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Effect of *Lactobacillus rhamnosus* GR-1 Supernatant on Cytokine and Chemokine Output From Human Amnion Cells Treated With Lipoteichoic Acid and Lipopolysaccharide

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Abstract

Preterm birth occurs in 9% to 13% of all human pregnancies and accounts for 80% of all neonatal morbidities and mortalities. Approximately 40% of all preterm births are idiopathic and about half are associated with infection and/or an activated inflammatory process. Further to studies showing anti-inflammatory effects of supernatant from the probiotic *Lactobacillus rhamnosus* GR-1 (GR-1), we tested its ability to modulate cytokine and chemokine production from amnion cells in response to stimulation by bacterial wall components, lipopolysaccharide (LPS), and lipoteichoic acid (LTA). Placentae were collected from women undergoing elective cesarean section at term. Amnion cells were cultured for 48 hours to confluence, serum starved for 12 hours, and then treated with GR-1 supernatant (1:20 dilution), followed after 12 hours by LPS (100 ng/mL) or LTA (10 ng/mL) for an additional 12 hours. Both LTA and LPS caused significant increases in the concentration of the pro-inflammatory cytokine, tumor necrosis factor α (TNF-α; 103.9 $\pm$ 67.5 pg/mL and 368.3 $\pm$ 65.7 pg/mL, respectively) in medium from cultured amnion cells compared to control (<4 pg/mL). There was no significant effect of GR-1 supernatant alone on TNF-α output, but there was significant reduction after LPS treatment. The basal output of the immunomodulatory cytokine, interleukin 6, was 613 $\pm$ 170 pg/mL and increased significantly after addition of GR-1 supernatant, LTA, LPS, and combinations of LTA/LPS with GR-1 supernatant. In conclusion, probiotic *L rhamnosus* GR-1 attenuates the effect of both LPS and LTA in stimulating the output of the pro-inflammatory cytokine TNF-α from mixed cultures of human amnion cells in keeping with previous findings in human trophoblast cells.

Keywords

cytokines, amnion, *lactobacillus*, preterm labor, probiotics

Introduction

Preterm birth occurs in 9% to 13% of all human pregnancies[1] and accounts for 80% of all neonatal morbidities and mortalities. Approximately 40% of all preterm births are idiopathic and about half are associated with infection and/or an activated inflammatory process.[1,2] Conditions such as bacterial vaginosis are characterized by a decline in endogenous lactobacilli in the vagina and may be associated with a 40% risk of preterm birth.[2] Many of the microbes present in amniotic fluid samples during intrauterine infection are of vaginal origin. However, before reaching the upper genital tract, bacteria and their by-products likely come into direct contact with the fetal membranes, the chorion and amnion. An alteration in the vaginal microbiota can trigger the body's innate immune system to produce an inflammatory response, characterized by increased levels of cytokines and chemokines derived from amnion and chorion that are secreted into the amniotic fluid.[3-7] Elevated levels of cytokines and chemokines cause recruitment of immune cells and increase prostaglandin production in the fetal

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membranes and decidua. The net result is the activation of downstream pathways leading to increased metalloproteinase expression with subsequent cervical ripening, membrane weakening, and myometrial activation.

The use of antibiotics to prevent preterm birth is largely ineffective and may even increase the risk of labor.[8,9] The use of probiotics, "live microorganisms which when administered in adequate amounts confer a health benefit on the host," may provide an alternative prophylactic treatment for women at risk of preterm labor (PTL).[10] The major species of *Lactobacillus* found in healthy vaginal flora are *L iners*, *L crispatus*, and *L jensenii*,[11-17] but the probiotic *L rhamnosus* GR-1 (GR-1) can multiply in the vagina[18] and when given orally to nonpregnant women can help restore a healthy vaginal microbiota.[19]

We have previously reported that a supernatant fraction from GR-1 prevents the expected increase of tumor necrosis factor α (TNF-α) in response to lipopolysaccharide (LPS) by placental and chorion trophoblasts, increases interleukin (IL)-10 output from these cells,[20,21] and partially prevents LPS-induced preterm delivery in mice.[22] In addition, we have shown that LPS-induced PTL in pregnant mice is mitigated by pretreatment with GR-1 supernatant in association with a downregulation of pro-inflammatory cytokines in uterine tissues, maternal plasma, and amniotic fluid. We therefore considered that, in addition to chorion and placenta, the amnion that occupies a critical position between the amniotic fluid and maternal tissues might be involved. Amnion is responsive to bacterial products and cytokines, thereby potentially playing an important role during infection and/or inflammation. The objective of the present study was to examine the ability of GR-1 to modulate cytokine and chemokine production from amnion cells in response to stimulation by bacterial wall components, LPS, and lipoteichoic acid (LTA).

Materials and Methods

Placenta Collection

All studies were approved by the Faculty of Medicine, University of Toronto, and Mount Sinai Hospital (Toronto, Canada) ethics review boards in accordance with the Canadian Tri-Council Policy Statement on Human Ethics Reviews (institutional review board #04-0018-U). Placentae were collected at Mount Sinai Hospital (Toronto, Canada) from women undergoing elective cesarean section at term (>37 weeks gestation), showing no signs of labor and without any indication of clinical infection. Indications for delivery by cesarean section included abnormal presentation of the fetus, cephalopelvic disproportion, or previous cesarean section. Written informed consent was obtained prior to tissue collection.

Amnion Cell Culture

Amnion was stripped from underlying choriodecidua, washed in saline, cut into fragments (1.5 cm^2) and digested in Dulbecco's Modified Eagle Medium (DMEM, GIBCO Invitrogen, Burlington, Ontario, Canada) + 0.1% trypsin (45 minutes) at 37°C. Tissue was then filtered (0.8-nm gauze) and newborn calf serum (Wisent Incorporated, St Bruno, Quebec, Canada) added to inhibit trypsin activity. After further digestion, cells were pooled, centrifuged at 192*g* for 5 minutes, and resuspended in culture medium (DMEM-F12; GIBCO Invitrogen) plus 10% fetal bovine serum (Wisent Inc) and 1% antibiotics. The remaining tissue was digested for 1 hour in DMEM + 0.1% collagenase (Roche Diagnostics, Mannheim, Germany), and the cell harvests pooled and counted using a Trypan Blue exclusion assay to ensure greater than 98% viability. These cultures contain both amnion epithelial and mesenchymal cells, since there is established interaction between these cell types.[23] The cells were plated to a density of 0.8×10^6 cells per well in a 24-well culture plate.

Lactobacillus Preparation

Lactobacillus rhamnosus GR-1 was grown anaerobically at 37°C in de Man, Rogosa, and Sharpe medium for 48 hours to reach the stationary growth phase. The culture medium was collected and centrifuged at 6000*g* for 10 minutes at 4°C. Residual bacteria were removed by filtration (0.22-μm pore size filter), and the supernatant divided into aliquots and frozen at −80°C.

Cell Treatments

Amnion cells were cultured for 48 hours (37°C; 5% CO_2: 95% O_2) to confluence. The cultures were serum starved for 12 hours, then treated with GR-1 supernatant (1:20 dilution), followed after 12 hours by LPS (100 ng/mL; Sigma-Aldrich, St Louis, Missouri) or LTA (10 ng/mL; Invivogen, San Diego, California) for an additional 12 hours. Amounts of GR-1, LPS, or LTA were determined from preliminary experiments (see below). Aliquots (100 μL) of medium were collected and frozen at −80°C.

Cytokine and Chemokine Measurements

Enzyme-Linked ImmunoSorbent Assay. In preliminary experiments, levels of IL-1β, IL-4, IL-6, IL-8, IL-10, granulocyte-macrophage colony stimulating factor (GM-CSF), and TNFα in conditioned media were determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit according to the manufacturer's instructions (Thermofisher Scientific, Burlington, Canada). Plate reading and curve fitting were performed on a plate reader (TECAN infinite M200) using Magellan6 software (Thermofisher Scientific, Burlington, Canada). The minimum detection limits were all <4.0 pg/mL.

Bioplex 27-cytokine/chemokine analysis. The cytokine output into culture supernatant was also determined by the Bio-Plex 200 system (Bio Rad Hercules, California) and Bio-Plex Human Cytokine 27-plex assay according to the manufacturers' instructions. Standard curves and the concentration of cytokines within samples were generated with the Bio-Plex version 4.1 software.

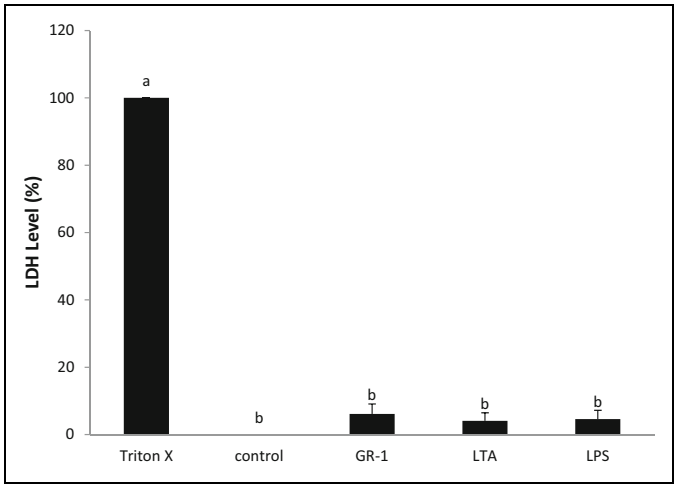

Figure 1. The effects of treatments on amnion cell toxicity. Lactate dehydrogenase (LDH), *Lactobacillus rhamnosus* GR-1 supernatant (GR-1), lipoteichoic acid (LTA), and lipopolysaccharide (LPS). Statistical significance ($P < .05$) is indicated by different letters.

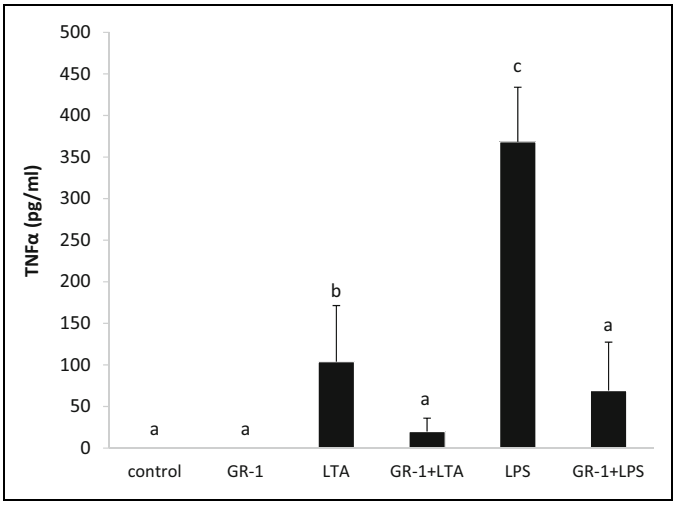

Figure 2. The effects of *Lactobacillus rhamnosus* GR-1 supernatant on tumor necrosis factor (TNF)-α concentration in human amnion cells. *Lactobacillus rhamnosus* GR-1 supernatant (GR-1), lipoteichoic acid (LTA), and lipopolysaccharide (LPS). Statistical significance ($P < .05$) is indicated by different letters.

Statistical Analysis

Data are presented as mean values ± standard error of the mean. Statistical significance between groups was determined using one-way analysis of variance followed by Student Newman-Keuls post hoc analysis using STAT32 version 2.0 statistical software.

Results

Viability of Cultured Cells

In order to determine whether treatments affected cell viability, levels of lactate dehydrogenase (LDH) were measured after 12 hours of incubation (Figure 1). None of the GR-1 supernatant, LTA, or LPS treatments resulted in significant changes in cytotoxicity compared to the control group. Triton X treatment, producing maximal cell death, was significantly different from all other groups ($n = 5$; $P < .05$).

Optimization of Time and Dose of Treatments

Preliminary studies were performed on mixed human amnion cell cultures treated with increasing LPS (0.1-1000 ng/mL) or increasing LTA doses (1-1000 ng/mL) for an incubation period of 12 hours. Interleukin 6 and IL-8 concentrations were determined by ELISA. Peak stimulation was found with LPS (100 ng/mL) and LTA (10 ng/mL) for IL-6 and IL-8 outputs. Time optimization studies were performed on the LTA and LPS doses for 4 to 24 hours; 12 hours was determined as the optimum time to reach maximum cytokine output. The GR-1 supernatant was tested at dilutions ranging between 1:10 and 1:100, with 1:20 being chosen as the optimum, as in previous studies with placental trophoblast primary cultures and mouse macrophages.[20-22]

Correlation of ELISA Versus Bioplex Method

In preliminary studies, both ELISA and Bioplex methodology were used to analyze cytokines and chemokines in the treatment groups. To compare these methods, the concentration of IL-6 was determined in the same samples by both procedures. The R^2 value was 0.8728 between the data sets, indicating a high correlation between the 2 methodologies (data not shown). Subsequent measurements reported in this manuscript utilized principally the Bioplex assay.

Effects of Lactobacillus rhamnosus GR-1 Supernatant on Cytokine and Chemokine Output by Amnion Cells Treated With LTA and LPS

Both LTA and LPS caused significant increases in the concentration of the pro-inflammatory cytokine, TNF-α (103.9 ± 67.5 pg/mL and 368.3 ± 65.7 pg/mL, respectively) in medium from cultured amnion cells compared to control (<4 pg/mL; $n = 5$; $P < .05$; Figure 2). There was no significant effect of GR-1 supernatant alone on TNF-α output, but it significantly reduced the stimulation seen after LPS treatment (both $P < .05$). In these experiments, IL-1β levels were undetectable across all treatment groups ($n = 8$).

Concentrations of the anti-inflammatory cytokines, IL-10 ($n = 8$) and IL-4 ($n = 6$), were below detectable limits (<4.0 pg/mL, <2.0 pg/mL) and were not altered in response to any one of LPS, LTS, or GR-1 supernatant treatments (data not shown).

Basal output of the immunomodulatory cytokine, IL-6, was 613 ± 170 pg/mL ($n = 12$) and increased significantly after addition of GR-1 supernatant, LTA, and LPS, and combinations of LTA/LPS with GR-1 supernatant (Figure 3, all treatments $P < .05$ compared to control). There were no significant differences between these treatment values.

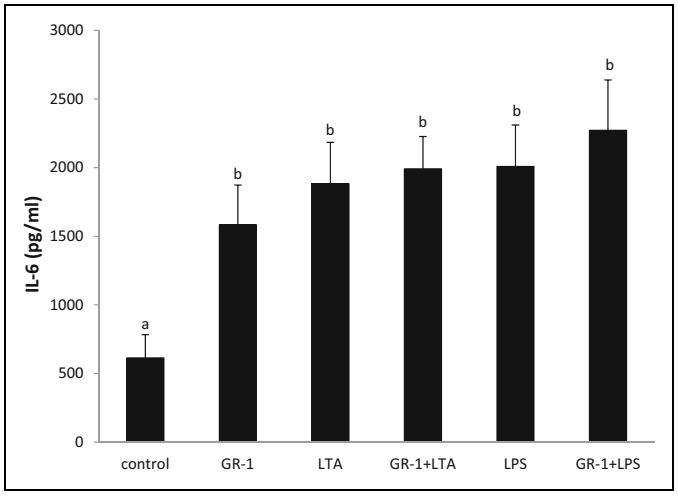

Figure 3. The effects of *Lactobacillus rhamnosus* GR-1 supernatant on interleukin (IL)-6 concentration in human amnion cells. *Lactobacillus rhamnosus* GR-1 supernatant (GR-1), lipoteichoic acid (LTA), and lipopolysaccharide (LPS). Statistical significance ($P < .05$) is indicated by different letters.

Basal outputs of chemokines monocyte chemotactic protein-1 (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES), and IL-8 were 1.1 ± 0.3 pg/mL (n = 8), 29.2 ± 14.4 pg/mL (n = 7), and 377 ± 110 pg/mL (n = 13), respectively (Figure 4). *Lactobacillus rhamnosus* GR-1 supernatant, but not LPS or LTA alone, significantly increased the output of MCP-1 ($P < .05$; Figure 4, bottom panel). The concentration of MCP-1 after GR-1 supernatant plus either LPS or LTA was not significantly different from that after GR-1 supernatant alone. A similar profile was seen in the effects of treatments on the concentration of RANTES; there was stimulation with GR-1 supernatant alone or with LTA or LPS, but no significant effect of either LTA or LPS alone (Figure 4, middle panel). Concentrations of IL-8 were significantly higher than control after treatment with GR-1 supernatant, LTA, LPS, and combinations of GR-1 supernatant with LTA or LPS (Figure 4, top panel), but none of the treatment groups were significantly different from any other.

Basal outputs of the immune cell-activating cytokines interferon (IFN)-γ and GM-CSF from amnion cells were 1.6 ± 0.7 pg/mL (n = 9) and 5.2 ± 5.2 pg/mL (n = 8), respectively. The output of IFN-γ was stimulated significantly by GR-1 supernatant alone and in combination with LTA or LPS (Figure 5, top panel, $P < .05$) but not by LTA or LPS alone. The output of GM-CSF was not altered significantly by GR-1 supernatant, LTA or LPS alone, but was significantly higher after treatment with GR-1 supernatant + LTA (Figure 5, bottom panel, $P < .05$).

Discussion

In this study, we have shown that the probiotic *L rhamnosus* GR-1 produces a substance(s) that attenuates the effect of both

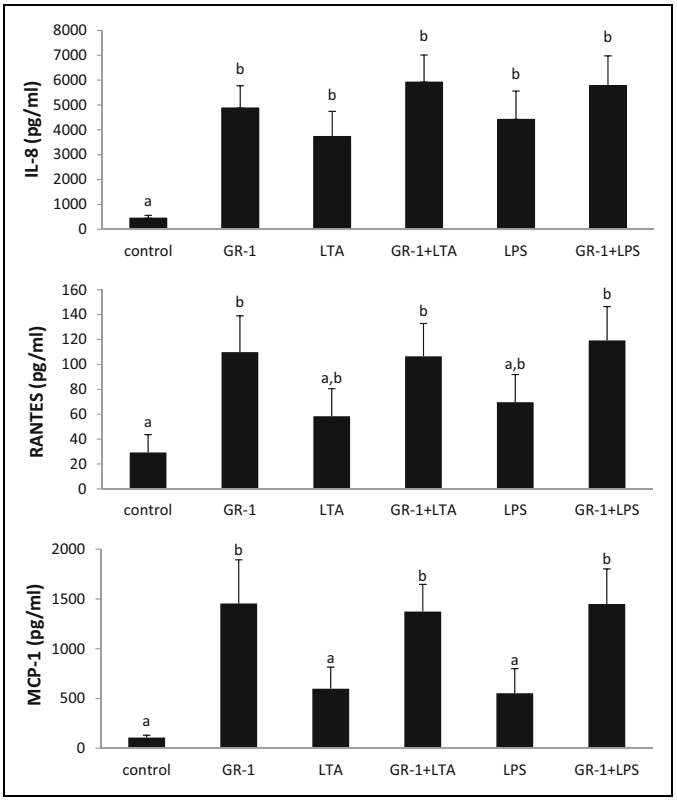

Figure 4. The effect of *Lactobacillus rhamnosus* GR-1 supernatant on chemokine concentrations in human amnion cells. *Lactobacillus rhamnosus* GR-1 supernatant (GR-1), lipoteichoic acid (LTA), and lipopolysaccharide (LPS). Statistical significance ($P < .05$) is indicated by different letters.

LPS and LTA in stimulating output of the pro-inflammatory cytokine TNF-α from mixed cultures of human amnion cells. Although we did not observe measurable amounts of the anti-inflammatory cytokines, IL-10 and IL-4, we did find that GR-1 supernatant increased the output of IL-6, chemokines MCP-1, RANTES, and IL-8, as well as IFN-γ. Significant stimulation of GM-CSF by GR-1 supernatant was found only in the presence of LTA. These findings suggest that cells within mixed amnion populations respond to endotoxins with altered output of cytokines and that *L rhamnosus* GR-1 is able to modify this response. The interaction with lactobacilli appears to initiate attenuation of some inflammatory mediators (TNF-α), yet also produces a protective anti-inflammatory response to endotoxin. Although extrapolation of these in vitro studies with cultured amnion cells are made with caution, the results suggest that this modulator ability could be important during pregnancy to prevent PTL.

We chose to study the effects on mixed amnion cell cultures since this most accurately reflects the in vivo environment. It is known that cytokine and chemokine production from amnion cell cultures can vary depending on the relative proportions of epithelial and mesenchymal cells,[23] which in turn can differ across preparations and with time. Cells were cultured for 12 hours in all experiments in order to minimize potential variability across experiments.

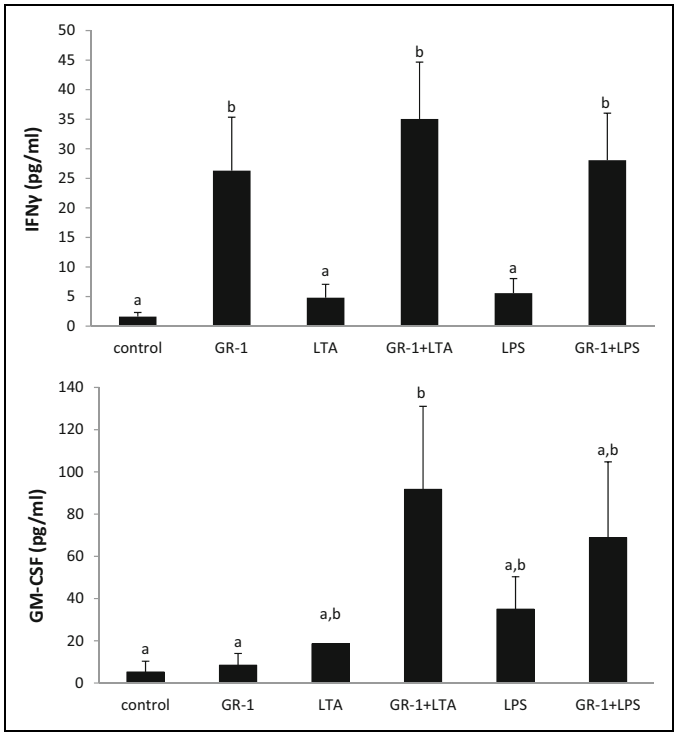

Figure 5. The effects of *Lactobacillus rhamnosus* GR-1 supernatant on immune cell activating cytokines. *Lactobacillus rhamnosus* GR-1 supernatant (GR-1), lipoteichoic acid (LTA), and lipopolysaccharide (LPS). Statistical significance ($P < .05$) is indicated by different letters.

The ability of lactobacilli to reduce the output of pro-inflammatory cytokines in amnion as well as in chorion and placental trophoblast cells adds to these previous findings.[20,21] In a recent report, Bloise et al[24] showed that heat-killed *L rhamnosus* GG stimulated IL-4 and IL-10 expression by human placental trophoblasts and attenuated LPS-induced TNF-α release, thus supporting our previous findings.[20,21] The GG and GR-1 strains are somewhat different genetically, even though they are both *L rhamnosus* species, but the latter appears superior in its ability to propagate in the vagina. Like the GG strain,[24] the GR-1 supernatant reduced TNF-α in amnion after treatment with LTA.

The increased output of the anti-inflammatory cytokine IL-10 after treatment with *L rhamnosus* GR-1 supernatant in trophoblast and the IL-10 responses of chorion were found in tissue from pregnancies carrying a female fetus.[20] In the present study, the IL-10 output was undetectable from amnion cells, regardless of the sex of the fetus and irrespective of GR-1 treatment. However, it is interesting that amnion cells responded to *L rhamnosus* GR-1 supernatant and to *L rhamnosus* GR-1 plus LTA/LPS with increased output of MCP-1 and RANTES. These are CXC chemokines known to be involved in recruitment of macrophages and neutrophils. Term labor, as well as PTL, is associated with increased flux of these cell types into the decidua[24] and fetal membranes.

The mechanism of GR-1 effects is of interest. We confirmed that *L rhamnosus* GR-1 has low or negligible toxicity on amnion cell cultures, based on LDH release. Further, the ability of *L rhamnosus* GR-1 to attenuate the effect of LPS or LTA on the TNF-α output by amnion does not appear to be attributable to IL-10 since levels of this cytokine remained undetectable. Interestingly, Bloise et al[24] showed that *L rhamnosus* GG increased urocortin (Ucn) output by trophoblast cells and Ucn could also attenuate LPS-induced TNF-α release. To date, the moieties within the *L rhamnosus* GR-1 supernatant that are responsible for the activity(ies) we describe have proved difficult to identify.

The finding that LPS and LTA stimulated TNF-α output in cultured human amnion cells confirms previous reports.[25,26] Pro-inflammatory cytokines, including TNF-α, increase production of prostaglandins in the amnion and upregulate metalloproteinases leading to rupture of the membranes, cervical ripening, and uterine contractions associated with PTL.[7,27-29] Downregulating TNF-α may interfere with activation in the amnion of these infection and/or inflammation-mediated processes associated with PTL. *Lactobacillus* supernatant also increased IL-6, IFN-γ, and GM-CSF in amnion cells providing a possible means of countering pro-inflammatory effects. These chemokines increase TNF-α receptor and/or interleukin-1 receptor antagonist (IL-1ra), further suggesting a potential route by which *L rhamnosus* GR-1 may minimize the effect of pro-inflammatory cytokines.[30-34]

Although *L rhamnosus* GR-1 supernatant downregulated key pro-inflammatory cytokine production in amnion cells, it also increased IL-6 to levels similar to those detected after LTA and LPS stimulation. Elevated IL-6 amniotic fluid levels are associated with intrauterine infection and are used as a diagnostic marker of women with threatened PTL.[35]

The increased release of CXC, MCP-1, and RANTES and the CC chemokine IL-8 compared to control groups suggests that GR-1 supernatant stimulates a chemokine environment capable of recruiting both monocytes and neutrophils to fetal membranes. The M1 macrophage phenotype is induced by TNF-α, IFN-γ, GM-CSF, and LPS, creating macrophages that exhibit strong pro-inflammatory properties.[36,37] In contrast, the M2 macrophage phenotype is stimulated by IL-4 and IL-13 creating macrophages that encourage attenuated pro-inflammatory responses and elevated anti-inflammatory responses.[36] *L rhamnosus* GR-1 supernatant increased levels of GM-CSF production, an anti-inflammatory cytokine that is also increased in placental trophoblast cells by similar treatments. Therefore, we suggest that immune cells are activated to a state capable of defending against pathogens, while the accompanying pro-inflammatory response is minimized by the effects of *L rhamnosus* GR-1. Further studies are required to determine the importance of this relationship in normal human pregnancy.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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