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TLR9 Activation Coupled to IL-10 Deficiency Induces Adverse Pregnancy Outcomes

Jessica E. Thaxton,* Roberto Romero,† and Surendra Sharma2*

Pregnancy outcome is severely compromised by intrauterine infections and inflammation. Although the pregnant uterine microenvironment is replete with innate immune cells and TLR expression, the mechanisms that facilitate adverse effects of their activation are largely unknown. In this study, we mimic the activation of TLR9 with its pathogenic ligand hypomethylated CpG and demonstrate that IL-10 proficiency protects against CpG-induced pregnancy complications. We show that fetal resorption and preterm birth are rapidly induced in IL-10−/− mice by low doses of CpG (~25 μg/mouse) when injected i.p. on gestational day 6 or gestational day 14, respectively. In contrast, wild-type mice failed to experience such effects at comparable doses, but pups born at term displayed craniofacial/limb defects in response to higher doses (~400 μg/mouse). Pregnancy complications in IL-10−/− mice were associated with unexpected and robust TLR9-triggered activation and amplification of uterine neutrophil and macrophage subpopulations followed by their migration to the placental zone. Furthermore, a dramatic increase in serum levels of mouse KC and TNF-α production by uterine F4/80+ cells, but not uterine NK or Gr-1+CD11b+ cells, was observed. Depletion of F4/80+ macrophages or neutralization of TNF-α rescued pregnancy to term. Our results have important implications for IL-10-mediated “uterine tolerance” against CpG-driven innate immune activation. The Journal of Immunology, 2009, 183: 1144–1154.

A common link for a significant proportion of early and late pregnancy maladies lies in intrauterine infections and inflammation (1, 2). In this regard, the role of the innate immune system at the maternal-fetal interface in response to normal pregnancy intrauterine milieu or inflammatory stimuli has attracted an abundance of recent interest. Although a vigorous uterine immune system juxtaposes the fetal tissue predominated by innate sentinels, NK cells and macrophages, it does not pose any intolerance to normal fetal development and survival of invading trophoblasts (3–5). We and others have proposed that uterine NK (uNK)3 cells produce angiogenic and pregnancy-compatible factors and are involved in local endovascular processes and regulation of trophoblast invasion (3, 6–8). On the other hand, in a mouse model, we have demonstrated that uNK cells become antagonistic to pregnancy in response to bacterial products (9, 10). Thus, the underlying mechanisms of diverse pathogen-mediated inflammatory events that may trigger cytotoxic activation of maternal immune cells and trophoblasts require further exploration.

A group of innate immune sentinel receptors known as TLRs are present not only on uterine leukocytes, but also on trophoblast cells, thus implying an active cross-talk between the placenta and local immunity (11–13). TLRs have evolved to recognize specific pathogen-associated molecular patterns enabling them to serve as the first line of defense in the innate immune system (14, 15). Although TLRs are ancient receptors without memory requirements for cells that express them, they harbor the ability to transduce both negative and positive signals depending on interactions with their immediate microenvironment (16). Moreover, pathogenic load and gene-environment interactions are likely to be overriding factors in the outcome of inflammation-induced positive versus negative cascades. Our recent studies provide support to this hypothesis in that very low doses of LPS cause adverse pregnancy outcomes in IL-10−/− mice compared with their wild-type (WT) counterparts (9, 10).

Systemic or intrauterine bacterial and viral infectious agents, and their breakdown products, are likely to lead to the excessive presence of pathogenic hypomethylated CpG DNA motifs which are recognized by TLR9 (17, 18). Few studies have focused on the role of TLR9-mediated immune activation during pregnancy. Using C57BL/6 mice, it has been shown that high doses of CpG cause deformities in pups born to treated dams (19). On the other hand, CpG has been used as an adjuvant to reduce negative fetal outcomes induced by Listeria monocytogenes in BALB/c pregnant dams (20). In other settings, it has been shown that direct injection of CpG DNA in neuroblastomas induces complete tumor rejection in mice and elicits long-term Th1-driven immunity (21). In addition, antitumor effects of CpG have been demonstrated in different intracranial models of syngeneic glioma (22, 23). These observations imply that CpG motifs are capable of triggering strong and polarized immune responses which may be beneficial or harmful depending on the intrinsic microenvironment.

References

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3. Abbreviations used in this paper: uNK, uterine NK; ODN, oligodeoxynucleotide; UMGc, uterine mononuclear and granular cells; DHR, dihydrorhodamine 123; gd, gestational day; ROS, reactive oxygen species; mKC, mouse KC.
Given the proposed widespread use of CpG as a treatment and vaccine tool among the general population, including pregnant individuals, it is important to determine whether overactivation of the immune system in response to nononcogenic doses of CpG can lead to negative pregnancy outcomes (20, 24–28). In this study, we examined the role of the CpG-TLR9 axis in a mouse model of pregnancy with a focus on the protective role of pregnancy-compatible cytokines such as IL-10. CpG-induced TLR9 activation has been associated mainly with stimulation of systemic immunity. Our observations allow us to elucidate a link between CpG-mediated activation of innate immune responses and IL-10 deficiency at the maternal-fetal interface that leads to adverse pregnancy outcomes.

Materials and Methods

Mice

Mice used in this study, C57BL/6 and C57BL/6 IL-10−/−, were obtained from The Jackson Laboratory. All mice were housed in a specific pathogen-free facility supervised by the Central Research Department of Rhode Island Hospital. All protocols were approved by the Lifespan Animal Welfare Committee and conducted according to its guidelines. Mice of 8–10 wks of age were mated and each experimental group contained at least three mice. The day of vaginal plug appearance was designated gestational day (gd) 0.

In vivo treatment of pregnant mice

WT and IL-10−/− mice received i.p. injections of CpG oligodeoxynucleotide (ODN 1826; InvivoGen) at doses of 15, 25, 100, 300, or 400 μg on gd6 or gd14. For IL-10−/− mice, suitable doses were 15 or 25 μg/gdam since higher doses caused maternal demise. Cellular depletions were performed with CpG injection on gd6 or gd14. One hundred microliters of anti-asialo GM1 (Wako) or nonimmune rabbit serum (Antibodies) was administered on gd4, 6, and 9 or gd9, 11, and 14 for NK cell depletion. Two hundred fifty micrograms of anti-Gr-1 (RB6-8C5; BD Biosciences) or isotype Ab (rat IgG1; BD Biosciences) was administered at gd5. Two hundred fifty micrograms of anti-F4/80 (BM8; E Bioscience) or isotype Ab (rat IgG2a, κ; E Bioscience) was given on gd5 and 7 or gd7 and 15. Competitive antagonist experiments were performed in IL-10−/− mice and i.p. injections of 100 μg or 50 μg of antagonist ODN (ODN 2088; InvivoGen) were given with 25 μg of CpG ODN on gd6. Monoclonal anti-TNF-α Ab (Gr81-2626; BD Pharmingen) was administered i.p. at 250 μg on gd5 and gd7 with CpG ODN injection on gd6 or on gd13 and gd15 with CpG ODN injection on gd14.

Cellular preparation

Uterine mononuclear and granular cells (UMGC) were obtained via mincing and mechanical dispersion of whole gd 8–9 or gd15 uteroplacental tissue in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin, and l-glutamine. Single-cell suspensions from uterine horns were run through a 100-μm cell strainer and subjected to density gradient separation in RPMI 1640 supplemented with 10% FBS, penicillin/streptomycin, and L-glutamine. Single-cell suspensions from uterine horns were run through a 100-μm cell strainer and subjected to density gradient separation (Fico-Lite LM (Atlanta Biologicals). Experiments were performed using Fico-Lite LM (Atlanta Biologicals). Isolated UMGC were washed in PBS and reconstituted in PBS containing 2% FBS (stain buffer). Combinations of Abs were added for extracellular staining for 30 min at 4°C, rinsed with stain buffer and acquired via flow cytometry (FACSCanto; BD Biosciences). Fluorochrome-conjugated isotype-matched Abs were used as controls. Abs to TNF-α (MP6-XT22) and IFN-γ (XM1G1.2) were purchased for intracellular staining (BD Biosciences). UMGC were washed with stain buffer and incubated in 96-well plates for 4–6 h with Brefeldin A (BD Biosciences), PMA (Calbiochem), and ionomycin (Calbiochem). Cells were washed twice with stain buffer and stained extracellularly as described above. For intracellular staining, UMGC were washed with Perm Wash (BD Biosciences) and fixed via flow cytometry (FACSCanto; BD Biosciences). Isolated UMGC were washed in PBS and resuspended in 1-ml tubes, allowed to clot for 30 min at room temperature, spun at 8000 rpm for 20 min at 4°C, and supernatants were collected and frozen for further analysis. TNF-α, IL-12, IFN-γ, mKC, MIP-1α, and MIP-2 were assayed using Quantikine ELISA kits (R&D Systems) and experiments were performed according to the manufacturer’s instructions. Separate serum samples were collected from each experimental treatment group (n = 9).

Immunohistochemistry

Fetoplacental units from varying experimental conditions were removed from uterine horns and placed in 4% buffered formalin or snap frozen in a cassette with OCT. Samples in the former treatment were paraffin embedded 24 h after fixation. Staining for mKC (KC Rabbit Polyclonal Ab; BioVision) was performed on paraffin-embedded tissue from gd9- or gd13-treated and control-matched samples as previously described (29). F4/80+ (MCA497R rat and mouse Ag; Serotec) cells were detected using paraffin-embedded tissue as described previously (30). Gr-1 (purified rat anti-mouse Ly6G and Ly6C; BD Biosciences) staining was performed on frozen gd9 and gd15 tissue cut into 10-μm sections as described previously (31).

Statistical analysis

Statistical significance of pregnancy outcomes was examined using the one-way ANOVA method. All experiments where flow cytometry plots were analyzed for n = 3 or more animals per condition and significance was assessed via the t test. Data are expressed as means ± SD. A p ≤ 0.05 was considered to be statistically significant.

Results

CpG ODN treatment induces fetal resorption and preterm birth in IL-10−/− mice

We studied the effect of CpG ODN treatment on pregnancy in either a fetal resorption or preterm birth model using C57BL/6 mice. The CpG ODN motif, CpG 1826, used in these studies has been widely used to trigger B or T cell-specific immunity (32, 33). To assess the ability of CpG ODN to induce fetal resorption, we injected i.p. varying doses of CpG ODN in IL-10−/− or WT mice on gd6. We initially tested a dose range from 5 to 400 μg/dam and found that the 25-μg dose was optimal in IL-10−/− mice to induce complete resorption of uterine horns. At the dose of 100 μg/dam or higher, we observed maternal wasting in IL-10−/− mice. As Fig. 1A demonstrates, fetal resorption was consistently observed in IL-10−/− mice in numerous matings (n = 37) at the 25-μg/dam dose. In contrast, no negative pregnancy effects were observed at this dose in WT mice (n = 8) as they maintained pregnancy to term (~20 days) and delivered healthy pups.

Next, we aimed to find a dose of CpG ODN that caused a negative pregnancy outcome in WT mice in response to gd6 administration. A range of doses up to 250 μg/dam did not exert any adverse consequences on pregnancy outcome or gestational length. However, at a dose of 400 μg/dam, a significant number of pups (43%) were born at term with cranial and distal limb malformations (Fig. 1C). These results are consistent with previously published data demonstrating similar deformities to pups born to dams treated with 300 μg of CpG ODN (19).

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ODN at these doses did not display teratogenic effects. We have previously shown that LPS administered to IL-10−/− and WT mice on gd14 induces preterm birth on gd17, albeit at 20-fold higher doses in WT mice (10). For preterm birth outcomes in response to CpG ODN administration, a similar approach was used. CpG ODN was administered i.p. on gd14 and mice were checked twice daily for preterm birth of newborns. As shown in Fig. 1B, IL-10−/− mice (n = 18) consistently delivered stillborn pups at a dose of 15 µg/dam within 24–48 h of injection. Lower doses of CpG ODN failed to induce preterm delivery. Similar to fetal resorption observations, WT mice (n = 4) failed to experience any negative consequences of CpG ODN administration at a comparable or higher dose in the range of 300 µg/dam (n = 8), except for deformities at higher doses similar to that shown in Fig. 1C.

Regarding fetal resorption in IL-10−/− mice, we noted that effects were rapid with severe uteroplacental pathology at 25 µg of CpG ODN (Fig. 1A). To characterize the kinetics and general pathology in IL-10−/− mice in response to CpG ODN administration, we visually assessed fetal resorption from gd7 through gd9. Fig. 2A demonstrates fetal resorption at 25 µg of CpG ODN as observed in uterine horns harvested as early as gd7 and the placental pathology appeared to increase in severity on gd8 and gd9. In contrast, control ODN-treated mice did not experience fetal resorption.
the pregnant mouse uterus by gd7 (4, 39, 40). These cells remain localized to the maternal side of uteroplacental tissue within the mesometrial triangle and decidua basalis. However, it is not yet clear whether these uterine immune cells acquire a cytotoxic phenotype and migrate to the placental zone to cause local damage. In this regard, we have recently shown for the first time that uNK cells are indeed activated in response to LPS administration and cause apoptosis in the placental region (10). It is possible that severe and rapid pathology in CpG ODN-treated mice (Figs. 1 and 2A) may be programmed by the mechanisms of cellular activation and placental injury.

There is abundant evidence in nonpregnant mice that CpG ODN treatment leads to activation of dendritic cells (41). We prepared single-cell suspensions of UMGC populations were isolated from IL10−/− (A and C) or WT (B and D) mice on gd9 or gd15 after treatment with control or CpG ODN. Cells were gated on CD45 populations for CD11b Gr-1 (A and B) and CD11b F4/80 (C and D); data shown are representative of multiple experiments using four animals per condition. A, IL10−/− mice showed a significant increase in the CD11b Gr-1 population on gd9 and gd15, p < 0.05. B, WT mice under similar conditions did not show any significant changes in this population. C, IL10−/− mice showed a significant increase in the CD11b F4/80 cell population on gd9 and gd15, p < 0.05, compared with control ODN-treated mice. D, WT mice under similar conditions did not show any significant changes in this population.
see any changes in the dendritic cell populations of IL-10−/− or WT mice treated with 25 μg of CpG ODN on gd6 (data not shown).

Interestingly, analysis of gd8 and 9 uterine mononuclear and granular cells from CpG ODN-treated IL-10−/− mice showed a significant increase in the CD11b+ Gr-1+ cellular population compared with vehicle-treated mice (9.3 ± 2.5 to 28.8 ± 4.4%; Fig. 3A). Similarly, we observed an increase in the CD11b+ GR1+ cellular population in IL-10−/− mice under preterm birth conditions when analyzed on gd15 (6 ± 1.4 to 23.7 ± 3.5%; Fig. 3A). A simultaneous increase occurred in CD11b− F4/80+ populations. When analyzed on gd8 and 9, this population increased from 11.1 ± 0.9% to 35.0 ± 4.0% and a similar increase occurred in the preterm birth condition, 9.5 ± 3.1% to 25 ± 2.6% (Fig. 3C). At the doses used to induce fetal resorption or preterm birth in IL-10−/− mice, we did not observe any significant changes in either of these cellular populations in WT mice (Fig. 3, B and D).

Analysis of uNK cells revealed a slight increase in NK1.1+ CD3− cells in CD45+ mononuclear and granular cells from gd8 and 9 and gd15 tissues from IL-10−/− but not WT mice. However, depletion of uNK cells using anti-asialo GM1 Ab proved ineffective in rescuing fetal resorption or preterm birth in IL-10−/− mice treated with CpG ODN (data not shown). Thus, we focused on the role of macrophages and neutrophils in adverse pregnancy outcomes because these cells were highly amplified in response to CpG ODN treatment.

**FIGURE 4.** CpG ODN treatment leads to elevated production of mKC in IL-10−/− mice. mKC was measured in sera collected from IL-10−/− or WT mice on gd9 (A) or gd15 (B). Data represent average values from nine different serum samples. IL-10−/− mice showed a significant increase in mKC levels above control ODN-treated mice (B). When analyzed on gd8 and 9, this population increased from 3.1% to 23.7% (Fig. 3A). Similarly, we observed an increase in the CD11b+ neutrophil population from 4.4% to 28.8% (Fig. 3D). mKC was measured in sera collected from IL-10−/− mice. mKC was evaluated by immunohistochemistry done on uterine tissue compared with control ODN-treated tissue on gd9 (C) and gd15 (D). M, Demarcates the mesometrial triangle, whereas P indicates the placental region.

mKC is significantly induced in CpG ODN-treated IL-10−/− mice

We addressed the possibility that a suitable chemokine was induced in response to CpG ODN treatment in IL-10−/− mice that led to the unscheduled recruitment and amplification of granulocyte and monocyte (CD11b+ GR1−, or CD11b+ F4/80+) cellular populations. Initial screening was done for serum levels of MIP-1α, MIP-2, and mKC using chemokine-specific ELISAs. No significant changes were observed for MIP-1α or MIP-2 in serum samples from control ODN or CpG ODN-treated mice (data not shown). However, mKC, the mouse homologue of human uNK cells and it has been proposed that this chemokine...
might be involved in recruitment of invading trophoblasts or macrophages for placental growth (6). We hypothesize that increased levels of mKC in response to CpG ODN motifs are responsible for the increased presence of CD11b<sup>+</sup>/Gr-1<sup>+</sup> and CD11b<sup>+</sup>/F4/80<sup>+</sup> cells in the uterine microenvironment.

Although an increase in serum mKC was observed, we wanted to determine that this protein was also produced locally at the maternal-fetal interface in response to CpG ODN. We performed mKC-specific immunostaining on paraffin-embedded sections of CpG ODN-treated uteroplacental tissue from IL-10<sup>−/−</sup> mice obtained on gd9 or gd15. Although tissue from control ODN-treated mice showed basal mKC staining in the mesometrial and developing placental regions on gd9 or gd15 (Fig. 4, C and D), CpG ODN treatment resulted in intense staining throughout the tissue organization collected from gd9 or gd15. Isotype control Ab failed to stain any specific regions of tissue sections (Fig. 4, C and D).

**CpG ODN-activated Gr-1<sup>+</sup> and F4/80<sup>+</sup> uterine cells migrate to the placenta in IL-10<sup>−/−</sup> mice**

As mentioned earlier, uterine immune cells remain localized to the maternal part of uteroplacental tissue (3, 5, 9, 40). In this study, we tested the hypothesis that CpG ODN treatment imparts migration of Gr-1<sup>+</sup> and F4/80<sup>+</sup> uterine cells to the placenta leading to adverse pregnancy outcomes. Gr-1<sup>+</sup> populations can be indicative of macrophages or neutrophils, thus we first aimed to specify the contribution of each of these particular cell types at the placental level. From tissues harvested on gd9 and gd15, we isolated mononuclear and granular cells and analyzed the CD45<sup>+</sup> population by FACS for macrophage or neutrophil markers F4/80 and Ly6G, respectively. F4/80 positivity was markedly increased in both the fetal resorption (10.5 ± 1.3 to 21.5 ± 1.3%) and preterm birth conditions over control levels (6.5 ± 1.3 to 21.0 ± 3.1%; Fig. 5A). We next examined whether F4/80<sup>+</sup> cells migrated to the placental zone. Uteroplacental tissue from gd9 was processed for immunohistochemical analysis of F4/80 positive staining (see Materials and Methods). As shown in Fig. 5C, no significant staining was observed with isotype Ab in CpG ODN-treated tissue. However, there was an abundance of F4/80<sup>+</sup> cells that were found not only in the mesometrial zone but also in the placental zone at gd9 (Fig. 5C). Control ODN-treated tissue showed low levels of F4/80<sup>+</sup> cells localized only to mesometrial zone (data not shown).

We next aimed to delineate the specific presence of neutrophils in uteroplacental tissue. As shown in Fig. 3, Gr-1<sup>+</sup> cells increased significantly in the CD45<sup>+</sup> uterine population from...
CpG ODN-treated IL-10\(^{-/-}\) mice. The Gr-1 receptor is composed of the Ly6G and Ly6C subreceptors (44). Although it is commonly accepted in the literature that Gr-1-positive cells are representative of neutrophils, we assessed these cells using the Ly6G Ab. As shown in Fig. 5B, Ly6G\(^+\) cells increased significantly from control levels in both gd9 (5.7 ± 1.7 to 53.2 ± 7.1%) and gd15 (7.8 ± 3.0 to 28.3 ± 6.5%) tissues. Immunohistochemical analysis further demonstrated migration of Gr-1\(^+\) cells well into the placental zone in tissue harvested on gd9 from CpG ODN-treated IL-10\(^{-/-}\) mice (Fig. 5D). Taken together, these data imply that migration of macrophages and neutrophils to the placenta may lead to pathological outcomes.

**TNF-\(\alpha\) functions as the regulator of CpG ODN-mediated pregnancy loss**

As discussed above, mKC was detected at high levels in serum of CpG ODN-treated IL-10\(^{-/-}\) mice, suggesting that it could contribute to pregnancy complications. However, experiments involving mKC neutralization did not result in rescue of pregnancy (data not shown). We hypothesized that a distinct cytotoxic factor(s) was produced by macrophages and/or neutrophils that induced adverse pregnancy outcomes in IL-10\(^{-/-}\) mice. CpG ODN is known to induce the expression of a variety of cytotoxic proteins, including IFN-\(\gamma\), TNF-\(\alpha\), and IL-12 (45). We measured the concentration of IFN-\(\gamma\), IL-12, and TNF-\(\alpha\) in serum samples collected on gd9 from mice treated with 25 \(\mu\)g of CpG ODN or control ODN on gd6 and in gd15 serum from mice treated with 15 \(\mu\)g of CpG ODN or control ODN on gd14. In both the fetal resorption (50.1 ± 15.0 to 168 ± 30.9 pg/ml) (Fig. 6A) and preterm birth conditions (51.0 ± 11.9 to 196.9 ± 36.1 pg/ml; Fig. 6B), we noted a significant increase in serum TNF-\(\alpha\) levels in CpG ODN-treated IL-10\(^{-/-}\) mice, but not in CpG ODN-treated WT mice. No significant differences were observed for IFN-\(\gamma\) and IL-12 between treated or untreated IL-10\(^{-/-}\) or WT mice (data not shown).

Due to the increased levels of this toxic cytokine in serum in response to CpG ODN, we aimed to determine whether TNF-\(\alpha\) was being produced within the uterine tissue of CpG ODN-treated IL-10\(^{-/-}\) mice. Uterine monocytes and granulocyte populations

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**FIGURE 6.** TNF-\(\alpha\) is the essential cytotoxic factor leading to fetal resorption or preterm birth in response to CpG ODN in IL-10\(^{-/-}\) mice. TNF-\(\alpha\) was measured in sera collected from IL-10\(^{-/-}\) or WT mice on gd9 (A) or gd15 (B). Data represent average values from nine different serum samples. IL-10\(^{-/-}\) mice (■) showed a significant increase in TNF-\(\alpha\) levels above control ODN-treated mice (□) on gd9 (A) and gd15 (B); *, \(p < 0.05\). C and D, UMG were collected on gd9 (C) or gd15 (D) from IL-10\(^{-/-}\) mice treated with control or CpG ODN to probe for the presence of TNF-\(\alpha\). Histograms are gated on CD45\(^+\) cells and are representative of data from multiple experiments using four animals per condition. IL-10\(^{-/-}\) mice showed a significant increase in TNF-\(\alpha\) cells on gd9 (C) and gd15 (D) compared with control ODN-treated mice, \(p < 0.05\). E, anti-TNF-\(\alpha\) mAb Ab, given i.p. on gd5 and 7, was used to neutralize TNF-\(\alpha\) activity induced by CpG ODN given on gd6. Data are representative of gd10 uterine horns treated with control ODN + anti-TNF-\(\alpha\) (top panel), CpG ODN + anti-TNF-\(\alpha\) (middle panel), or CpG ODN alone (bottom panel). Data represent multiple experiments using four animals per condition.
were isolated and intracellular staining for TNF-α was performed as described in Materials and Methods. Uterine CD45<sup>+</sup> cells from CpG ODN-treated tissue showed a marked increase in intracellular TNF-α production compared with those from control ODN-treated tissue from gd9 (8.25 ± 1.7 to 40.5 ± 8.3%, Fig. 6C). Similar results were obtained from uterine cells isolated from gd15 tissue (6.25 ± 2.0 to 21.5 ± 3.3%; Fig. 6D).

Since we noted a significant increase in TNF-α both in serum and in uterine CD45<sup>+</sup> cells in CpG ODN-treated IL-10<sup>−/−</sup> mice, we aimed to determine whether this cytokine played a critical role in adverse pregnancy outcomes. We used a neutralizing Ab for in vivo inactivation of TNF-α. In the context of early pregnancy loss, we injected anti-TNF-α Ab on gd5 and gd7 in combination with a CpG ODN injection on gd6. First, we noted healthy litters were born at term in mice treated at the aforementioned time points. Correspondingly, mice treated with CpG ODN on gd6 showed intact fetal-placental units on gd10 in response to anti-TNF-α treatment (Fig. 6E). Similarly, mice treated with CpG ODN on gd14 gave birth to healthy litters at term when given injections of anti-TNF-α Ab on gd13 and 15 (Table I).

**Gr-1<sup>+</sup> cellular depletion in IL-10<sup>−/−</sup> mice does not lead to pregnancy rescue or reduced TNF-α production**

We sought to examine the cellular origin of TNF-α and the role that CD11b<sup>+</sup>Gr-1<sup>+</sup> populations played in CpG ODN-induced fetal demise in IL-10<sup>−/−</sup> mice. We first assessed the effects of depletion of Gr-1<sup>+</sup> cells. We used an anti-Gr-1<sup>+</sup> Ab which was administered i.p. in one dose on gd5 with a CpG ODN injection on gd6 followed by euthanization of animals on gd9 to determine whether rescue from fetal resorption ensued. As shown in Fig. 7A, anti-Gr-1<sup>+</sup> Ab treatment depleted Gr-1<sup>+</sup> cells in CpG ODN-treated IL-10<sup>−/−</sup> mice. However, this event was not associated with the depletion of a CD45<sup>+</sup>TNF-α<sup>+</sup>-producing population as assessed simultaneously by intracellular FACS staining (Fig. 7A). In addition, depletion of Gr-1<sup>+</sup> cells did not rescue pregnancy. These observations suggest that the large infiltrates of Ly6G<sup>+</sup>Gr-1<sup>+</sup> cells in response to CpG ODN treatment (Fig. 5, B and D) play a secondary role in induction of CpG ODN-mediated fetal resorption or preterm birth.

**Evidence of respiratory burst in uterine Gr-1<sup>+</sup>CD11b<sup>+</sup> cells from CpG ODN-treated IL-10<sup>−/−</sup> mice**

Although uterine Gr-1<sup>+</sup> cells did not produce TNF-α (Fig. 7A) and their depletion did not lead to pregnancy rescue, it is still not clear whether these cells were activated and harbor a cytotoxic phenotype. In this regard, we examined production of ROS by Gr-1<sup>+</sup> uterine cells because it is a specific phenotype of these cells upon activation (46, 47). To assess the production of ROS, we used DHR in flow cytometric analysis. This molecule increases its fluorescent intensity upon oxygenation and has recently been used to

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**Table I. Rescue of CpG ODN-induced preterm birth in IL-10<sup>−/−</sup> mice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>n</th>
<th>gd Birth&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p</th>
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<tr>
<td>C57BL/6 IL-10&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>CpG ODN + Ab&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
<td>15.5 ± 0.5</td>
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<tr>
<td></td>
<td>Control ODN + Ab&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
<td>20.0 ± 1.0</td>
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<tr>
<td></td>
<td>CpG ODN + Ab&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>20.6 ± 0.8</td>
<td>&lt;0.001</td>
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<td>19.7 ± 1.1</td>
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<tr>
<td></td>
<td>CpG ODN + F4/80</td>
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<td>20.1 ± 0.8</td>
<td>&lt;0.001</td>
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</table>

<sup>a</sup> gd of birth was assessed visually by the presence of live or dead pups delivered after gd14 treatment.

**FIGURE 7.** Gr-1 depletion does not rescue CpG ODN-mediated negative fetal outcomes in IL-10<sup>−/−</sup> mice, but Gr-1<sup>+</sup> cells actively produce ROS. A, Anti-Gr-1 mAb was used to deplete Gr-1<sup>+</sup> uterine cells. Dot plots are gated on CD45<sup>+</sup> cells for CD11b<sup>+</sup>Gr-1<sup>+</sup> populations. Data represent multiple experiments using four animals per condition. Although Gr-1<sup>+</sup> cells were successfully depleted below control levels, no rescue of pregnancy resulted. TNF-α from these CD45<sup>+</sup> cells was evaluated and histograms show that the presence of TNF-α under the given conditions was not abrogated. B, A role for the increasing CD11b<sup>+</sup>Gr-1<sup>+</sup> populations in response to CpG ODN was evaluated via DHR fluorescence. The two gates on dot plots correspond to the cell populations displayed in the histograms: pink is CD45<sup>+</sup>Gr-1<sup>+</sup> and blue is CD45<sup>−</sup>Gr-1<sup>−</sup>CD11bb<sup>e/b</sup>. Data are representative of results from gd9 control or CpG ODN-treated IL-10<sup>−/−</sup> mice. Control ODN-treated samples (top panel) or CpG ODN-treated samples (bottom panel) show a mean fluorescent intensity shift of DHR in the blue population of CpG ODN-treated animals only, indicative of respiratory burst.
measure production of ROS in several studies (31). CD45<sup>−</sup>CD11b<sup>+</sup>Gr-1<sup>−</sup> cells were typed from gd9 or gd15 uteroplacental tissue from control ODN- or CpG ODN-treated IL-10<sup>−/−</sup> mice and stained simultaneously with DHR as described in Materials and Methods. Uterine CD45<sup>−</sup>CD11b<sup>+</sup>Gr-1<sup>−</sup>cellular populations from CpG ODN-treated tissue harvested on gd9 (Fig. 7B) or gd15 (data not shown) show a significant increase in DHR fluorescence, as calculated by an increase in mean fluorescent intensity compared with that seen in tissues from control ODN-treated mice. These data clearly suggest that uterine Gr-1<sup>−</sup> cells were depleted, we analyzed CD45<sup>−</sup> uterine mononuclear and granular cell population by FACS for the presence of an F4/80<sup>−</sup> population. As shown in Fig. 7B, uterine F4/80<sup>−</sup> cells were successfully depleted below those of control levels. CpG ODN, however, amplified the proportional presence of F4/80<sup>−</sup> cells upward of 20%, similar to results shown in Figs. 3 and 5. Importantly, we assessed intracellular TNF-α production from CpG ODN-treated mice depleted or not depleted for F4/80<sup>−</sup> cells from gd9 uteroplacental tissues. In the F4/80<sup>−</sup>-depleted mice, there was a lack of intracellular staining for TNF-α compared with levels seen in response to CpG ODN alone (Fig. 8B). These data suggest a causative role of uterine macrophages in inducing adverse pregnancy outcomes in CpG ODN-treated IL-10<sup>−/−</sup> mice.

Discussion

It is widely accepted that the innate immune system is essential as a dynamic factor that could be both protective and detrimental to the maternal-fetal stasis (3, 8–10). Although its role is poorly understood, the relative presence of the TLR system in the placental microenvironment further adds to this complexity. Growing evidence clearly suggests that TLR-mediated activation through endogenous signals may indeed provide pregnancy-compatible responses (12, 48). On the other hand, it is not yet fully understood how the TLR machinery will respond to external signals with origins from intrauterine infections and inflammation, particularly when coupled with intrinsic deficiencies in molecules that are critical to normal pregnancy outcome. Two such scenarios are the bacterial and viral DNA breakdown products represented by unmethylated CpG ODN motifs and dsRNA.

In this study, we have examined the gestational age-dependent consequences of CpG ODN administration in pregnant WT and IL-10<sup>−/−</sup> mice. We observed that when injected i.p. on gd6 or gd14, CpG ODN caused rapid fetal resorption or preterm stillbirth in IL-10<sup>−/−</sup> mice, respectively (Fig. 1). In contrast, a similar low dose or a 10-fold higher CpG ODN dose failed to exert any adverse pregnancy effects in WT mice. CpG ODN-mediated consequences in IL-10<sup>−/−</sup> mice were TLR9 specific as an antagonistic CpG ODN ligand-rescued pregnancy in response to pathogenic CpG ODN (Fig. 2B). These observations point to a protective role of IL-10 at the maternal-fetal interface when challenged with bacterial or viral pathogens that may give rise to CpG-like breakdown products.

Several key features were noted in pregnant IL-10<sup>−/−</sup> mice when challenged with the pathogenic CpG ODN motif. First, the induction of fetal resorption or preterm birth was rapid and severe (Fig. 2A). In comparison, WT mice only experienced teratogenic effects at a very high dose (~400 μg/dam) in a portion of the litter at term. Second, the functional presence of uterine CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages and CD11b<sup>+</sup>Gr-1<sup>−</sup> neutrophils (Fig. 3), not CD11c<sup>−</sup> dendritic cells or NK1.1<sup>−</sup>/CD3<sup>−</sup> NK cells, was significantly increased. This influx was critically associated with a drastic increase in serum and the local presence of mKC (Fig. 4). Uterine macrophages, not Gr-1<sup>−</sup> neutrophils, exhibited a significantly high
production of TNF-α (Fig. 8). Importantly, both cell types uncharacteristically migrated to the placental zone (Fig. 5). Finally, depletion of F4/80+ cells (Fig. 8) or in vivo neutralization of TNF-α (Fig. 6d) in CpG-treated IL-10−/− mice blunted fetal resorption and preterm birth and rescued pregnancy to term (Table I).

It is important to mention that depletion of Gr-1+ cells did not lead to rescue of pregnancy nor did it reduce the presence of TNF-α-producing uterine cells (Fig. 7). Thus, when encountered in an abortion- or preterm birth-prone environment such as IL-10 deficiency, TLR9-triggered cytokative activation of macrophages and TNF-α production has severe pathological effects on pregnancy. Neutrophils may influence pregnancy outcome mainly through production of ROS (Fig. 7). These results are important in light of the numerous reports on the association of bacterial and viral infections with early and late pregnancy loss in humans (1, 2, 49, 50). In connection with these data, recent cohort studies have demonstrated that women with abnormally low IL-10 production are more susceptible to systemic and intrauterine infections, leading to increased incidence of pregnancy loss (51–54).

TLR9, localized in intracellular compartments, is expressed in both innate and adaptive immune cells and has been shown to be present in uterine immune cells (11, 14, 15). Curiously, human placental explants did not show any response to CpG compared with agonists for other TLRs (12), suggesting that in humans the maternal immune cells are the major target for CpG-mediated effects. It is possible that TLR9 remains localized to the endoplasmic reticulum of the trophoblast in a nonfunctional form, whereas it becomes activated by localizing to and undergoing cleavage in endolysosomes in uterine macrophages and neutrophils (55, 56). TLR9 signaling has been shown to be essential for recruitment and activation of NK and dendritic cells in many settings of parasitic and bacterial infections (57). TLR9−/− mice have been shown to lack cytokative activation of these cells following infection with Leishmania major (58). Since we did not observe cytokative activation of uNK cells, it is possible that these cells behave differently in response to pathogenic TLR9 agonists than circulating NK cells.

Our findings of high levels of mKC are critical for implications toward understanding the balance between maternal and fetal health. IL-8 has been shown to be imperative for proper trophoblast cell invasion in humans (6). On the other hand, dysregulated production of this chemokine in response to a TLR9-mediated inflammatory insult could prove to be highly detrimental by way of the robust recruitment of cytotoxic macrophages and neutrophils.

We have shown an increased incidence of two cellular populations in response to CpG-mediated pathogens in IL-10−/− mice, namely, macrophages and neutrophils (Figs. 3 and 5). Although the response of these cells to this TLR9 ligand is widely expected (59, 60), this is the first time it has been documented in the context of pregnancy and the uterine microenvironment. We have elucidated novel mechanisms involving these two cell types as first responders to CpG-mediated inflammatory insult, and we have parsed apart their individual roles in this system. Importantly, we have linked the role of macrophages with the production of TNF-α in response to CpG challenge during pregnancy (Fig. 8). Based on our recent findings with other TLR ligands (Refs. 9 and 10; our unpublished observations), we propose that TNF-α is a common final product in response to a spectrum of TLR agonists that induce adverse pregnancy outcomes in response to systemic administration. As shown in Fig. 6 and Table I, neutralization of TNF-α resulted in rescue of pregnancy to term, suggesting that renewed focus should be assigned to the cellular producers of this cytokotoxin in response to a particular TLR that is activated. An understanding of the cellular diversity of TNF-α production at the maternal-fetal interface may be essential in providing proper therapeutic interventions in the context of pregnancy complications.

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Disclosures

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References


