K6* (Mitogen-Activated Protein Kinase Kinase 6), and *PIK3CD* (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta) that are downstream pathways in EGFR signaling [49–51] (Figure 1). Further study on the interaction between NF-κB and EGFR signaling would help gain insights on the mechanisms of GBS-induced senescence in the fetal membranes.

Although this is the first study to examine the specific interplay between GBS infection, senescence of the fetal membrane, and IL-1 signaling, there are a few limitations. To study the contribution of IL-1 from the choriodecidua, we separated choriodecidua from amnion; cultured choriodecidual punch biopsies with GBS; collected and filter sterilized culture media; and exposed human amnion epithelial cells in culture to the choriodecidual conditioned media. Although it is possible that GBS may directly produce cellular senescence in the amnion epithelium, previous studies suggest that amnion epithelial cells are incapable of producing IL-1 [52, 53]. Instead, IL-1β likely serves as a paracrine signaling molecule between the choriodecidua and the amnion epithelium and regulates secretion of human beta defensin-2 in amnion epithelial cells [12]. This supports a role for the choriodecidua as a source of IL-1 and an activator of senescence pathways in amnion epithelial cells. Nonetheless, the direct impact of GBS on senescence of the amnion will be explored in future studies. Second, IL-1 signaling does not fully explain the GBS-mediated senescence and SASP in this study. GBS stimulates the secretion of biomolecules including cytokines (IL-1α, IL-1β, TNF-α, IL-6, and IL-8), MMPs, and prostaglandins from the fetal membranes [12, 20], which are part of SASP. Therefore, combinational or synergistic effects of various molecules may explain the stronger responses in GBS CD-treated amnionic epithelial cells compared with IL-1α or IL-1β treatment alone.

In addition, recent studies propose that extracellular vesicle (EV)-mediated senescence and senescence-associated inflammation as a novel mechanism of signaling in human fetal membrane [15, 54, 55]. Inflammatory signals from senescent fetal membranes may be propagated via EV to the uterus and cervix and trigger premature parturition [15]. A recent study shows that maternal decidual and myometrial cell-derived exosomes exposed to pathological stimuli induce inflammatory response in amnion epithelial cells and chorionic trophoblast cells, indicating bidirectional signaling at the fetal–maternal interface via EV [14]. Future characterization of EVs from GBS-exposed fetal membrane and/or from GBS alone will broaden our understanding of GBS-induced senescence and adverse pregnancy outcomes.

In summary, our study sheds light on the role of IL-1 signaling in GBS-mediated senescence and SASP factor secretion in human fetal membranes in relevance with adverse pregnancy outcomes. Further investigations focusing on the molecular mechanisms in the regulation of GBS-induced senescence in the human placenta or fetal membrane are warranted. Understanding the intricate interplay between these cytokines and senescence may provide crucial insights into the pathogenesis of adverse pregnancy outcomes associated with GBS infection and potentially offer targets for therapeutic interventions.

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Author contributions

H-RP, KAH, and RLC conceived and designed research; MCC helped obtaining human placenta; H-RP and KAH performed experiments; H-RP, KAH, and SMH analyzed data and interpreted results of experiments; H-RP prepared figures; H-RP, KAH, and SMH drafted manuscript; all authors edited and revised manuscript; all authors approved final version of manuscript.

Conflict of Interest: The authors have no conflicts of interest to declare.

Supplementary Data

Supplementary data are available at *BIOLRE* online.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

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