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Letter

Environmental Toxicant Exposure Paralyzes Human Placental Macrophage Responses to Microbial Threat

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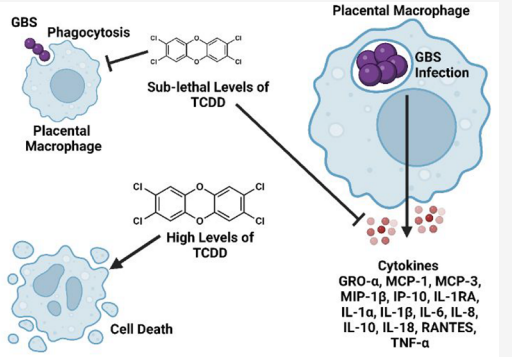
ABSTRACT: Exposure to environmental toxicants (such as dioxins) has been epidemiologically linked to adverse reproductive health outcomes, including placental inflammation and preterm birth. However, the molecular underpinnings that govern these outcomes in gravid reproductive tissues remain largely unclear. Placental macrophages (also known as Hofbauer cells) are crucial innate immune cells that defend the gravid reproductive tract and help promote maternal−fetal tolerance. We hypothesized that exposure to environmental toxicants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) could alter placental macrophage responses to inflammatory insults such as infection. To test this, placental macrophages were cultured in the presence or absence of TCDD and then infected with the perinatal pathogen Group B *Streptococcus* (GBS). Our results indicate that TCDD is lethal to placental macrophages at and above a 5 nM concentration and that sublethal dioxin exposure inhibits phagocytosis and cytokine production. Taken together, these results indicate that TCDD paralyzes placental macrophage responses to bacterial infection.



KEYWORDS: *environmental toxicants, TCDD, GBS, placental macrophages, antimicrobial responses*

Industrial manufacturing and human chemical warfare have resulted in the contamination of the environment with a wide array of environmental toxicants.[1] Many of these contaminants are long-lasting molecules, referred to as "forever chemicals", which persist in environmental reservoirs.[1,2] Some of these persistent environmental contaminants, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are potent endocrine-disrupting compounds that can affect reproduction in humans.[3−6] Indeed, murine model studies of TCDD exposure *in utero* revealed that the F1 offspring have an altered risk for adverse pregnancy outcomes, including susceptibility to perinatal infection with pathogenic bacteria.[3−6]

Streptococcus agalactiae, or Group B *Streptococcus* (GBS), is a Gram-positive bacterial pathogen that can cause perinatal infections.[7−10] GBS colonization of the rectovaginal niche represents a strong risk factor for invasive infection and cognate disease outcomes, including chorioamnionitis, preterm prelabor rupture of membranes, preterm birth, maternal sepsis, and neonatal sepsis.[7−10] Approximately 10−40% of pregnant patients will test positive for GBS colonization during pregnancy.[11,12] Although universal screening and early interventions to treat GBS infection have reduced early-onset neonatal sepsis incidence, late-onset disease and adverse pregnancy outcomes that occur prior to screening, such as preterm birth, remain an ongoing problem in the clinic.[13,14]

During infection of a pregnant individual, GBS invades the gravid reproductive tract tissues and encounters sentinel innate immune cells, such as placental macrophages (also referred to as Hofbauer cells).[15,16] Placental macrophages defend the gravid reproductive tract from infectious assault and promote maternal−fetal tolerance to maintain a healthy pregnancy.[17−19] Previous work indicates that placental macrophages can phagocytose GBS and secrete a repertoire of cytokines and chemokines in response to infection.[20−24] However, the role of environmental factors, such as toxicant exposure, in these responses remains largely obscure. We hypothesized that the placental macrophage exposure to TCDD could alter innate immune responses to infection. To that end, we utilized an *ex vivo* primary human placental macrophage culture assay to

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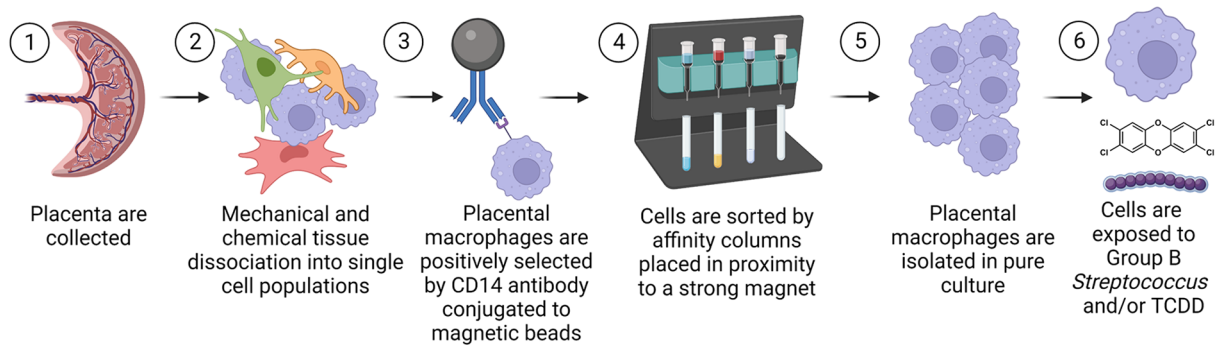

Figure 1. Isolation of placental macrophages. De-identified placental tissue was collected from non-laboring patients who delivered healthy, full-term infants by Caesarian section. Villous core tissue was mechanically separated and enzymatically digested. Isolation of $CD14^+$ cells was performed via a magnetic MACS Cell Separation system. Human placental macrophage cells were isolated in pure culture before being used in assays with TCDD and/or GBS. Image created with BioRender.

evaluate the contribution of TCDD exposure to macrophage responses to GBS. Our results revealed that TCDD is toxic to placental macrophages at concentrations of 5 nM or above, but at sublethal concentrations TCDD inhibits macrophage phagocytosis of GBS and cytokine responses to infection.

TCDD Is Toxic to Placental Macrophages. To evaluate the effect of TCDD on placental macrophage viability, macrophages were isolated from human placenta (Figure 1) and cultured for 4 h in medium alone or supplemented with increasing concentrations of TCDD, reflective of physiological exposures (0.005, 0.025, 0.1, 1, 5, 10, and 25 nM).[3,5] Viability was then assessed by the ATPLite assay to quantitate cellular ATP levels. Exposure to 0.005, 0.025, 0.1, or 1 nM TCDD resulted in ATP levels that were statistically indistinguishable from the untreated control samples (Figure 2). However,

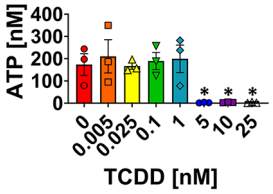

Figure 2. Analysis of human placental macrophage viability by the ATPLite assay. Macrophages were cultured in medium alone (0) or supplemented with increasing concentrations (0.005, 0.025, 0.1, 1, 5, 10, and 25 nM) of TCDD for 4 h, then viability was assessed by ATPLite assay to quantitate cellular ATP levels. Exposure to 5, 10, or 25 nM TCDD resulted in a statistically significant decrease in cellular ATP ($P \leq 0.05$, one-way ANOVA with Dunnet's *post hoc* multiple corrections test).

exposure to 5, 10, or 25 nM TCDD resulted in a 74-fold, 52-fold, or 58-fold decrease, respectively, in cellular ATP levels compared to untreated samples ($P = 0.0475$, $P = 0.0491$, $P = 0.0485$, respectively, one-way ANOVA with Dunnet's multiple corrections test). Comparatively, THP-1 macrophage cell viability has been shown to be impaired beginning at 60 nM TCDD, suggesting placental macrophages exhibit higher sensitivity than THP-1 immortalized cell lines to TCDD.[25]

Sublethal TCDD Exposure Inhibits Placental Macrophage Phagocytosis and Internalization of GBS. To evaluate the contribution that TCDD exposure makes in placental macrophage antimicrobial responses, we evaluated placental macrophage phagocytosis of fluorescently labeled GBS (Figure 3A). Four hours of exposure to 0.025 nM TCDD resulted in a 17-fold reduction in intracellular bacterial

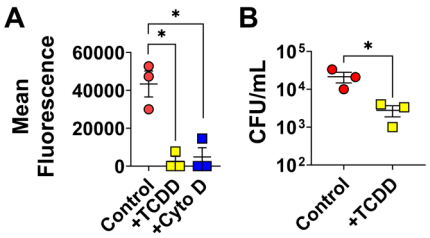

Figure 3. Evaluation of human placental macrophage phagocytosis and internalization of GBS. (A) To evaluate placental macrophage phagocytosis, fluorescently labeled GBS cells were cultured in medium alone (Control), 0.025 nM TCDD (TCDD), or 5 μM of the known phagocytosis inhibitor cytochalasin D (Cyto D) as a positive control. After the extracellular signal was quenched with trypan blue stain, intracellular bacterial fluorescence was measured. TCDD and Cyto D inhibited placental macrophage phagocytosis of GBS compared to untreated controls, a result that was statistically significant ($^*P \leq 0.05$, one-way ANOVA with Tukey's *post hoc* multiple comparisons test). (B) To evaluate intracellular bacterial numbers, gentamicin protection and quantitative culture assays were performed. Our results indicated that TCDD exposure resulted in a significant reduction in intracellular GBS within placental macrophages ($^*P \leq 0.05$, Mann–Whitney U analysis, $N = 3$ biological replicates from separate placental samples from different donors, error bars indicate standard error of the mean).

fluorescence compared to untreated controls ($P = 0.0031$, one-way ANOVA with Tukey's *post hoc* multiple comparisons test). Treatment with the known phagocytosis inhibitor cytochalasin D resulted in a 9-fold reduction in intracellular bacterial fluorescence compared to untreated controls ($P = 0.0041$, one-way ANOVA with Tukey's *post hoc* multiple comparisons test) and was statistically indistinguishable from TCDD treatment, underscoring that sublethal concentrations of TCDD potently inhibit phagocytosis. Additionally, placental macrophage cell viability in the presence of GBS infection after 4 h of treatment with 0.025 nM TCDD was evaluated; no significant difference in cellular ATP was observed across these treatments (Supporting Information). We also performed gentamicin protection and quantitative culture assays to enumerate the number of bacteria inside placental macrophages (Figure 3B). Our results indicated that TCDD exposure resulted in an 8-fold reduction in intracellular GBS

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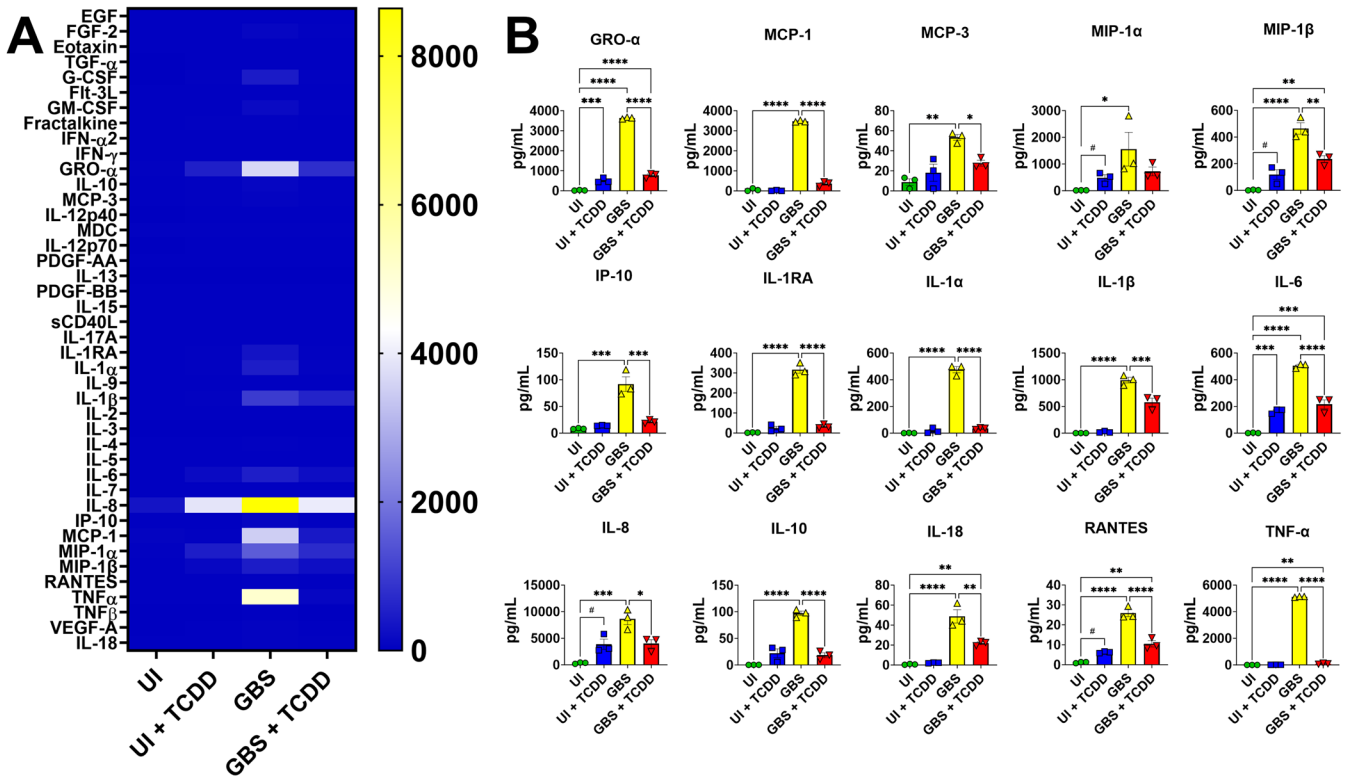

Figure 4. Analysis of cytokine and chemokine production by human placental macrophages. Placental macrophages were cultured in medium alone or medium supplemented with TCDD (+TCDD) prior to infection with GBS (+GBS), and uninfected (UI) negative controls were also included. Supernatants were collected and analyzed by multiplex assay to quantify the cytokines and chemokines produced. (A) Heat map analysis of cytokine production by human placental macrophages. Blue indicates low expression (0 pg/mL), white is medium expression (4000 pg/mL), and yellow is high expression (8000+ pg/mL). Values used to calculate the heat map were derived from the mean of three biological replicates. (B) Individual bar graphs of cytokines significantly altered by GBS infection and/or TCDD exposure (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, one-way ANOVA with Tukey's *post hoc* multiple comparisons test; #$P < 0.05$, Student's t test). Bars indicate mean ± standard error, with individual data points from different biological replicates derived from separate placental samples overlaid upon the bars ($N = 3$).

within placental macrophages, a significant decrease as determined by Mann−Whitney U analysis ($P = 0.0500$). Taken together, these results indicate that TCDD treatment blunts placental macrophage ability to phagocytose and internalize GBS. Li and colleagues observed that TCDD exposure (up to 50 nM) resulted in lower expression of adhesion molecules such as ICAM-1, VCAM-1, and CD11b, decrease in cell pseudopodia, and cognate repression of F-actin expression in THP-1 macrophage cells.[26] Because changes in cell adherence, pseudopodia function, and cytoskeletal remodeling are critical for phagocytosis, it is possible that TCDD affects these pathways in both THP-1 and placental macrophage cell types.

Sublethal TCDD Exposure Inhibits Placental Macrophage Cytokine Responses to GBS Infection. To initiate innate and adaptive immune responses to defend the host against invasive infections, placental macrophages secrete cytokines and chemokines in response to infection. Our previous work indicates that human placental macrophages secrete a repertoire of cytokines and chemokines in response to GBS infection, including granulocyte-colony stimulating factor, granulocyte-macrophage-colony stimulating factor, GRO-α, interleukin (IL)-1RA, IL-1α, IL-1β, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, and tumor necrosis factor (TNF)-α.[20,21] In our current study, we observed statistically significant increases in placental macrophage production of GRO-α ($P < 0.0001$), MCP-1 ($P < 0.0001$), MCP-3 ($P = 0.0011$), MIP-1α ($P = 0.0441$), MIP-1β ($P < 0.0001$), IP-10 ($P = 0.0001$), IL-RA ($P < 0.0001$), IL-1α ($P < 0.0001$), IL-1β ($P < 0.0001$), IL-6 ($P < 0.0001$), IL-8 ($P = 0.005$), IL-10 ($P < 0.0001$), IL-18 ($P < 0.0001$), RANTES ($P < 0.0001$), and TNF-α ($P < 0.0001$) in GBS-infected human placental macrophages compared to uninfected controls (one-way ANOVA with Tukey's *post hoc* multiple comparisons test) (Figure 4). TCDD exposure in uninfected placental macrophages resulted in significant increases in GRO-α ($P = 0.0011$), MIP-1α ($P = 0.0087$), MIP-1β ($P = 0.0167$), IL-8 ($P = 0.0112$), and RANTES ($P < 0.0001$) production compared to untreated samples (Student's t test, one-tailed with Welch's correction), as well as IL-6 ($P < 0.0001$, one-way ANOVA), indicating TCDD exposure is sufficient to initiate a low-level cytokine and chemokine response, although the magnitude of these changes was smaller than those initiated by infection with GBS. Similar results were reported by Tanha and colleagues, who observed elevated IL-6 production by peripheral blood mononuclear cells in response to TCDD exposure.[27] Additionally, Selvam and colleagues reported that TCDD exposure in RAW 264.7 macrophage cells enhanced production MIP-1α and MIP-1β.[28] Recent work published by Sciullo et al. indicates TCDD can induce IL-8 expression in the U937 human macrophage cell line.[29] Taken together, these results indicate that TCDD exposure can alter

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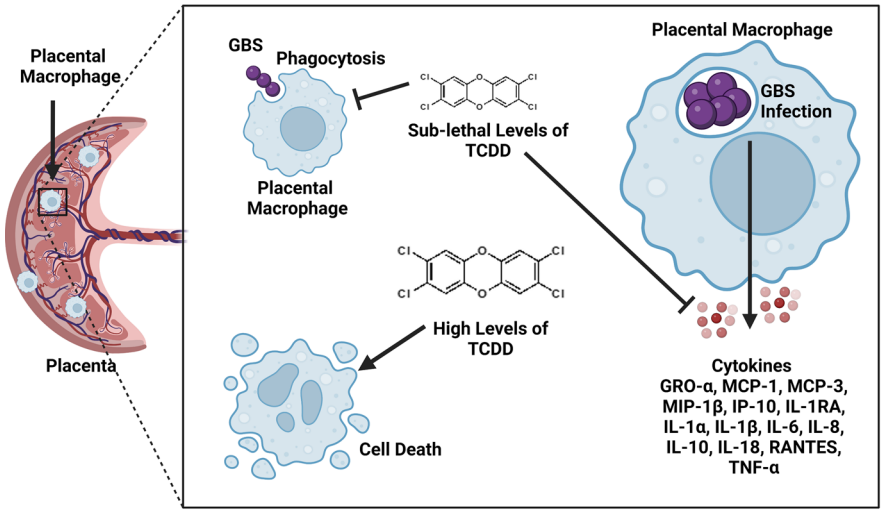

Figure 5. Conceptual diagram of the effects of TCDD exposure on human placental macrophages. Exposure to high levels of TCDD (at or above 5 nM) results in placental macrophage cell death. Exposure to sublethal doses of TCDD (at 0.025 nM) results in decreased placental macrophage phagocytosis, internalization of GBS, and inhibition of cytokine secretion in response to infection. Image created with BioRender.

cytokine and chemokine secretion in a wide variety of macrophage cell types.

Interestingly, in our study, exposure to TCDD significantly blunted placental macrophage production of GRO-α ($P < 0.0001$), MCP-1 ($P < 0.0001$), MCP-3 ($P = 0.0285$), Mip-1β ($P = 0.0034$), IP-10 ($P = 0.0004$), IL-RA ($P < 0.0001$), IL-1α ($P < 0.0001$), IL-1β ($P = 0.0010$), IL-6 ($P < 0.0001$), IL-8 ($P = 0.0166$), IL-10 ($P < 0.0001$), IL-18 ($P = 0.0024$), RANTES ($P < 0.0001$), and TNF-α ($P < 0.0001$) in response to GBS infection. TCDD has been shown to attenuate inflammatory responses in other infection-related inflammation models, such as a pertussis toxin-mediated systemic inflammation model and a mouse model of influenza A infection.[30,31] Domínguez-Acosta and colleagues also reported that TCDD exposure significantly decreased production of the pro-inflammatory cytokines TNF-α, IL-6, and IL-12 after macrophage activation with lipopolysaccharide/interferon.[32] This study identified the target of TCDD, the aryl hydrocarbon receptor (AhR), as a negative regulator of the innate and adaptive immune responses to infection and proposed an interaction between AhR and RelA/p65 (the major nuclear factor (NF)-κB subunit) which plays a crucial role in immune responses to infection.[32] Our study showed a TCDD-blunted placental macrophage inflammatory response to infection (Figure 5), which could indicate similar TCDD targeting of the AhR receptor, but further confirmation of this requires additional studies.

Most human exposures to dioxins occur as a consequence of eating contaminated food.[33] Of the 50 foods most commonly associated with high TCDD contamination, 16 of these are consumed in disproportionately high quantities by pregnant people, thereby enhancing the risk of exposure in this population.[34] Additionally, *in vivo* animal and *ex vivo* tissue studies indicate that TCDD accumulates in placental tissues and that concentrations of TCDD are higher in placental tissue than in peripheral blood.[35−37] Augustowska et al. observed a 36% bioaccumulation of TCDD to a final concentration of 0.101 ng/mL in placental tissue (~314 nM) under *ex vivo* exposure conditions, underscoring that TCDD has the capacity to bioaccumulate at the maternal−fetal interface, where placental macrophages reside, in higher concentrations than we utilized for our study. Indeed, our study has several limitations, including the use of a single cell type in an *ex vivo* model and constant single-dose exposure that may or may not be reflective of *in vivo* concentrations.

In conclusion, we report that exposure to the environmental toxicant TCDD alters human placental macrophages functions. Specifically, exposure to high levels of TCDD (at or above 5 nM) results in placental macrophage cell death. Exposure to sublethal doses of TCDD (at 0.025 nM) results in decreased placental macrophage phagocytosis and internalization of GBS. Sublethal TCDD exposure also inhibits secretion of numerous cytokines, including GRO-α, MCP-1, MCP-3, MIP-1β, IP-10, IL-1RA, IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-18, RANTES, and TNF-α in response to GBS infection.

METHODS

Bacterial Strains and Culture Conditions. *S. agalactiae* strain GB00112 (GB112) is a capsular serotype III and sequence type 17 strain isolated by post-partum rectovaginal swabs.[38] Bacterial strains were grown on tryptic soy agar plates supplemented with 5% sheep blood (blood agar) plates and sub-cultured in Todd-Hewitt broth (THB) at 37 °C.

Ethics Statement. This study was carried out with approval from the Vanderbilt University Medical Center Institutional Review Board (VUMC IRB #181998), and patients were enrolled in the study with written informed consent.

Isolation of Primary Human Placental Macrophage Cells. De-identified placental tissue was collected from non-laboring patients who delivered healthy, full-term infants by Caesarian section at Vanderbilt University Medical Center under VUMC IRB #181998. All placental macrophages were isolated according to our previously published methods.[20,21] Briefly, villous core tissue was mechanically separated and enzymatically digested with hyaluronidase, collagenase, and DNase (Sigma-Aldrich) and suspended in Roswell Park Memorial Institute (RPMI) medium supplemented with L-glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fetal bovine serum (referred to as "modified

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RPMI") supplemented with antibiotics, and antifungal factors (Figure 1). Cells were filtered and collected by centrifugation, and isolation of $CD14^+$ cells was performed via a magnetic MACS Cell Separation system with CD14 microbeads (Miltenyi Biotec). Cells were cultured overnight in modified RPMI supplemented with 1% antibiotic/antimycotic solution (ThermoFisher) at 37 °C in 5% carbon dioxide. The following day, cells were suspended in fresh antibiotic/antimycotic-free modified RPMI for experimental assays.

Assessment of Placental Macrophage Viability. Placental macrophage cells were seeded at a density of 50,000 cells per well in a polystyrene, 96-well culture plate in RPMI supplemented with L-glutamine, HEPES, plus 10% charcoal stripped fetal bovine serum alone or supplemented with increasing concentrations (0.005, 0.025, 0.1, 1, 5, 10, and 25 nM) of TCDD and then incubated for 4 h in a humidified atmosphere at 37 °C and 5% CO_2. Supernatants were removed, and the ATPLite assay (PerkinElmer) was performed on the remaining cells to quantify cellular ATP levels as a proxy for cell viability. Briefly, 50 μL of cell lysis buffer was added to each well and incubated on an orbital shaker for 5 min at room temperature. Then 50 μL of substrate solution was applied to each well and incubated on an orbital shaker for 5 min at room temperature. The solution was transferred to a black-bottom plate, and luminescence was measured using a Bio-Tek Synergy HT plate reader. A standard curve was used to calculate the ATP concentration from each sample.

Evaluation of Placental Macrophage Phagocytosis of GBS. Phagocytosis assays were performed as previously described.[39] Briefly, placental macrophages were seeded into black-bottom 96-well plates and exposed to 0.025 nM TCDD for 4 h, and untreated controls were also maintained before supernatants were removed and fresh RPMI supplemented with L-glutamine, HEPES, plus 10% charcoal stripped fetal bovine serum was added. As a positive control for phagocytosis inhibition, cells were pretreated with 5 μM cytochalasin D for 30 min. Bacteria were grown to stationary phase in Todd-Hewitt broth overnight before being washed three times with labeling buffer (0.1 M $NaHCO_3$, pH 9.2). FITC was added to a final concentration of 0.25 mg/mL to the bacteria in labeling buffer and incubated with shaking in dark conditions for 1 h. Bacteria were washed three times with labeling buffer, and the OD_{600} was measured to determine cell density (with a coefficient of 1 OD_{600} = 10^9 bacteria). FITC-labeled GBS was co-cultured with placental macrophage cells at a multiplicity of infection (MOI) of 10 bacterial cells to each placental macrophage (10:1) for 2 h in static conditions at 37 °C in 5% carbon dioxide. Co-cultures were washed three times with sterile phosphate buffered saline (PBS) before extracellular bacterial fluorescence was quenched with trypan blue stain. Intracellular bacterial fluorescence was measured at an excitation wavelength of 495 nm and emission at 519 nm with a Bio-Tek Synergy HT plate reader.

Gentamicin Protection Assays to Quantitate Intracellular Bacteria. To enumerate intracellular GBS, placental macrophages were exposed to 0.025 nM TCDD for 4 h, and untreated controls were also maintained before supernatants were removed and fresh RPMI supplemented with L-glutamine, HEPES, plus 10% charcoal stripped fetal bovine serum was added. GBS was co-cultured with placental macrophage cells at an MOI of 10 bacterial cells to each placental macrophage (10:1) for 1 h in static conditions at 37 °C in 5% carbon dioxide. Co-cultures were washed with sterile media, resuspended in fresh RPMI medium containing 100 μg/mL of gentamicin (Sigma) to kill extracellular bacteria, and further incubated for 1 h at 37 °C. Co-cultures were washed three times with sterile PBS, lysed in 1 mL of distilled water, serially diluted in PBS, and plated on blood agar medium to enumerate the number of viable intracellular bacteria.

Evaluation of Cytokine and Chemokine Production. To evaluate cytokine and chemokine production, multiplex assays were employed as previously described.[20,21] Briefly, placental macrophages were cultured in medium alone (modified RPMI) or medium supplemented with 0.025 nM TCDD for 4 h. Supernatants were removed, and fresh modified RPMI was added. GBS was co-cultured with placental macrophage cells at an MOI of 10 bacterial cells to each placental macrophage (10:1) for 24 h in static conditions at 37 °C in 5% carbon dioxide. Supernatants were collected, 10 mg/mL of penicillin was added, and samples were passed through a 0.22 μm filter. Samples were frozen at −80 °C until analyses were performed. Samples were analyzed by Eve Technologies via a multiplex cytokine array (Eve Technologies).

Statistical Analyses. Statistical analysis of data with more than two groups was performed using one-way ANOVA with Tukey's or Dunnet's *post hoc* correction for multiple comparisons; all reported P values were adjusted to account for multiple comparisons. For parametric data with two groups, a one-tailed Student's t test with Welch's correction was used. For nonparametric tests (such as CFU data which is log-transformed prior to analysis), one-tailed Mann−Whitney U analyses were performed. $P \leq 0.05$ was considered significant. All data analyzed in this work were derived from at least three biological replicates (representing placental macrophages derived from different patient placental samples). Statistical analyses were performed by using GraphPad Prism 9 (GraphPad Software Inc.).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.3c00490.

Figure S1, evaluation of placental macrophage cellular ATP after GBS infection ± TCDD treatment, and methods (PDF)

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Notes

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