Mammalian pregnancy requires protection against immunological rejection of the developing fetus bearing discordant paternal antigens. Immune evasion in this developmental context entails silenced expression of chemoattractant proteins (chemokines), thereby preventing harmful immune cells from penetrating the maternal-fetal interface. Here, we demonstrate that fetal wastage triggered by prenatal *Listeria monocytogenes* infection is driven by placental recruitment of CXCL9-producing inflammatory neutrophils and macrophages that promote infiltration of fetal-specific T cells into the decidua. Maternal CD8+ T cells with fetal specificity upregulated expression of the chemokine receptor CXCR3 and, together with neutrophils and macrophages, were essential for *L. monocytogenes*–induced fetal resorption. Conversely, decidual accumulation of maternal T cells with fetal specificity and fetal wastage were extinguished by CXCR3 blockade or in CXCR3-deficient mice. Remarkably, protection against fetal wastage and in utero *L. monocytogenes* invasion was maintained even when CXCR3 neutralization was initiated after infection, and this protective effect extended to fetal resorption triggered by partial ablation of immune-suppressive maternal Tregs, which expand during pregnancy to sustain fetal tolerance. Together, our results indicate that functionally overriding chemokine silencing at the maternal-fetal interface promotes the pathogenesis of prenatal infection and suggest that therapeutically reinforcing this pathway represents a universal approach for mitigating immune-mediated pregnancy complications.
CXCR3 blockade protects against *Listeria monocytogenes* infection–induced fetal wastage

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Mammalian pregnancy requires protection against immunological rejection of the developing fetus bearing discordant paternal antigens. Immune evasion in this developmental context entails silenced expression of chemoattractant proteins (chemokines), thereby preventing harmful immune cells from penetrating the maternal-fetal interface. Here, we demonstrate that fetal wastage triggered by prenatal *Listeria monocytogenes* infection is driven by placental recruitment of CXCL9–producing inflammatory neutrophils and macrophages that promote infiltration of fetal-specific T cells into the decidua. Maternal CD8+ T cells with fetal specificity upregulated expression of the chemokine receptor CXCR3 and, together with neutrophils and macrophages, were essential for *L. monocytogenes*–induced fetal resorption. Conversely, decidual accumulation of maternal T cells with fetal specificity and fetal wastage were extinguished by CXCR3 blockade or in CXCR3-deficient mice. Remarkably, protection against fetal wastage and in utero *L. monocytogenes* invasion was maintained even when CXCR3 neutralization was initiated after infection, and this protective effect extended to fetal resorption triggered by partial ablation of immune-suppressive maternal Tregs, which expand during pregnancy to sustain fetal tolerance. Together, our results indicate that functionally overriding chemokine silencing at the maternal-fetal interface promotes the pathogenesis of prenatal infection and suggest that therapeutically reinforcing this pathway represents a universal approach for mitigating immune-mediated pregnancy complications.

Introduction

Stillbirth, defined as fetal loss in the second half of pregnancy, remains a pressing global health problem, with devastating medical and psychosocial consequences. The World Health Organization estimates that 2.6 million cases occur annually (1). Although the etiology in most cases is multifactorial and not definitively identified, maternal infection is undoubtedly an important causative factor (2–4). In this regard, while pathogens best implicated in causing stillbirth are limited to a handful of ubiquitous bacterial, viral, or parasitic microbes capable of intracellular invasion and/or genital-urinary colonization (2–6), the rapid kinetics whereby fetal injury can occur coupled with this microbial heterogeneity still preclude the use of antimicrobials targeting individual pathogens as a means of therapy or prevention. Accordingly, dissecting the underlying pathophysiology causing fetal wastage represents an important perquisite for new strategies aimed at more universal protection against infection-induced pregnancy complications.

Among pathogens that cause prenatal infection, the Gram-positive intracellular bacterium, *Listeria monocytogenes*, has unique predilection for disseminated maternal infection, with often mortal consequences for the developing fetus (7, 8). For example, in 178 cases of prenatal *L. monocytogenes* infection, 20% of pregnancies terminated in abortion or stillbirth, and 68% of live offspring were infected (9). This predisposition for fetal wastage and disseminated *L. monocytogenes* infection during pregnancy is not limited to only humans but widely reiterated across mammalian species, including nonhuman primates (10), ruminants (11, 12), and rodents (13–15). Interestingly, our recent studies using mice bearing allogeneic pregnancies designed to recapitulate the natural heterogeneity between maternal MHC haplotype antigens and fetal MHC haplotype antigens indicate that prenatal *L. monocytogenes* infection–induced fetal resorption may not require direct in utero bacterial invasion (16). Instead, overriding suppression by expanded maternal FOXP3+ regulatory CD4+ T cells (Tregs) by attenuated *L. monocytogenes* that do not cross the placental-fetal barrier triggers sterile fetal wastage, along with expansion and IFN-γ production by maternal T cells with fetal specificity (16–18). Direct associations between blunted expansion of maternal Tregs or their dampened suppressive properties are also recognized increasingly in many idiopathic pregnancy complications linked with disruptions in fetal tolerance (e.g., preeclampsia, spontaneous abortion, prematurity) (19–24). This necessity for expanded maternal Tregs modeled in animal pregnancy shows that even partial transient depletion of FOXP3+ cells to levels before pregnancy unleashes expansion and activation of IFN-γ–producing maternal CD8+ effector T (Tc1) and CD4+ helper T (Th1) cells with fetal specificity that share striking commonality with disruptions in fetal tolerance instigated by prenatal *L. monocytogenes* infection (25, 26). Thus, overriding fetal tolerance, with ensuing activation of maternal immune components with fetal specificity, may play universal roles in the pathogenesis of pregnancy complications.
Recent pioneering observations revealed how silenced expression of Th1/Tc1-inducing chemokines (e.g., CXCL9 and CXCL10) among decidual cells creates an immunological barrier that restricts harmful IFN-γ-producing maternal T cells from gaining access to the maternal-fetal interface (27). Limiting T cell access to the decidua in healthy pregnancy explains protection against fetal loss, despite high circulating levels of activated maternal T cells with defined fetal specificity (27, 28). Collectively, these findings suggest that, if maternal Th1/Tc1 cells unleashed by fractured fetal tolerance drive fetal wastage, dysregulation of decidual chemokine expression silencing could play a pivotal role in the immune pathogenesis of ensuing pregnancy complications (27). Limiting T cell access to the decidua in healthy pregnancy explains protection against fetal loss, despite high circulating levels of activated maternal T cells with defined fetal specificity (27, 28). Collectively, these findings suggest that, if maternal Th1/Tc1 cells unleashed by fractured fetal tolerance drive fetal wastage, dysregulation of decidual chemokine expression silencing could play a pivotal role in the immune pathogenesis of ensuing pregnancy complications. In turn, establishing commonality in the pathophysiology that drives fetal wastage after prenatal infection and noninfectious disruptions in fetal tolerance may reveal new therapeutic targets for reinforcing protection for the fetus against unintentional attack by maternal immune components.

Herein, the immune pathogenesis of fetal injury triggered by infectious and noninfectious disruptions in fetal tolerance was investigated using mouse pregnancy, in which OVA is transduced into a surrogate fetal antigen. We found that prenatal L. monocytogenes infection unleashes the recruitment of Th1/Tc1 chemokine–producing inflammatory cells to the decidua, circumventing the normally protective immunological barrier restricting fetal-specific T cells from the maternal-fetal interface. Reciprocally, neutralizing CXCR3, the receptor for Th1/Tc1-inducing chemokines CXCL9, CXCL10, and CXCL11, before or shortly after prenatal L. monocytogenes infection, efficiently protects against fetal wastage. Interestingly, protective benefits conferred by CXCR3 blockade extend to immune-mediated fetal wastage induced by intrapartum depletion of maternal Tregs. Thus, dissecting the underlying immune pathogenesis of prenatal infection reveals chemokine signaling as a new therapeutic target for averting pregnancy complications and preventing stillbirth.

Results
Maternal CD8+ T cells and IFN-γ are essential for prenatal L. monocytogenes infection–induced fetal wastage. To investigate whether maternal adaptive immune components are essential for infection-induced fetal wastage, pregnancy outcomes were evaluated in RAG2-deficient mice completely lacking T and B cells after L. monocytogenes prenatal infection initiated at midgestation (E11.5) during allogeneic pregnancy. To bypass infection susceptibility in the absence of “innate” T cells (29, 30), an attenuated ΔactA L. monocytogenes strain that cannot cause productive infection due to defects in intercellular spread, while still retaining the ability to fracture fetal tolerance and induce sterile fetal resorption, was used (16, 18). Remarkably, we found that fetal resorption with loss of live pups induced by L. monocytogenes ΔactA prenatal infection among immune-competent C57BL/6 mice was reduced in isogenic RAG2-deficient mice compared with uninoculated mice (Figure 1A). Thus, maternal adaptive immune components are essential for L. monocytogenes infection–induced fetal wastage.

Considering that prenatal infection with virulent or ΔactA–attenuated L. monocytogenes each primes expansion of maternal T cells with fetal specificity (16), pregnancy outcomes were evaluated after depletion of CD4+ and CD8+ T cells individually or concurrently 1 day prior to L. monocytogenes inoculation to more specifically investigate the necessity for each T cell subset in
Infection-induced fetal wastage requires maternal CD8+ T cells with fetal specificity. To address whether maternal CD8+ T cells with fetal specificity drive fetal wastage after prenatal infection, pregnancy outcomes were evaluated among T cell receptor (TCR) transgenic mice containing CD8+ T cells with fixed monoclonal specificity. In particular, P14 and OT-1 TCR transgenic mice with exclusive CD8+ T cell specificity to MHC class I–restricted lymphocytic choriomeningitis virus–glycoprotein amino acids 33–41 (LCMV-GP 33–41) and OVA257–264 peptides, respectively (34, 35), were maintained on a RAG2-deficient background to exclude potential recognition of other antigens through dual TCR expression (36) and reconstituted with bulk splenocytes from CD8α-deficient mice to restore a polyclonal repertoire of CD4+ T and B cells. Using these mice with defined monoclonal CD8+ T cell specificity, we found that L. monocytogenes infection–induced fetal wastage and IFN-γ production by maternal CD8+ T cells were each eliminated if maternal CD8+ T cells did not have overlapping fetal specificity (pregnancies in P14 female mice containing CD8+ T cells with LCMV-GP33–41 specificity impregnated by BALB/c males or transgenic male mice engineered to constitutively express OVA as a transmembrane protein ubiquitously in all cells behind the β-actin promoter on the BALB/c background [BALB/c-OVA] (25, 37) or pregnancies in OT-1 female mice containing CD8+ T cells with OVA257–264 specificity impregnated by BALB/c males) (Figure 2). On the other hand, fetal resorption, loss of live pups, and IFN-γ production by maternal CD8+ T cells each rebounded when maternal CD8+ T cell specificity was engineered to

Figure 2. Prenatal infection-induced fetal resorption requires maternal CD8+ T cells with fetal specificity. (A) Percentage of resorbed fetuses and number of live pups 5 days after L. monocytogenes ΔactA or L. monocytogenes ΔactA OVA infection (each 10^7 CFU) initiated midgestation (E11.5) among P14 or OT-1 TCR transgenic mice during allogeneic pregnancies after mating with BALB/c or BALB/c-OVA males. Ten days before mating, P14 and OT-1 TCR transgenic mice maintained on a RAG2-deficient background were reconstituted with polyclonal CD4+ T and B cells from splenocytes of CD8α-deficient mice. (B) Representative FACS plots and composite data showing the percentage IFN-γ production after PMA/ionomycin stimulation among maternal CD8+ splenocytes recovered 5 days after L. monocytogenes ΔactA or L. monocytogenes ΔactA OVA infection (each 10^7 CFU) for the mice described in A. Each symbol indicates the data from a single mouse, and these results, containing 6–8 mice per group, are representative of 3 independent experiments, each with similar results. Error bars represent mean ± 1 SEM.
Decidual accumulation of maternal CD8+ T cells with fetal specificity after prenatal *L. monocytogenes* infection. Considering the recently described protective role for locally repressed Th1/Tc1 chemokine expression that restricts harmful IFN-γ-producing inflammatory cells, patches of fetal-OVA-specific CD8+ T cells were scattered throughout the decidua basalis and junctional zone selectively for OVA+ concepti after prenatal *L. monocytogenes* infection, whereas CD90.1+ staining cells were absent in both sham-infected controls and OVA concepti regardless of infection (Figure 3A). Infection-induced accumulation of maternal CD8+ T cells with fetal-OVA specificity in the placenta was verified by immunohistochemistry using anti-CD90.1 antibodies. Along with diffuse infiltration of mixed polymorphonuclear and lymphocytic inflammatory cells, patches of fetal-OVA-specific CD8+ T cells were scattered throughout the decidua basalis and junctional zone selectively for OVA+ concepti after prenatal *L. monocytogenes* infection, whereas CD90.1+ staining cells were absent in both sham-infected controls and OVA concepti regardless of infection (Figure 3B and data not shown).

To further investigate the possibility that maternal T cells enriched within highly vascular decidual tissue could reflect contamination by intravascular cells, staining with fluorochrome-labeled antibody against the pan-leukocyte CD45 antigen injected intravenously immediately prior to euthanasia was evaluated (42). This analysis showed that, while cells recovered from the blood
as qualitative shifts in CD45+ decidual leukocytes were observed reaching ~4-fold expanded levels, with sharply enriched proportions of CD11b+Ly6C<sup>int</sup> neutrophils by 72 hours after *L. monocytogenes* ΔactA infection (Figure 4, A and B). Thus, decidual accumulation of maternal CD8+ T cells with fetal specificity after prenatal *L. monocytogenes* infection parallels the influx of neutrophils and other leukocyte subsets to the maternal-fetal interface. Given repressed expression of Th1/Tc1 chemokines among decidual stromal cells previously shown to prevent maternal CD8+ T cells from infiltrating the maternal-fetal interface (27), we investigated whether decidual accumulation of fetal-specific CD8+ T cells triggered by prenatal *L. monocytogenes* infection circumvents chemokine expression silencing. Within the first 72 hours after *L. monocytogenes* ΔactA infection, expression of the prototypical Th1/Tc1 chemokine, CXCL9, increased progressively among cells recovered from the decidua compared with those from the adjacent myometrium (Figure 4C). Interestingly, however, CXCL9-producing decidual cells were comprised almost exclusively of CD11b+Ly6C<sup>int</sup> neutrophils and F4/80+ macrophages, whereas CXCL9 expression remained at background levels among CD45− stromal cells and CD8+ T cells (Figure 4D).

To address the necessity for these specific CXCL9-producing cell subsets in infection-induced fetal wastage, we evaluated the effects of their in vivo depletion on fetal resorption and decidual infiltration by CD8+ T cells with fetal specificity. We found uniformly stained positive and paraaortic lymph node cells consistently did not bind intravenously injected anti-CD45.2 antibody (positive and negative controls), approximately 70% of CD8+ T cells recovered from the decidua also did not bind intravenously injected antibody, suggesting this cellular majority is of nonvascular origin (Supplemental Figure 2). Interestingly, however, regardless of staining with intravenously injected anti-CD45.2 antibody, CD90.1+ cells with surrogate fetal-OVA specificity were equally enriched among CD8+ T cells recovered from the decidua compared with blood or lymph node cells, demonstrating massive enrichment of fetal-OVA-specific CD8+ T cells among both tissue resident and intravascular decidual cells. Together, these results show that prenatal *L. monocytogenes* infection–induced fetal wastage parallels robust accumulation of maternal CD8+ T cells with fetal specificity at the maternal-fetal interface.

Decidual infiltration of CXCL9-expressing leukocytes and CXCR3-expressing CD8+ T cells after prenatal *L. monocytogenes* infection. To further define shifts in cells that infiltrate the decidua after prenatal *L. monocytogenes* infection, the number and composition of cells in this tissue was evaluated. Within the first 24 hours after *L. monocytogenes* ΔactA inoculation, the absolute number of decidual cells increased by ~2-fold; the increased cells were composed almost exclusively of CD45+ leukocytes with composition similar to those in uninfected control mice (Figure 4, A and B, and Supplemental Figure 3). As infection progressed, quantitative data showing average results from 5 to 10 mice per group per time point are representative of 3 independent experiments, each with similar results. Error bars represent mean ± 1 SEM.
that administration of anti-Gr1 antibody (RB6-8C5) (43), which depletes both neutrophils and inflammatory macrophages 1 day prior to *L. monocytogenes* infection, significantly diminished the frequency of fetal resorption and decidual accumulation of maternal CD8+ T cells with surrogate fetal-OVA specificity (Figure 5). In turn, expression of the CXCL9 receptor, CXCR3, was also sharply upregulated among fetal-OVA specific CD8+ T cells recovered from both the decidua and periphery (Figure 6). Taken together, these results show that the immunological barrier enforced in healthy both the decidua and periphery (Figure 6). Taken together, these of chemokine-producing inflammatory cells and maternal CD8+ T cells with surrogate fetal-OVA specificity (Figure 5). In order to derive these deleterious cells.

CXCR3 deprivation protects against *L. monocytogenes* infection-induced fetal wastage. Considering that multiple Th1/Tc1-inducing chemokines, including CXCL9, CXCL10, and CXCL11, share the common receptor CXCR3 (44), complementary loss-of-function studies targeting CXCR3 were performed to more definitively establish the necessity for decidual T cell infiltration in the pathogenesis of prenatal infection. For CXCR3-deficient mice or mice treated with CXCR3-neutralizing antibody prior to prenatal infection, fetal wastage triggered by *L. monocytogenes* ΔactA was reduced to background levels compared with that in each group of CXCR3-replete no infection control mice (Figure 7, A and B). Decidual accumulation of maternal CD8+ T cells with fetal-OVA specificity for mice bearing allogeneic pregnancies after mating with BALB/c-OVA-expressing males was similarly averted by in vivo CXCR3 neutralization (Figure 7C). Importantly, near-complete elimination of decidual fetal-specific CD8+ T cell accumulation with CXCR3 deprivation cannot be explained by diminished priming of maternal CD8+ T cells with fetal-OVA specificity, as these cells were found in similar proportions in the paraaortic lymph node draining the uterus for anti-CXCR3 antibody-treated mice compared with isotype control antibody-treated mice (Figure 7C). Thus, despite nonessential roles for CXCR3 in priming systemic expansion of maternal CD8+ T cells with fetal specificity, stimulation through this chemokine receptor is required for *L. monocytogenes* ΔactA infection–induced fetal wastage and decidual infiltration of these deleterious cells.

CXCR3 neutralization protects against fetal wastage triggered by virulent *L. monocytogenes* prenatal infection. Given the efficiency whereby CXCR3 blockade protects against attenuated *L. monocytogenes* ΔactA–induced fetal resorption, the protective benefits of CXCR3 neutralization on fetal wastage and in utero fetal invasion after virulent *L. monocytogenes* prenatal infection were evaluated. While systemic expansion of immune-suppressive maternal Tregs required for sustaining fetal tolerance confers susceptibility to disseminated *L. monocytogenes* infection (25), placental-fetal tropism appears to be an equally decisive contributor, since other pathogens with more restricted tissue distribution (e.g., influenza A) that prime robust antigen-specific CD8+ T cell expansion do not

![Figure 5. Depletion of CXCL9-producing neutrophils and macrophages protects against *L. monocytogenes* infection–induced fetal wastage.](image1)

**Figure 5. Depletion of CXCL9-producing neutrophils and macrophages protects against *L. monocytogenes* infection–induced fetal wastage.** (A) Percentage of resorbed fetuses 5 days after *L. monocytogenes* ΔactA (107 CFU) infection initiated midgestation (E11.5) among C57BL/6 female mice bearing allogeneic pregnancies after mating with BALB/c males that were administered anti-Gr1 compared with isotype control antibody (500 μg per mouse) 1 day prior to infection. (B) Representative FAC5 plots and composite data showing the percentage of fetal-OVA257–264–specific cells (CD90.1+) among CD8+ T cells recovered from the decidua 3 days after *L. monocytogenes* ΔactA (107 CFU) infection for C57BL/6 female mice bearing allogeneic pregnancies after mating with BALB/c-OVA males that were administered anti-Gr1 compared with isotype control antibody (500 μg per mouse) 1 day prior to infection. Each symbol reflects the data from a single mouse, and these results, containing 5–7 mice per group, are representative of 3 independent experiments, each with similar results. Error bars represent mean ± 1 SEM. Differences between the indicated groups were evaluated using a 2-tailed unpaired Student’s t test.

![Figure 6. Prenatal *L. monocytogenes* infection selectively primes CXCR3 expression by maternal CD8+ T cells with fetal specificity.](image2)

**Figure 6. Prenatal *L. monocytogenes* infection selectively primes CXCR3 expression by maternal CD8+ T cells with fetal specificity.** Representative plots and composite analysis showing relative expression of CXCR3 by OVA257–264–specific (CD90.1+) CD8+ T cells recovered from the decidua or spleen among C57BL/6 female mice bearing allogeneic pregnancies after mating with BALB/c-OVA mice compared with BALB/c males 3 days after *L. monocytogenes* ΔactA (107 CFU) infection initiated midgestation (E11.5) and controls without infection. Each symbol reflects the data from a single mouse, and these results, containing 5–11 mice per group, are representative of 3 independent experiments, each with similar results. Error bars represent mean ± 1 SEM. Differences between the indicated groups were evaluated using the 1-way ANOVA statistical test. MFI, mean fluorescent intensity.
cause fetal wastage, even with high dosage maternal infection during allogeneic pregnancy (Supplemental Figure 4 and refs. 45–48). Accordingly, a relatively high virulent \( \text{L. monocytogenes} \) dosage (\( 10^4 \text{ CFU strain 10403s} \)), representing only a half-log\(_{10} \) reduction from the LD\(_{50} \) for mice bearing allogeneic pregnancies, shown previously to cause consistent fetal resorption with in utero bacterial invasion, was used to investigate the effects of CXCR3 deprivation (16, 25). Remarkably, CXCR3-neutralizing antibody compared with isotype control antibody administered 1 day prior to virulent \( \text{L. monocytogenes} \) infection initiated midgestation efficiently mitigated fetal resorption, loss of live pups, and bacterial invasion into individual concepti (Figure 8A). Protection against fetal wastage paralleled near-complete elimination of decidual fetal-OVA-specific CD8\(^+\) T cell accumulation, without significant shifts in increased CXCL9 expression levels by CD11b\(^+\)Ly6C\(^{int}\) neutrophils and F4/80\(^+\) macrophages (Figure 8, B and C). Together, these results suggest that the detrimental properties of fetal-specific CD8\(^+\) T cell access to the decidua, shown for attenuated \( \text{L. monocytogenes} \Delta\text{actA} \), extend to fetal wastage triggered by virulent \( \text{L. monocytogenes} \) prenatal infection.

The necessity for fetal-specific CD8\(^+\) T cells in fetal wastage after virulent \( \text{L. monocytogenes} \) infection was further addressed in complementary studies using mice bearing syngeneic pregnancies (C57BL/6 males mated with isogenic females). We reasoned that, if activated maternal CD8\(^+\) T cells with specificity to immunologically discordant paternal antigen expressed by the developing fetus and their infiltration into the decidua drive the immune pathogenesis of prenatal infection-induced fetal wastage, artificially eliminating discordance between maternal-paternal (fetal) MHC haplotype antigens in syngeneic matings would override the protective effects of CXCR3 neutralization. In agreement with this hypothesis, prenatal \( \text{L. monocytogenes} \) infection–induced fetal resorption and in utero bacterial invasion were markedly reduced in mice bearing syngeneic pregnancies compared with those bearing allogeneic pregnancies, whereas CXCR3 blockade had no significant effects on diminished rates of fetal wastage during syngeneic pregnancy (Supplemental Figure 5). Importantly, the ineffectiveness of CXCR3 neutralization in syngeneic pregnancies compared with allogeneic pregnancies cannot be explained by diminished susceptibility to prenatal \( \text{L. monocytogenes} \) infection, because CXCR3 blockade also showed no protective benefits, despite more uniform fetal wastage and in utero bacterial invasion achieved by increasing the dosage of virulent \( \text{L. monocytogenes} \) (5-fold) used for infection during syngeneic pregnancy (Supplemental Figure 5). Thus, in the more physiological context of discordance between maternal-fetal MHC haplotype antigens recapitulated in mouse allogeneic pregnancy, preventing decidual accumulation of maternal CD8\(^+\) T cells with foreign fetal specificity by CXCR3 neutralization protects against fetal resorption and in utero pathogen invasion after virulent \( \text{L. monocytogenes} \) prenatal infection.

To further address whether protection against fetal wastage shown for CXCR3 neutralization beginning prior to infection extends to ongoing prenatal infection, pregnancy outcomes were evaluated after initiating CXCR3 blockade 12 and 24 hours after virulent \( \text{L. monocytogenes} \) inoculation at midgestation. Strikingly, near-complete protection with regards to fetal resorption, number of live pups, in utero \( \text{L. monocytogenes} \) fetal invasion frequency, and decidual accumulation of maternal CD8\(^+\) T cells with fetal-OVA specificity were each only slightly diminished with initiating anti-CXCR3 antibody 12 hours after infection compared with CXCR3 blockade before infection (Figure 8, A and B). On the other hand, these protective properties were eliminated if CXCR3 blockade was delayed until 24 hours after infection, as fetal resorption, loss of live pups, \( \text{L. monocytogenes} \) fetal invasion, and accumulation of fetal-specific CD8\(^+\) T cells in the decidua each rebounded to levels indistinguishable from those of control mice without anti-CXCR3 antibody treatment (Figure 8, A and B).
infection and noninfectious etiologies that blunt expansion of immune-suppressive maternal Tregs and fracture fetal tolerance (16, 25, 51), related studies addressed whether the protective benefits conferred by CXCR3 blockade shown for prenatal *L. monocytogenes* infection extend to noninfectious disruptions in fetal tolerance. To mimic blunted expansion of maternal FOXP3+ Tregs increasingly linked with human pregnancy complications (19–24), a strategy that exploits the X-linked inheritance of Foxp3 and random inactivation of this chromosome in female Foxp3DTR/WT mice heterozygous for coexpression of the high-affinity human diphtheria toxin (DT) receptor with FOXP3 to consistently achieve partial transient depletion of maternal FOXP3+ cells to levels before pregnancy was used (25, 52, 53). This analysis showed that partial transient ablation of bulk maternal FOXP3+ Tregs triggers markedly increased CXCL9 expression by decidual neutrophils (CD11b+Ly6Cint) and macrophages (F4/80+CD11b−), along with expanded accumulation of fetal-OVA-specific CD8+ T cells at the maternal-fetal interface (Figure 10, A and B). These immune cell shifts in the decidua parallel sharply increased rates of fetal wastage among Foxp3DTR/WT mice bearing allogeneic pregnancies administered DT (Figure 10C and refs. 25, 26). To investigate the

To extrapolate how these time points in mouse pregnancy may apply to a potential therapeutic window after infection for intervention in human pregnancy, we compared how delayed initiation of the current clinical gold standard therapy for *L. monocytogenes* infection (ampicillin) affects pregnancy outcomes after prenatal *L. monocytogenes* infection (7, 49, 50). Interestingly, this analysis showed protection against fetal wastage achieved with ampicillin occurred with similar efficacy compared with CXCR3 neutralization at each time point after prenatal *L. monocytogenes* infection — both conveyed near-complete protection when initiated 12 hours after infection, whereas benefits are uniformly dissipated if delayed until 24 hours after infection (Figures 8 and 9). Together, these results demonstrate that CXCR3 blockade beginning prior to infection is highly efficacious in averting negative sequelae of prenatal infection, whereas initiating CXCR3 blockade shortly after infection shows therapeutic benefits comparable to the current gold standard clinical antimicrobial therapy for prenatal listeriosis.

CXCR3 neutralization protects against fetal resorption triggered by maternal FOXP3+ cell depletion. Given the potentially shared pathophysiology between fetal wastage induced by prenatal infection and noninfectious etiologies that blunt expansion of immune-suppressive maternal Tregs and fracture fetal tolerance (16, 25, 51), related studies addressed whether the protective benefits conferred by CXCR3 blockade shown for prenatal *L. monocytogenes* infection extend to noninfectious disruptions in fetal tolerance. To mimic blunted expansion of maternal FOXP3+ Tregs increasingly linked with human pregnancy complications (19–24), a strategy that exploits the X-linked inheritance of Foxp3 and random inactivation of this chromosome in female Foxp3DTR/WT mice heterozygous for coexpression of the high-affinity human diphtheria toxin (DT) receptor with FOXP3 to consistently achieve partial transient depletion of maternal FOXP3+ cells to levels before pregnancy was used (25, 52, 53). This analysis showed that partial transient ablation of bulk maternal FOXP3+ Tregs triggers markedly increased CXCL9 expression by decidual neutrophils (CD11b+Ly6Cint) and macrophages (F4/80+CD11b−), along with expanded accumulation of fetal-OVA-specific CD8+ T cells at the maternal-fetal interface (Figure 10, A and B). These immune cell shifts in the decidua parallel sharply increased rates of fetal wastage among Foxp3DTR/WT mice bearing allogeneic pregnancies administered DT (Figure 10C and refs. 25, 26). To investigate the
therapeutic benefits of CXCR3 blockade in this noninfectious context, fetal resorption and loss of live pups were compared among DT-treated Foxp3DTR/WT mice administered CXCR3-neutralizing antibody prior to or or 12 or 24 hours after DT administration. Remarkably, CXCR3 neutralization initiated before or 12 hours after initiating DT treatment conferred near-complete protection against fetal wastage induced by partial depletion of maternal FOXP3+ Tregs (Figure 10C). Conversely, the protective benefits on fetal resorption and loss of live pups were mitigated when CXCR3 blockade was delayed until 24 hours after DT administration (Figure 10C). Thus, protection against fetal wastage conferred by CXCR3 neutralization initiated before or shortly after prenatal L. monocytogenes infection extends to disruptions in fetal tolerance triggered by partial depletion of maternal FOXP3+ Tregs.

Discussion

The maternal immune system is charged with two monumen
tal responsibilities during pregnancy: avert rejection of the fetus bearing foreign paternal antigens, while simultaneously maintain
ing immunity against pathogenic microbes (51). This is accomplished, in large part, by anatomically segregating antigenically discordant fetal tissue and concentrating immune silencing mechanisms where they are needed most, at the maternal-fetal interface (54). In this regard, many nonoverlapping strategies that restrict maternal immune cell access to the decidua and sup
press local activation of potentially harmful immune components have been identified. These include diminished or skewed MHC expression by trophoblast cells (55, 56), impaired decidual comple
ment activation and deposition (57), tryptophan catabolism through indoleamine 2,3-dioxygenase (58), expanded immune-
suppressive maternal FOXP3+ Tregs (25, 59), uterine entrap
ment of antigen-presenting cells (60), and decidual exclusion of IFN-γ-producing Th1/Tc1 T cells through chemokine gene silencing (27). However, an inevitable consequence of intensely focused immune silencing in this fashion is vulnerability to infection by pathogens capable of breaching this “immune-privileged” site (5). Therefore, it is not surprising that microbes with tropism for infecting trophoblasts and other placental cells have widely been shown to cause pregnancy complications (3–5).

Along with direct in utero pathogen invasion, our recent find
ings suggest that overriding normally silenced maternal immune components that prevent fetal rejection by prenatal infection also contribute to the immune pathogenesis of fetal wastage (16, 51). For example, after L. monocytogenes prenatal infection, fetal resorption directly parallels systemic expansion and activation of maternal T cells with fetal specificity, despite infection parameters that preclude direct in utero fetal invasion (16). Based on these observations, we proposed a model whereby infection-induced disruptions in fetal tolerance instigate fetal wastage by drawing maternal immune cells and circulating pathogen to the mater
nal-fetal interface (16, 51). However, underlying questions regarding the specific maternal immune components responsible for fetal injury, their necessity for fetal antigen recognition, and decidual infiltration in driving fetal wastage have remained elusive. More
over, chemokine silencing that restricts IFN-γ-producing Th1/Tc1 T cells from the maternal-fetal interface (27) could mitigate fetal injury, despite systemic disruptions in fetal tolerance triggered by prenatal infection.

These outstanding issues are reconciled with results described in the current study. In particular, the necessity for maternal CD8+ T cells with fetal specificity and their recruitment to the decidua in infection-induced fetal wastage is definitively established using transgenic mouse tools that fix maternal T cell specificity and complementary loss-of-function approaches for averting decidual infiltration by maternal T cells with fetal specificity. Consistent with previously described CXCL9 repression, enforced through histone methylation and other epigenetic modifications among decidual stromal cells (27), only background levels of CXCL9 expression were found among nonleukocyte CD45+ cells isolated from the decidua after prenatal infection. Nonetheless, CXCL9–producing CD11b+Ly6Cint neutrophils and F4/80+ macrophages that infiltrate this tissue after L. monocytogenes prenatal infection functionally circumvent chemokine expression silencing at the maternal-fetal interface. In turn, maternal CD8+ T cells with fetal specificity selectively upregulate expression of the CXCL9

Figure 9. Ampicillin administration early after virulent L. monocytog
enes prenatal infection protects against fetal wastage. Percentage of resorbed fetuses, number of live pups, and frequency of L. monocytogenes recovery from each conceptus 5 days after virulent L. monocytogenes (105 CFU) infection initiated midgestation (E11.5) among female C57BL/6 mice bearing allogeneic pregnancies after mating with BALB/c males that were administered ampicillin in the drinking water beginning 12 or 24 hours after infection compared with controls maintained on autoclaved drinking water without supplementation. Each symbol indicates the data from a single mouse, and these results, containing 7–8 mice per group, are representative of 3 independent experiments, each with similar results. Error bars represent mean ± 1 SEM.
Figure 10. CXCR3 blockade protects against fetal wastage and decidual fetal-specific CD8+ T cell accumulation triggered by partial depletion of maternal FOXP3+ Tregs. (A) Mean fluorescent intensity after staining with anti-CXCL9 compared with isotype control antibody among neutrophils (CD11b Ly6Cint) and macrophages (F4/80+ CD11b−) recovered from the decidua 3 days after initiating DT treatment to E11.5 Foxp3DTR/WT female mice on the C57BL/6 background bearing allogeneic pregnancies after mating with BALB/c-OVA males. (B) Representative FACS plots and composite data showing the percentage of fetal-OVA-specific specific cells (CD90.1+) among CD8+ T cells recovered from the decidua 3 days after initiating DT treatment compared with mice with no DT treatment described in A. (C) Percentage of resorbed fetuses and number of live pups for Foxp3DTR/WT female mice on the C57BL/6 background bearing allogeneic pregnancies after mating with BALB/c males that were administered anti-CXCR3 antibody (500 μg per mouse) 24 hours before or 12 or 24 hours after initiating sustained daily DT treatment (E11.5), compared with controls with no DT or anti-CXCR3 antibody treatment. Each symbol indicates the data from a single mouse, and these results, containing 3 to 8 mice per group, are representative of 3 independent experiments, each with similar results. Error bars represent mean ± 1 SEM.

chemokine receptor, CXCR3, and are recruited to the decidua. For immunity to microbes such as L. monocytogenes and viral pathogens (e.g., herpes simplex virus and LCMV) that each reside within the host cell intracellular compartment, these findings are consistent with recruitment of protective pathogen-specific T cells to non-gestational female reproductive tissue after exogenous CXCL9 administration or resident memory CD8+ T cell reactivation (61, 62). These results are also in agreement with prior descriptions of cellular infiltration, along with elevated expression of proinflammatory cytokines such as G-CSF, TNF-α, IL-6, IFN-γ, and CXCL9 at the maternal-fetal interface after infection with other bacterial or parasitic pathogens (e.g., Salmonella, Plasmodium, Toxoplasma, or Leishmania spp.) that cause vertical transmission (63–71).

These findings further underscore the necessity for sustained immune tolerance systemically and, perhaps more importantly, at the maternal-fetal interface in maintaining pregnancy. In response to prenatal pathogens that cause disseminated infection and directly invade the decidua, systemic as well as local immune-suppressive pathways are likely dampened as a means for more efficient pathogen eradication (16, 17, 51). In turn, even partial disruptions in tolerance to immunologically discordant antigens expressed by the developing fetus unleash the activation and decidual recruitment of maternal effector T cells with fetal specificity. By contrast, we show that prenatal infection with influenza A virus with distribution confined to respiratory tissue and only isolated case reports of placental-fetal invasion (45, 46, 72–76) does not cause fetal resorption after infection in mice bearing allogeneic pregnancy. Nonetheless with regards to pathogens like L. monocytogenes that cause systemic infection with tropism for decidual invasion, our data now demonstrate a necessity for Th1/Tc1-inducing chemokines in the pathogenesis of infection-induced fetal wastage. Blockade of the Th1/Tc1 chemokine receptor, CXCR3, not only eliminates CD8+ T cell infiltration into the decidua, but also protects against in utero bacterial invasion and infection-induced fetal resorption. Taken together, these results establish that Th1/Tc1 T cell recruitment via CXCR3 not only promotes but is essential for the immune pathogenesis of fetal wastage and in utero fetal infection after L. monocytogenes prenatal infection.

By delaying CXCR3 neutralization after the initiation of L. monocytogenes infection, sustained protective benefits that stem from disrupting this chemokine receptor pathway were also revealed. We found that initiating CXCR3 blockade within the first 12 hours after prenatal infection conferred near-complete protection against fetal resorption, loss of live pups, and in utero L. monocytogenes fetal invasion comparable to CXCR3 neutralization prior to infection. Conversely, these beneficial outcomes were eliminated when CXCR3 blockade was delayed until 24 hours after infection. Although it is difficult to extrapolate how this timing after infection during the sharply accelerated kinetics of murine pregnancy may relate to protection against human pregnancy complications, it is encouraging that protection against L. monocytogenes infection-induced fetal wastage conferred by CXCR3 blockade occurred with similar kinetics and efficacy compared with ampicillin, the clinical gold standard treatment of prenatal L. monocytogenes infection (7, 49, 50). However, considering the inciting pathogen is undefined initially in most cases of prenatal infection, this more universal approach for reversing immune-mediated preg-
nancy complications may offer more comprehensive benefits compared with antimicrobial agents targeting individual pathogens.

Protection conferred by CXCR3 neutralization shown for pathogen-induced fetal wastage extends to mitigating fetal resorption and loss of live pups triggered by partial depletion of maternal immune-suppressive FOXP3+ Tregs. Similar to prenatal L. monocytogenes infection, partial transient ablation of maternal Tregs to levels before pregnancy primes expansion and IFN-γ production among fetal-specific maternal effector T cells (25, 26, 53) — illustrating the sustained systemic expansion of this immune-suppressive T cell subset is essential for maintaining fetal tolerance. Fetal wastage triggered by partial depletion of maternal FOXP3+ cells in mice directly parallels blunted expansion of maternal Tregs in the peripheral blood and decidua in human pregnancy complications associated with disruptions in fetal tolerance (e.g., preeclampsia, spontaneous abortion) (19–24), whereas overriding local immune-suppressive pathways in place to sustain fetal tolerance likely play decisive roles in the pathogenesis of fetal injury, considering the absence of fetal wastage after infection with influenza A that does not directly invade the maternal-fetal interface. Accordingly, this newfound pathway, whereby decidual chemokine expression silencing becomes functionally circumvented, may drive the underlying pathogenesis of fetal wastage after infectious as well as idiopathic disruptions in fetal tolerance, possibly representing subclinical or undiagnosed local infection (2–4). Reciprocally, restoring repressed chemokine function at the maternal-fetal interface through CXCR3 neutralization may provide more universal protection against unintentional attack by maternal immune components with fetal specificity. Based on these exciting preclinical findings, additional investigation establishing whether circumventing maternal T cell infiltration to the decidua through CXCR3 neutralization represents a new therapeutic approach for reinforcing fetal tolerance and protection against infectious and noninfectious causes of immune-mediated pregnancy complications is warranted.

Methods

Mice. C57BL/6 (B6, H-2b), BALB/c (H-2k), and RAG2-deficient mice on the B6 background were purchased from the National Cancer Institute or The Jackson Laboratory and used at between 6 to 8 weeks of age. The use of transgenic mice that ubiquitously express OVA protein behind the constitutively active β-actin promoter to impregnate nontransgenic females transforming OVA into a surrogate fetal antigen has been described previously (28, 37, 40). OVA-expressing mice were backcrossed >10 generations to BALB/c mice in our studies to establish allogeneic pregnancy (16, 25). P14 TCR transgenic mice, in which all CD8+ T cells have exclusive monoclonal specificity to LCMV-GFp34.5 Peptide (34) on a RAG2-deficient B6 background, were purchased from Taconic Farms. OT-I TCR transgenic mice, in which all CD8+ T cells have exclusive monoclonal specificity to OVA peptide (35), were maintained on a RAG2-deficient CD90.1 congenic background. Foxp3ΔΔ mice and the use of Foxp3ΔΔ/WT female mice to investigate the necessity for sustained maternal Treg expansion during allogeneic pregnancy have been described previously (25, 26, 52, 53). For partial transient depletion of maternal Tregs, Foxp3ΔΔ/WT females impregnated by BALB/c males were administered purified DT (Sigma-Aldrich) daily (0.5 μg first dose, followed by 0.1 μg per dose thereafter), beginning at midgestation (E11.5) for 5 consecutive days, and the frequency of fetal resorption was evaluated E16.5 as previously described (25, 26). Where indicated, pregnant mice were administered autoclaved drinking water supplemented with ampicillin (0.5 mg/ml) (Sigma-Aldrich).

L. monocytogenes and influenza A infections. The wild-type virulent L. monocytogenes strain 10403s, the isogenic ΔactA-attenuated strain DPL1942, and recombinant L. monocytogenes ΔactA-OVA have each been described previously (16, 38, 39, 77). For infection, L. monocytogenes were grown to early log phase (OD600 0.1) in brain-heart infusion media at 37°C, washed, and diluted with saline to 200 μl and injected via the lateral tail vein at the following dosages (L. monocytogenes ΔactA, 105 CFU; L. monocytogenes 10403s, 104 CFU) as described previously (16). For each infection, the inoculum was verified by plating serial dilutions onto agar media. For enumerating recoverable L. monocytogenes CFU, individual concepti (placentas and fetuses), along with spleens, livers, lungs, kidneys, and brains, were sterilely dissected, homogenized in saline containing 0.05% Triton X, and spread onto agar plates as described previously (16, 25). Influenza A virus serotype H1N1 strain PR8 has been described previously (78) and was provided by Monica Malone McNeal (Cincinnati Children’s Hospital). For infection, mice were anesthetized with ketamine and xylazine and inoculated intranasally with 107 PFU suspended in 30 μl sterile saline. Five days after infection, viral recovery was evaluated by plating serial dilutions of each organ homogenate onto Madin-Darby canine kidney cells (cell line MDCK.2 from ATCC, clone CRL-2936) and enumerating plaques 18 hours thereafter (79).

Tissue harvest, antibodies, and flow cytometry. Single-cell suspensions of splenocytes and paraaortic lymph node cells were prepared by gentle tissue dissociation between frosted glass slides, and rbc lysis was performed in ammonium chloride buffer. Decidua and myometrium were isolated as previously described (60). Specifically for flow cytometry, uteri were dissected in ice-cold HBSS, and the myometrium was peeled away from each individual conceptus and placenta. Placentas were then removed from the fetuses and separated at the interface of the labyrinth and junctional zone for analysis of the decidua (decidua basalis and junctional layers), placed in rbc lysis buffer, and dissociated into single-cell suspension by grinding between frosted glass slides. Thereafter, DMEM was added to quench the lysis reaction, and samples were filtered through a 70-μm cell strainer and pelleted by centrifugation (530 g for 5 minutes). The tissue cell pellets were then suspended in DMEM media supplemented with 10% fetal bovine serum and used for cell surface and intracellular staining. For histological analysis, the uterine wall and placenta were harvested en bloc and fixed in 10% paraformaldehyde (4°C for 12 to 16 hours). Thereafter, the tissue was embedded in paraffin, cut into 5-μm sections, deparaffinized in xylene, and rehydrated with ethanol. Endogenous fluorescence was reduced using 0.1% sodium borohydride. Nonspecific protein interactions were blocked using goat serum (5%) and bovine serum albumin (1%). Sections were then incubated with PE-conjugated anti-CD90.1 antibody (clone OX-7, BioLegend) for 1 hour at room temperature and mounted with VECTASHIELD media with DAPI (Vector laboratories). For cytokine production, cells were stimulated with PMA/ionomycin for 5 hours in media supplemented with brefeldin A (GolgiPlug, BD Biosciences) prior to cell surface and intracellular staining. For CXCL9 expression, cells recovered from the decidua or myometrium were incubated in media supplemented with brefeldin A for 5 hours, followed by cell surface and intracellular staining using anti-CXCL9 antibody (clone 2F5.5, eBioscience). Other
fluorophore-conjugated antibodies used for cell surface and intracellular staining were anti-CD4 (clone GK1.5, eBioscience, or clone RM4-4, BioLegend), anti-CD8α (clone 53-6.7, eBioscience), anti-CD8β (clone H35-7.2, eBioscience), anti-NK1.1 (clone PKH36, BD Pharmingen), anti-CD90.1 (clone H1S51, eBioscience), anti-CD45.1 (clone A20, eBioscience), anti-45.2 (clone 104, BioLegend), anti-62L (clone ME1-14, eBioscience), anti-CD11b (clone M1/70, BioLegend), anti-CD11c (clone N418, BioLegend), anti-CD11c (clone RA3-6B2, BioLegend), anti-F4/80 (clone BM8, eBioscience), anti-CCR3 (clone CXCR3-173, BioLegend), and IFN-γ (clone XMG1.2, eBioscience). Purified antibodies for in vivo T cell depletion (anti-CD4, clone GK1.5; anti-CD8, clone 2.43; both from BioXcell), IFN-γ neutralization (clone XMG1.2, BioXcell), neutrophil and macrophage depletion (anti-Gr1, clone RB6-8C5, BioXcell), CXCR3 neutralization (clone CXCR3-173, BioXcell), and rat and hamster IgG isotype control antibodies were administered intraperitoneally (500 μg each antibody per mouse).

Adoptive cell transfers. OVA-specific CD8+ T cells were isolated from CD90.1 congenic OT-1 mice and injected intravenously into CD90.2 recipients (105 donor OT-1 CD8+ T cells) 1 day prior to L. monocytogenes infection or DT administration (to Foxp3DTR/WT mice) at midgestation (E11.5). Thereafter, accumulation of OVA-specific T cells in each tissue was evaluated by gating on CD90.1 donor cells among CD90.2 recipient cells as described previously (16, 25).

Intravascular staining. To discriminate between tissue resident and intravascular leukocytes, staining with fluorochrome-conjugated anti-CD45.2 antibody after intravenous injection immediately prior to euthanasia was performed as described previously (42). Specifically, 3 μg PE-conjugated anti-CD45.2 antibody (clone 104, BioLegend) was injected through the lateral tail vein 3 minutes prior to euthanasia and tissue harvest.

Statistics. Differences in fetal resorption frequency, number of live pups, percentage of infected concepti, and cell numbers were analyzed using an unpaired 2-tailed Student t test (2 groups) or 1-way ANOVA (>2 groups), with P values of less than 0.05 considered as statistical significant.

Study approval. All experiments involving the use of animals were performed under Cincinnati Children’s Hospital Institutional Animal Care and Use Committee–approved protocols.

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