CXCR3 blockade protects against *Listeria monocytogenes* infection–induced fetal wastage

Vandana Chaturvedi,[1] James M. Ertelt,[1] Tony T. Jiang,[1] Jeremy M. Kinder,[1] Lijun Xin,[1] Kathryn J. Owens,[2] Helen N. Jones,[2] and Sing Sing Way[1]

[1]Division of Infectious Disease and [2]Division of Surgery, Cincinnati Children's Hospital Perinatal Institute, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA.

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.

Conflict of interest: The authors have declared that no conflict of interest exists.
Submitted: August 16, 2014; **Accepted:** January 22, 2015.
Reference information: *J Clin Invest*. 2015;125(4):1713–1725. doi:10.1172/JCI78578.

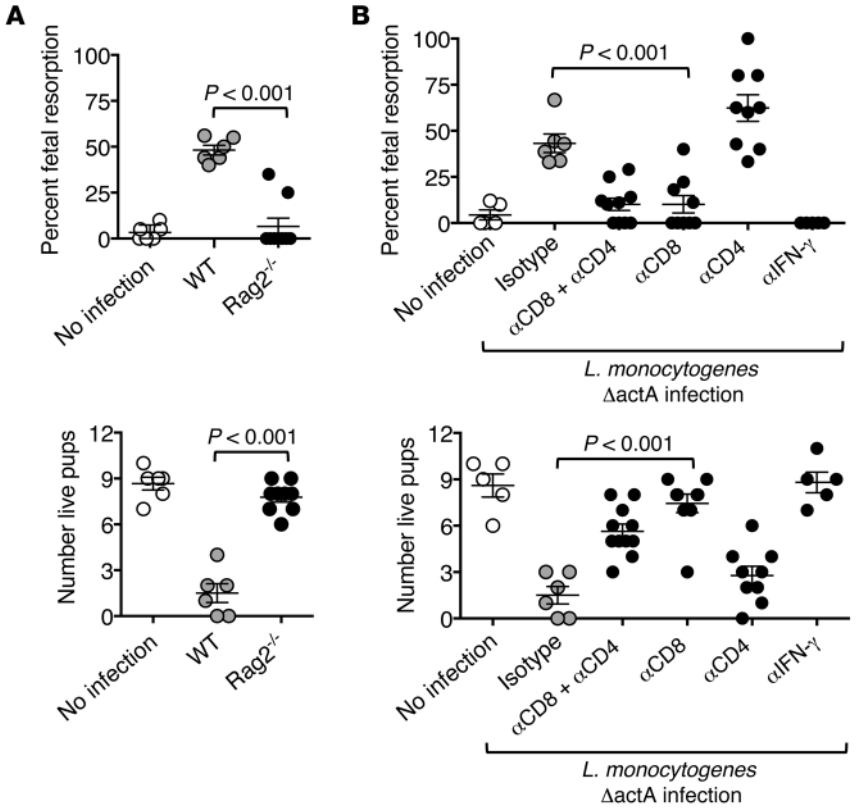

Figure 1. Maternal CD8⁺ T cells are essential for prenatal *L. monocytogenes* infection–induced fetal wastage. (**A**) Percentage of resorbed fetuses and number of live pups 5 days after *L. monocytogenes* ΔactA (10^7 CFU) infection initiated midgestation (E11.5) among C57BL/6 wild-type mice compared with RAG2-deficient female mice bearing allogeneic pregnancies after mating with BALB/c males and controls without infection. (**B**) Percentage of resorbed fetuses and number of live pups 5 days after *L. monocytogenes* ΔactA (10^7 CFU) infection initiated midgestation (E11.5) among C57BL/6 female mice bearing allogeneic pregnancies after mating with BALB/c males that were treated with anti-CD4 and/or anti-CD8 or anti–IFN-γ antibody compared with rat IgG control antibody (500 μg per mouse) 1 day prior to infection and controls without infection. Each symbol indicates the data from a single mouse, and these results, containing 5–9 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.

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 pivotally important 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 transformed 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 to background levels found in uninfected control pregnancies (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

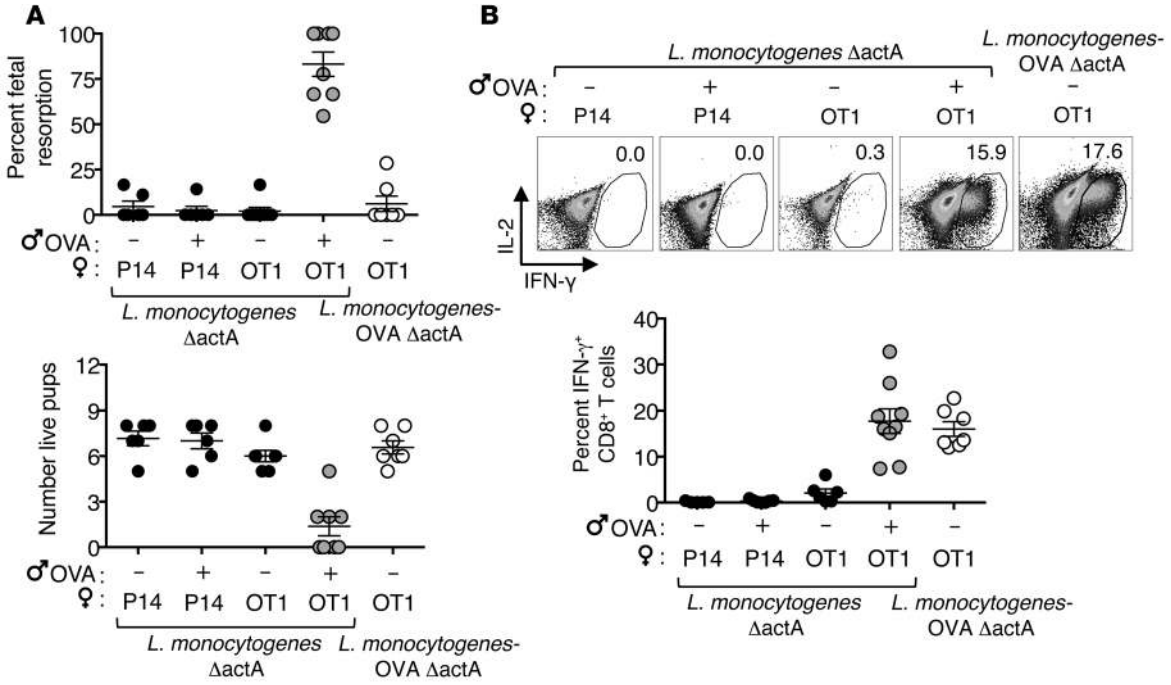

Figure 2. Prenatal infection-induced fetal resorption requires maternal CD8⁺ T cells with fetal specificity. (**A**) Percentage of resorbed fetuses and number of live pups 5 days after *L. monocytogenes* ΔactA or *L. monocytogenes* ΔactA OVA infection (each 10^7 CFU) initiated midgestation (E11.5) among 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.

infection-induced fetal wastage. Pregnancy did not affect the efficiency of T cell depletion using well-characterized monoclonal antibodies (31), and the frequency of fetal resorption and loss of live pups were sharply reduced with depletion of both CD4⁺ and CD8⁺ T cells prior to *L. monocytogenes* infection, in line with protection from fetal wastage in RAG2-deficient mice (Figure 1 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI78578DS1). Interestingly, depletion of CD8⁺ T cells alone conferred similar protective benefits, with fetal resorption and loss of live pups reduced to background levels, comparable to those in uninfected control mice (Figure 1B). By contrast, fetal resorption and loss of live pups increased significantly when CD4⁺ T cells were depleted in isolation, consistent with disruptions in fetal tolerance that occurred after eliminating immune-suppressive Tregs restricted to the CD4⁺ subset of peripheral T cells (Figure 1B and refs. 32, 33). To further investigate the necessity for IFN-γ cytokine produced by maternal CD8⁺ T cells primed by prenatal *L. monocytogenes* infection (16), pregnancy outcomes were evaluated in mice treated with anti–IFN-γ–neutralizing antibody. These experiments showed that IFN-γ neutralization also efficiently reversed *L. monocytogenes* ΔactA–induced fetal wastage, as only background levels of fetal resorption, with negligible loss of live pups, were observed (Figure 1B). Collectively, these results indicate that maternal CD8⁺ T cells and IFN-γ are each essential for fetal wastage triggered by prenatal *L. monocytogenes* ΔactA infection.

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 $OVA_{257–264}$ 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-$GP_{33–41}$ 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 $OVA_{257–264}$ 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

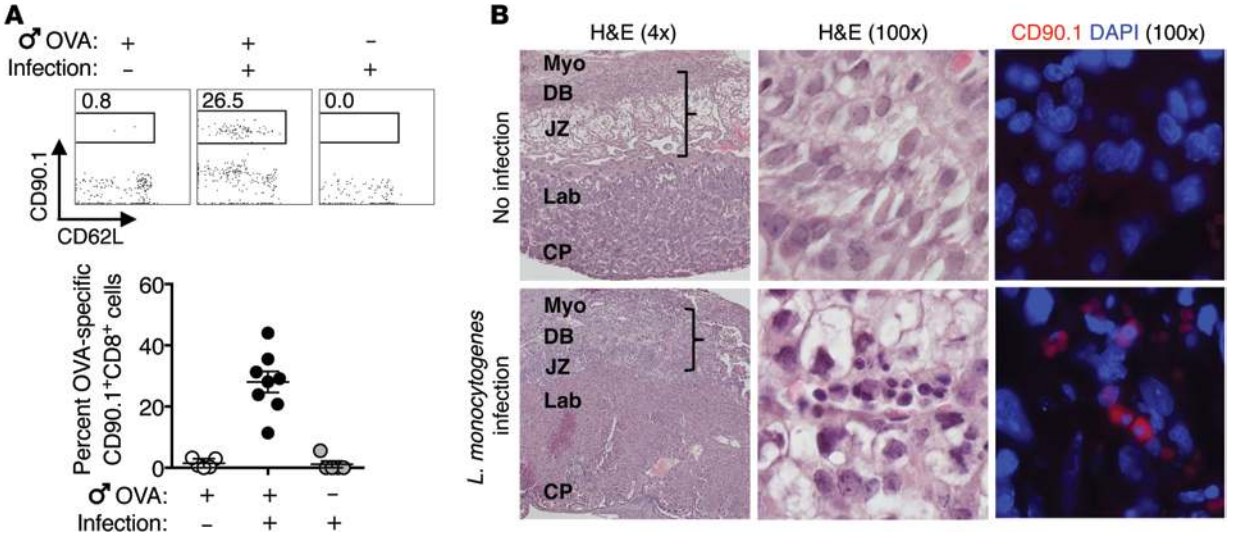

Figure 3. Placental accumulation of maternal CD8⁺ T cells with fetal specificity triggered by prenatal *L. monocytogenes* infection. (**A**) Representative FACS plots and composite data showing the percentage of fetal-$OVA_{257–264}$-specific (CD90.1⁺) cells among CD8⁺ T cells recovered from the decidua 3 days after *L. monocytogenes* ΔactA (10^7 CFU) infection initiated midgestation (E11.5) among C57BL/6 female mice during allogeneic pregnancies after mating with BALB/c-OVA males compared with nontransgenic BALB/c males and controls without infection. Each symbol indicates the data from a single mouse, and these results, containing 5–8 mice per group, are representative of 3 independent experiments, each with similar results. (**B**) Representative histological analysis of the placentas recovered from mice described in **A** showing no infection control compared with *L. monocytogenes* ΔactA (10^7 CFU) infection among OVA⁺ concepti after H&E staining, along with anti-CD90.1 (red) and DAPI nuclear immunofluorescence staining. High-magnification fields (original magnification, ×100) show placental tissue intersecting the decidua basalis (DB) and junctional zone (JZ). Brackets in the low-magnification fields (original magnification, ×4) indicate the source of decidual tissue harvested for analysis by flow cytometry. These images are representative of 10 individual placentas analyzed per group from 3 separate litters. Myo, myometrium; Lab, labyrinth; CP, chorionic plate. Error bars represent mean ± 1 SEM.

overlap with fetal expressed antigen (OT-1 female mice containing maternal CD8⁺ T cells with $OVA_{257–264}$ specificity impregnated by BALB/c-OVA males) (Figure 2). To further investigate whether protection from fetal wastage in pregnancies without overlap in specificity between maternal CD8⁺ T cells and fetal antigen could be explained by less activated CD8⁺ T cells, pregnancy outcomes were enumerated in OT-1 female mice after infection with recombinant *L. monocytogenes* expressing OVA as a pathogen-associated antigen that primes expansion of IFN-γ–producing OVA-specific CD8⁺ T cells (38, 39). During allogeneic pregnancies, after mating with nontransgenic BALB/c males, fetal wastage remained at background levels, despite robust IFN-γ production by activated maternal CD8⁺ T cells with nonfetal OVA specificity (Figure 2). Thus, maternal CD8⁺ T cell recognition of fetal antigen is essential for infection-induced fetal injury, whereas nonspecific activation of maternal CD8⁺ T cells in isolation is insufficient to cause fetal wastage.

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 T cells from the maternal-fetal interface (27), together with the unambiguous necessity for maternal CD8⁺ T cells with fetal specificity in infection-induced fetal wastage, the potential for decidual accumulation of fetal-specific CD8⁺ T cells after prenatal *L. monocytogenes* infection was investigated. To more precisely identify maternal T cells with fetal specificity, $OVA_{257–264}$-specific CD8⁺ T cells from the aforementioned OT-1 TCR transgenic mice maintained on the CD90.1 congenic background were tracked after adoptive transfer into CD90.2⁺ female recipients during allogeneic pregnancies after mating with BALB/c-OVA males (16, 25, 40). Interestingly, CD8⁺ T cells with surrogate fetal-OVA specificity (identified by expression of the CD90.1 congenic marker) were found to accumulate by >20-fold in the decidua compared with background levels in uninfected control mice within the first 3 days after prenatal *L. monocytogenes* ΔactA infection (Figure 3A). Here, a slightly earlier time point after infection was used to bypass the inconsistent recovery of decidual tissue that occurs with fetal wastage 5 days after infection (41). Importantly, decidual accumulation of maternal CD8⁺ T cells reflects a targeted response to fetal antigen stimulation, because OVA-specific CD90.1⁺ CD8⁺ T cells remained at background levels after *L. monocytogenes* ΔactA infection in mice impregnated by BALB/c (non-OVA-expressing) males (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 CD90.1⁺ 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

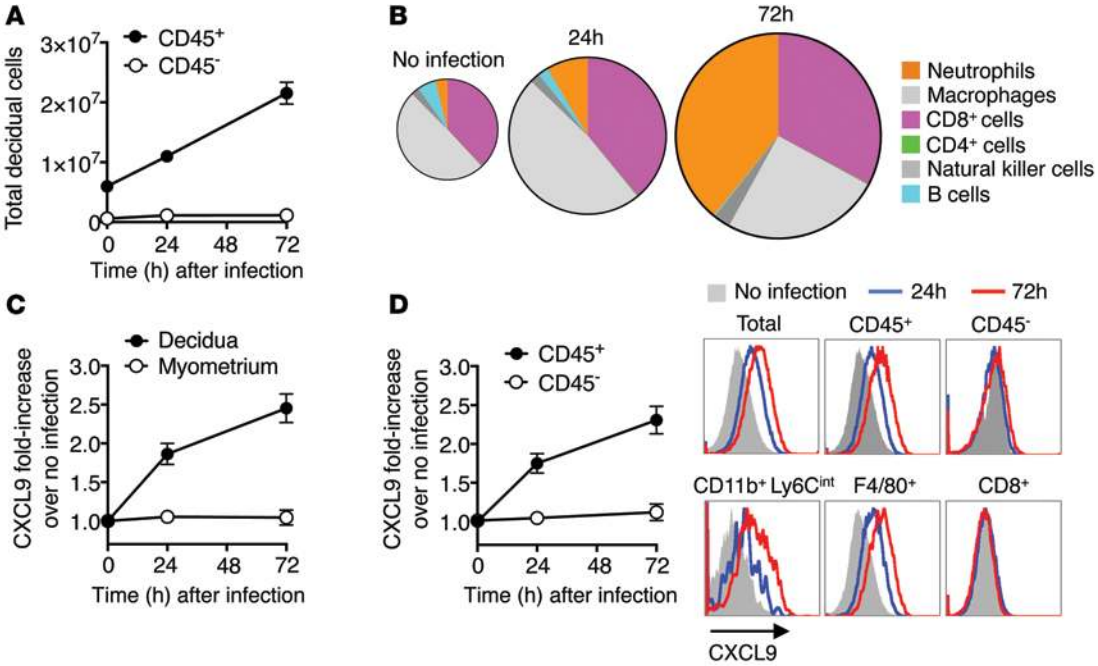

Figure 4. CXCL9-producing inflammatory cells accumulate in the decidua after prenatal *L. monocytogenes* infection. (**A**) Number of CD45$^+$ leukocyte and CD45$^-$ nonleukocyte stromal cells recovered from the decidua at each time point after *L. monocytogenes* ΔactA (10^7 CFU) infection initiated midgestation (E11.5) among C57BL/6 female mice during allogeneic pregnancies after mating with BALB/c males. (**B**) Pie chart illustrating quantitative accumulation and quantitative shifts in each CD45$^+$ leukocyte subset recovered from the decidua for mice described in **A**. Individual leukocyte subsets were delineated after gating on CD45$^+$ cells and identified as neutrophils (CD11b$^+$Ly6C^{int}), macrophages (F4/80$^+$CD11b$^-$), natural killer cells (NK1.1$^+$CD4$^-$CD8$^-$), B cells (B220$^+$CD4$^-$CD8$^-$), CD4 cells (CD4$^+$CD8$^-$), and CD8 cells (CD8$^+$CD4$^-$). (**C**) Relative CXCL9 expression among cells recovered from the decidua compared with adjacent myometrium after *L. monocytogenes* ΔactA (10^7 CFU) infection for mice described in **A**. (**D**) Relative CXCL9 expression among CD45$^+$ compared with CD45$^-$ decidual cells and representative histogram plots showing CXCL9 expression by each cell type before (gray shaded) and 24 (blue line) or 72 (red line) hours after *L. monocytogenes* ΔactA infection. These 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.

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 as well as qualitative shifts in CD45$^+$ decidual leukocytes were observed reaching ~4-fold expanded levels, with sharply enriched proportions of CD11b$^+$Ly6C^{int} 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^{int} 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

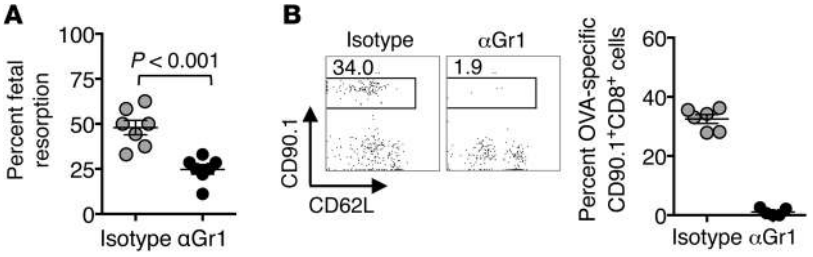

Figure 5. Depletion of CXCL9-producing neutrophils and macrophages protects against *L. monocytogenes* infection–induced fetal wastage. (**A**) Percentage of resorbed fetuses 5 days after *L. monocytogenes* ΔactA (10^7 CFU) infection initiated midgestation (E11.5) among C57BL/6 female mice bearing allogeneic 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 FACS plots and composite data showing the percentage of fetal-$OVA_{257–264}$-specific cells ($CD90.1^+$) among $CD8^+$ T cells recovered from the decidua 3 days after *L. monocytogenes* ΔactA (10^7 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.

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 pregnancy through Th1/Tc1 chemokine expression silencing is circumvented by *L. monocytogenes* prenatal infection that drives influx of chemokine-producing inflammatory cells and maternal $CD8^+$ T cells with fetal specificity to the maternal-fetal interface.

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

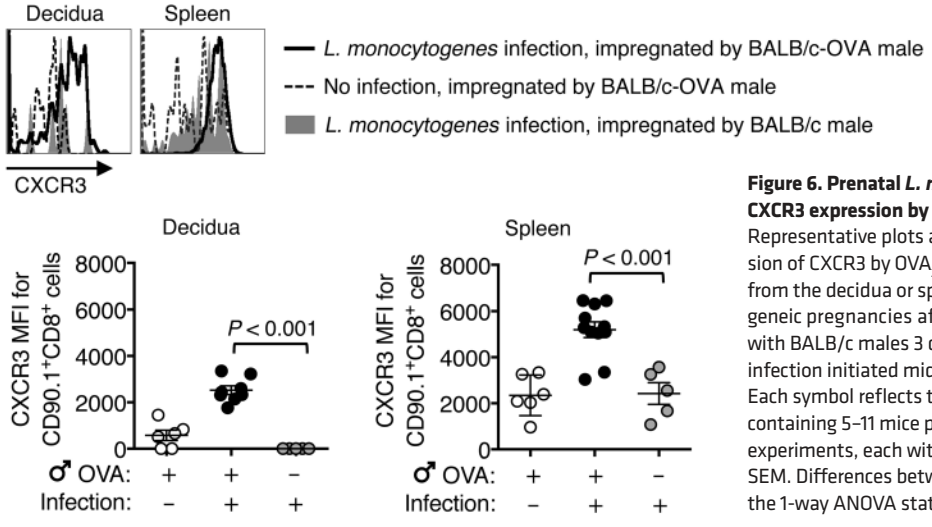

Figure 6. Prenatal *L. monocytogenes* infection selectively primes CXCR3 expression by maternal $CD8^+$ T cells with fetal specificity. Representative plots and composite analysis showing relative expression of CXCR3 by $OVA_{257–264}$-specific ($CD90.1^+$) $CD8^+$ T cells recovered from the decidua 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 (10^7 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.

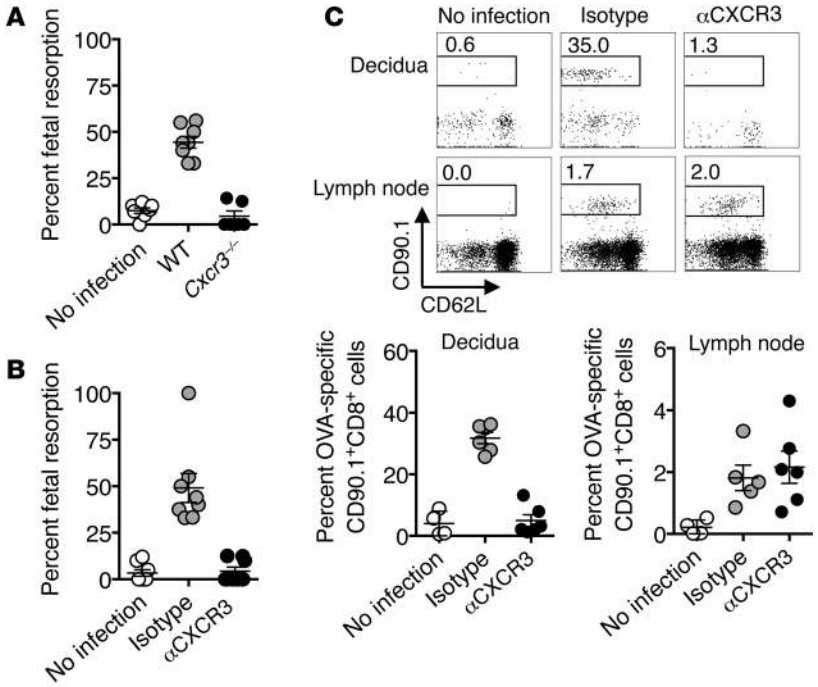

Figure 7. CXCR3 deprivation protects against prenatal *L. monocytogenes* infection–induced fetal wastage. (**A**) Percentage of resorbed fetuses among C57BL/6 mice compared with isogenic CXCR3-deficient female mice 5 days after *L. monocytogenes* ΔactA (10^7 CFU) infection initiated midgestation (E11.5) during allogeneic pregnancies after mating with BALB/c males and controls without infection. (**B**) Percentage of resorbed fetuses among C57BL/6 female mice 5 days after *L. monocytogenes* ΔactA (10^7 CFU) infection initiated midgestation (E11.5) among C57BL/6 female mice during allogeneic pregnancies after mating with BALB/c males that were administered anti-CXCR3 compared with isotype control antibody (500 μg per mouse) 1 day prior to infection and controls without infection. (**C**) Representative FACS plots and composite data showing the percentage of fetal-$OVA_{257–264}$-specific cells ($CD90.1^+$) among $CD8^+$ T cells recovered from the decidua or paraaortic lymph node 3 days after *L. monocytogenes* ΔactA (10^7 CFU) infection initiated midgestation (E11.5) for C57BL/6 female mice during allogeneic pregnancies after mating with by BALB/c-OVA males. Each symbol indicates the data from a single mouse, and these results, containing 4–8 mice per group, are representative of 3 independent experiments, each with similar results. Error bars represent mean ± 1 SEM.

cause fetal wastage, even with high dosage maternal infection during allogeneic pregnancy (Supplemental Figure 4 and refs. 45–48). Accordingly, a relatively high virulent *L. monocytogenes* dosage (10^4 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 *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 *L. monocytogenes* ΔactA, extend to fetal wastage triggered by virulent *L. monocytogenes* prenatal infection.

The necessity for fetal-specific $CD8^+$ T cells in fetal wastage after virulent *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 *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 *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 *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 *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 *L. monocytogenes* inoculation at midgestation. Strikingly, near-complete protection with regards to fetal resorption, number of live pups, in utero *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, *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).

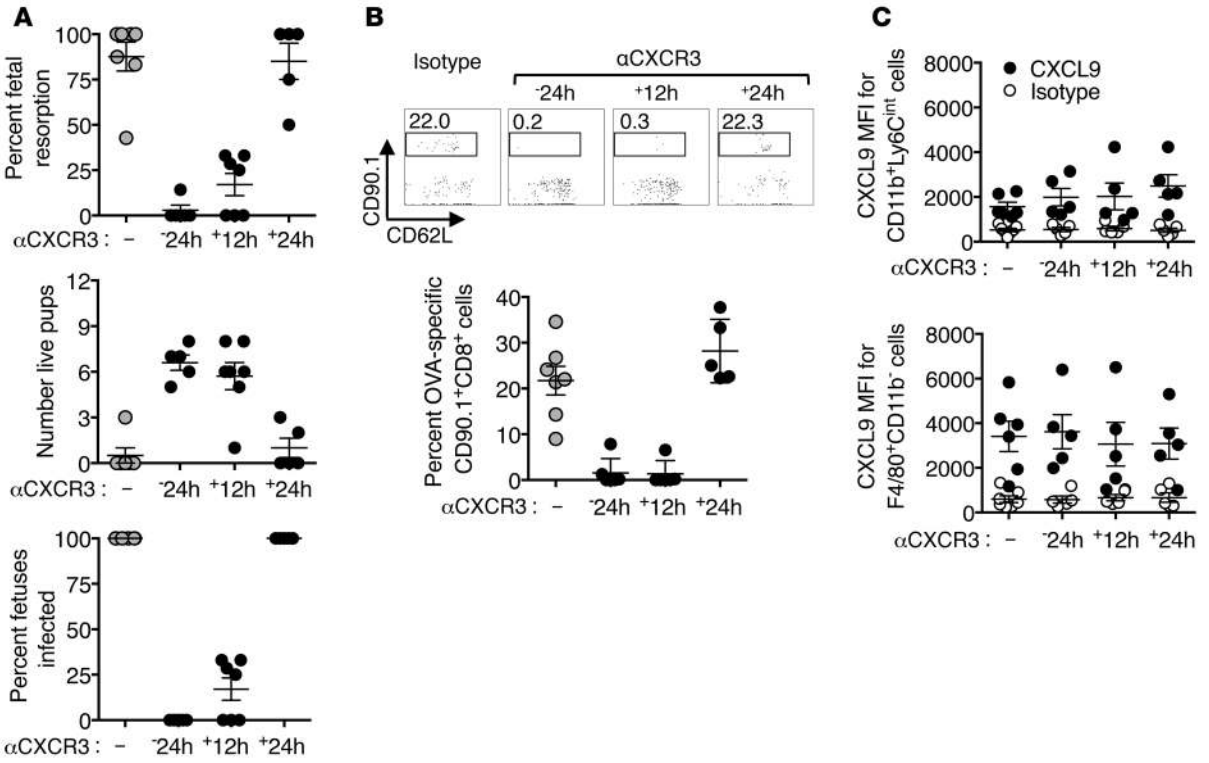

Figure 8. CXCR3 blockade initiated before or shortly after virulent *L. monocytogenes* prenatal infection protects against fetal wastage and mitigates decidual fetal-specific CD8⁺ T cell accumulation. (**A**) Percentage of resorbed fetuses, number of live pups, and frequency of *L. monocytogenes* recovery from each concepti 5 days after virulent *L. monocytogenes* (10^4 CFU) infection initiated midgestation (E11.5) among C57BL/6 female mice 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 infection compared to controls without antibody treatment. (**B**) Representative FACS plots and composite data showing the percentage of fetal-$OVA_{257–264}$-specific cells ($CD90.1^+$) among $CD8^+$ T cells recovered from the decidua 3 days after virulent *L. monocytogenes* (10^4 CFU) infection initiated midgestation (E11.5) among C57BL/6 female mice bearing allogeneic pregnancies after mating with BALB/c-OVA males that were administered anti-CXCR3 antibody (500 μg per mouse) 24 hours before or 12 or 24 hours after infection compared to controls without antibody treatment. (**C**) Mean fluorescent intensity after staining with anti-CXCL9 compared with isotype control antibody among neutrophils ($CD11b^+Ly6C^{int}$) and macrophages ($F4/80^+$ $CD11b^-$) recovered from the decidua 3 days after virulent *L. monocytogenes* (10^4 CFU) infection for mice described in **B**. Each symbol indicates 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.

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 $Foxp3^{DTR/WT}$ 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 ($CD11b^+$ $Ly6C^{int}$) 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 $Foxp3^{DTR/WT}$ mice bearing allogeneic pregnancies administered DT (Figure 10C and refs. 25, 26). To investigate the

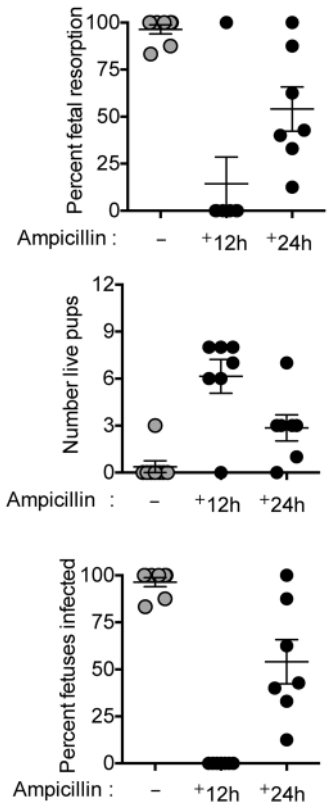

Figure 9. Ampicillin administration early after virulent *L. monocytogenes* prenatal infection protects against fetal wastage. Percentage of resorbed fetuses, number of live pups, and frequency of *L. monocytogenes* recovery from each concepti 5 days after virulent *L. monocytogenes* (10^4 CFU) infection 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.

therapeutic benefits of CXCR3 blockade in this noninfectious context, fetal resorption and loss of live pups were compared among DT-treated *Foxp3*$^{DTR/WT}$ mice administered CXCR3-neutralizing antibody prior to 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 monumental responsibilities during pregnancy: avert rejection of the fetus bearing foreign paternal antigens, while simultaneously maintaining 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 suppress local activation of potentially harmful immune components have been identified. These include diminished or skewed MHC expression by trophoblast cells (55, 56), impaired decidual complement activation and deposition (57), tryptophan catabolism through indoleamine 2,3-dioxygenase (58), expanded immune-suppressive maternal FOXP3$^+$ Tregs (25, 59), uterine entrapment 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 findings 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 maternal-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. Moreover, 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$^+$Ly6C^{int} 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

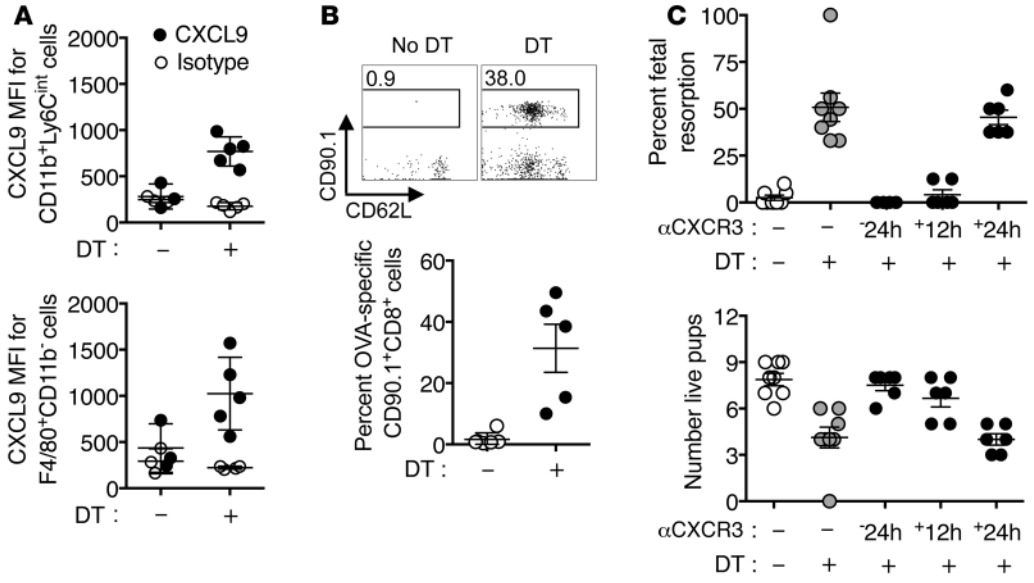

Figure 10. CXCR3 blockade protects against fetal wastage and decidual fetal-specific CD8⁺ T cell accumulation triggered by partial depletion of maternal FOXP3⁺ Tregs. (**A**) Mean fluorescent intensity after staining with anti-CXCL9 compared with isotype control antibody among neutrophils ($CD11b^+$ $Ly6C^{int}$) and macrophages ($F4/80^+$ $CD11b^-$) recovered from the decidua 3 days after initiating DT treatment to E11.5 *Foxp3*$^{DTR/WT}$ 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-$OVA_{257–264}$-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 *Foxp3*$^{DTR/WT}$ 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 nongestational 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 invasion 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-2^b), BALB/c (H-2^d), 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-GP$_{33–41}$ peptide (34) on a RAG2-deficient B6 background, were purchased from Taconic Farms. OT-1 TCR transgenic mice, in which all CD8$^+$ T cells have exclusive monoclonal specificity to OVA$_{257–264}$ peptide (35), were maintained on a RAG2-deficient CD90.1 congenic background. *Foxp3^{DTR}* mice and the use of *Foxp3$^{DTR/WT}$* 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, *Foxp3$^{DTR/WT}$* 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 (OD_{600} 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, 10^7 CFU; *L. monocytogenes* 10403s, 10^4 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 10^3 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 concepti and placentas. 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 H35-17.2, eBioscience), anti-NK1.1 (clone PK136, BD Pharmingen), anti-CD90.1 (clone HIS51, eBioscience), anti-CD45.1 (clone A20, eBioscience), anti-45.2 (clone 104, BioLegend), anti-62L (clone MEL-14, eBioscience), anti-CD11b (clone M1/70, BioLegend), anti-CD11c (clone N418, BioLegend), anti-B220 (clone RA3-B62, BioLegend), anti-F4/80 (clone BM8, eBioscience), anti-CXCR3 (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-I mice and injected intravenously into $CD90.2^+$ recipients (10^5 donor OT-1 $CD8^+$ T cells) 1 day prior to *L. monocytogenes* infection or DT administration (to $Foxp3^{DTR/WT}$ 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.

Acknowledgments

We thank Adrian Erlebacher (New York University), David Haslam (Cincinnati Children's Hospital), and Joseph Qualls (Cincinnati Children's Hospital) for helpful discussions and Beverly Strong (Cincinnati Children's Hospital) for assistance with placental dissections. This work is supported, in part, by the NIH National Institute of Allergy and Infectious Diseases through grant awards R01-AI087830, R01-AI100934, and R21-AI112186. S.S. Way holds an Investigator in the Pathogenesis of Infectious Disease award from the Burroughs Wellcome Fund. This manuscript is dedicated in fond memory of Judith E. Ertelt.

Address correspondence to: Sing Sing Way, 3333 Burnet Avenue, MLC 7017, Cincinnati Children's Hospital, Cincinnati, Ohio 45229, USA. Phone: 513.636.7603; E-mail: singsing.way@cchmc.org.

1. Kelley M. Counting stillbirths: women's health and reproductive rights. *Lancet*. 2011;377(9778):1636–1637.
2. Goldenberg RL, Thompson C. The infectious origins of stillbirth. *Am J Obstet Gynecol*. 2003;189(3):861–873.
3. Gibbs RS. The origins of stillbirth: infectious diseases. *Semin Perinatol*. 2002;26(1):75–78.
4. Goldenberg RL, McClure EM, Saleem S, Reddy UM. Infection-related stillbirths. *Lancet*. 2010;375(9724):1482–1490.
5. Robbins JR, Bakardjiev AI. Pathogens and the placental fortress. *Curr Opin Microbiol*. 2012;15(1):36–43.
6. Kourtis AP, Read JS, Jamieson DJ. Pregnancy and infection. *N Engl J Med*. 2014;370(23):2211–2218.
7. Silver HM. Listeriosis during pregnancy. *Obstet Gynecol Surv*. 1998;53(12):737–740.
8. Lamont RF, et al. Listeriosis in human pregnancy: a systematic review. *J Perinat Med*. 2011;39(3):227–236.
9. Mylonakis E, Paliou M, Hohmann EL, Calderwood SB, Wing EJ. Listeriosis during pregnancy: a case series and review of 222 cases. *Medicine (Baltimore)*. 2002;81(4):260–269.
10. Smith MA, et al. Nonhuman primate model for Listeria monocytogenes-induced stillbirths. *Infect Immun*. 2003;71(3):1574–1579.
11. Linde K, Fthenakis GC, Lippmann R, Kinne J, Abraham A. The efficacy of a live Listeria monocytogenes combined serotype 1/2a and serotype 4b vaccine. *Vaccine*. 1995;13(10):923–926.
12. Gitter M, Richardson C, Boughton E. Experimental infection of pregnant ewes with Listeria monocytogenes. *Vet Rec*. 1986;118(21):575–578.
13. Bakardjiev AI, Stacy BA, Fisher SJ, Portnoy DA. Listeriosis in the pregnant guinea pig: a model of vertical transmission. *Infect Immun*. 2004;72(1):489–497.
14. Guleria I, Pollard JW. The trophoblast is a component of the innate immune system during pregnancy. *Nat Med*. 2000;6(5):589–593.
15. Redline RW, Lu CY. Role of local immunosuppression in murine fetoplacental listeriosis. *J Clin Invest*. 1987;79(4):1234–1241.
16. Rowe JH, Ertelt JM, Xin L, Way SS. Listeria monocytogenes cytoplasmic entry induces fetal wastage by disrupting maternal $Foxp3^+$ regulatory T cell-sustained fetal tolerance. *PLoS Pathog*. 2012;8(8):e1002873.
17. Ertelt JM, et al. $Foxp3^+$ regulatory T cells impede the priming of protective $CD8^+$ T cells. *J Immunol*. 2011;187(5):2569–2577.
18. Le Monnier A, et al. ActA is required for crossing of the fetoplacental barrier by Listeria monocytogenes. *Infect Immun*. 2007;75(2):950–957.
19. Prins JR, et al. Preeclampsia is associated with lower percentages of regulatory T cells in maternal blood. *Hypertens Pregnancy*. 2009;28(3):300–311.
20. Santner-Nanan B, et al. Systemic increase in the ratio between $Foxp3^+$ and IL-17-producing $CD4^+$ T cells in healthy pregnancy but not in preeclampsia. *J Immunol*. 2009;183(11):7023–7030.
21. Sasaki Y, et al. Proportion of peripheral blood and decidual CD4(+) CD25(bright) regulatory T cells in pre-eclampsia. *Clin Exp Immunol*. 2007;149(1):139–145.
22. Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S. Decidual and peripheral blood $CD4^+CD25^+$ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. *Mol Hum Reprod*. 2004;10(5):347–353.
23. Schober L, Radnai D, Schmitt E, Mahnke K, Sohn C, Steinborn A. Term and preterm labor: decreased suppressive activity and changes in composition of the regulatory T-cell pool. *Immunol Cell Biol*. 2012;90(10):935–944.
24. Jiang TT, et al. Regulatory T cells: new keys for further unlocking the enigma of fetal tolerance and pregnancy complications. *J Immunol*. 2014;192(11):4949–4956.
25. Rowe JH, Ertelt JM, Aguilera MN, Farrar MA, Way SS. Foxp3(+) regulatory T cell expansion required for sustaining pregnancy compromises host defense against prenatal bacterial pathogens. *Cell Host Microbe*. 2011;10(1):54–64.
26. Rowe JH, Ertelt JM, Xin L, Way SS. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. *Nature*. 2012;490(7418):102–106.
27. Nancy P, Tagliani E, Tay CS, Asp P, Levy DE, Erlebacher A. Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface. *Science*. 2012;336(6086):1317–1321.
28. Perchellet AL, Jasti S, Petroff MG. Maternal $CD4^+$ and $CD8^+$ T cell tolerance towards a fetal minor histocompatibility antigen in T cell receptor transgenic mice. *Biol Reprod*. 2013;89(4):102.
29. Berg RE, Cordes CJ, Forman J. Contribution of $CD8^+$ T cells to innate immunity: IFN-γ secretion induced by IL-12 and IL-18. *Eur J Immunol*. 2002;32(10):2807–2816.
30. O'Donnell H, et al. Toll-like receptor and inflam-

masome signals converge to amplify the innate bactericidal capacity of T helper 1 cells. *Immunity*. 2014;40(2):213–224.
31. Johanns TM, et al. Early eradication of persistent Salmonella infection primes antibody-mediated protective immunity to recurrent infection. *Microbes Infect*. 2011;13(4):322–330.
32. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nat Immunol*. 2003;4(4):330–336.
33. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003;299(5609):1057–1061.
34. Pircher H, Burki K, Lang R, Hengartner H, Zinkernagel RM. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. *Nature*. 1989;342(6249):559–561.
35. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. *Cell*. 1994;76(1):17–27.
36. Manning TC, Rund LA, Gruber MM, Fallarino F, Gajewski TF, Kranz DM. Antigen recognition and allogeneic tumor rejection in $CD8^+$ TCR transgenic/RAG(–/–) mice. *J Immunol*. 1997;159(10):4665–4675.
37. Ehst BD, Ingulli E, Jenkins MK. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am J Transplant*. 2003;3(11):1355–1362.
38. Foulds KE, Zenewicz LA, Shedlock DJ, Jiang J, Troy AE, Shen H. Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. *J Immunol*. 2002;168(4):1528–1532.
39. Way SS, Havenar-Daughton C, Kolumam GA, Orgun NN, Murali-Krishna K. IL-12 and type-I IFN synergize for IFN-γ production by CD4 T cells, whereas neither are required for IFN-γ production by CD8 T cells after Listeria monocytogenes infection. *J Immunol*. 2007;178(7):4498–4505.
40. Erlebacher A, Vencato D, Price KA, Zhang D, Glimcher LH. Constraints in antigen presentation severely restrict T cell recognition of the allogeneic fetus. *J Clin Invest*. 2007;117(5):1399–1411.
41. Clark DR, et al. Perinatal Listeria monocytogenes susceptibility despite preconceptual priming and maintenance of pathogen-specific CD8(+) T cells during pregnancy. *Cell Mol Immunol*. 2014;11(6):595–605.
42. Anderson KG, et al. Intravascular staining for discrimination of vascular and tissue leukocytes. *Nat Protoc*. 2014;9(1):209–222.
43. Shi C, Hohl TM, Leiner I, Equinda MJ, Fan X, Pamer EG. Ly6G+ neutrophils are dispensable for defense against systemic Listeria monocytogenes infection. *J Immunol*. 2011;187(10):5293–5298.
44. Bromley SK, Mempel TR, Luster AD. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat Immunol*. 2008;9(9):970–980.
45. Irving WL, et al. Influenza virus infection in the second and third trimesters of pregnancy: a clinical and seroepidemiological study. *BJOG*. 2000;107(10):1282–1289.
46. Ramphal R, Donnelly WH, Small PA. Fatal influenzal pneumonia in pregnancy: Failure to demonstrate transplacental transmission of influenza virus. *Am J Obstet Gynecol*. 1980;138(3):347–348.
47. Tripp RA, Hou S, McMickle A, Houston J, Doherty PC. Recruitment and proliferation of $CD8^+$ T cells in respiratory virus infections. *J Immunol*. 1995;154(11):6013–6021.
48. Gianfrani C, Oseroff C, Sidney J, Chesnut RW, Sette A. Human memory CTL response specific for influenza A virus is broad and multispecific. *Hum Immunol*. 2000;61(5):438–452.
49. Fleming AD, Ehrlich DW, Miller NA, Monif GR. Successful treatment of maternal septicemia due to Listeria monocytogenes at 26 weeks' gestation. *Obstet Gynecol*. 1985;66(3 suppl):52S–53S.
50. Kalstone C. Successful antepartum treatment of listeriosis. *Am J Obstet Gynecol*. 1991;164(1 pt 1):57–58.
51. Rowe JH, Ertelt JM, Xin L, Way SS. Regulatory T cells and the immune pathogenesis of prenatal infection. *Reproduction*. 2013;146(6):R191–203.
52. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol*. 2007;8(2):191–197.
53. Samstein RM, Josefowicz SZ, Arvey A, Treuting PM, Rudensky AY. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell*. 2012;150(1):29–38.
54. Erlebacher A. Immunology of the maternal-fetal interface. *Annu Rev Immunol*. 2013;31:387–411.
55. Zuckermann FA, Head JR. Expression of MHC antigens on murine trophoblast and their modulation by interferon. *J Immunol*. 1986;137(3):846–853.
56. Hunt JS, Andrews GK, Wood GW. Normal trophoblasts resist induction of class I HLA. *J Immunol*. 1987;138(8):2481–2487.
57. Xu C, Mao D, Holers VM, Palanca B, Cheng AM, Molina H. A critical role for murine complement regulator crry in fetomaternal tolerance. *Science*. 2000;287(5452):498–501.
58. Munn DH, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science*. 1998;281(5380):1191–1193.
59. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. *Nat Immunol*. 2004;5(3):266–271.
60. Collins MK, Tay CS, Erlebacher A. Dendritic cell entrapment within the pregnant uterus inhibits immune surveillance of the maternal/fetal interface in mice. *J Clin Invest*. 2009;119(7):2062–2073.
61. Shin H, Iwasaki A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature*. 2012;491(7424):463–467.
62. Schenkel JM, Fraser KA, Vezys V, Masopust D. Sensing and alarm function of resident memory CD8(+) T cells. *Nat Immunol*. 2013;14(5):509–513.
63. Chattopadhyay A, Robinson N, Sandhu JK, Finlay BB, Sad S, Krishnan L. Salmonella enterica serovar Typhimurium-induced placental inflammation and not bacterial burden correlates with pathology and fatal maternal disease. *Infect Immun*. 2010;78(5):2292–2301.
64. Dong S, Kurtis JD, Pond-Tor S, Kabyemela E, Duffy PE, Fried M. CXC ligand 9 response to malaria during pregnancy is associated with low-birth-weight deliveries. *Infect Immun*. 2012;80(9):3034–3038.
65. Zhang H, Hu X, Liu X, Zhang R, Fu Q, Xu X. The Treg/Th17 imbalance in Toxoplasma gondii-infected pregnant mice. *Am J Reprod Immunol*. 2012;67(2):112–121.
66. Krishnan L, Guilbert LJ, Wegmann TG, Belosevic M, Mosmann TR. T helper 1 response against Leishmania major in pregnant C57BL/6 mice increases implantation failure and fetal resorptions. Correlation with increased IFN-γ and TNF and reduced IL-10 production by placental cells. *J Immunol*. 1996;156(2):653–662.
67. Avila-Garcia M, Mancilla-Ramirez J, Segura-Cervantes E, Farfan-Labonne B, Ramirez-Ramirez A, Galindo-Sevilla N. Transplacental transmission of cutaneous Leishmania mexicana strain in BALB/c mice. *Am J Trop Med Hyg*. 2013;89(2):354–358.
68. Osorio Y, et al. Congenital transmission of experimental leishmaniasis in a hamster model. *Am J Trop Med Hyg*. 2012;86(5):812–820.
69. Eltoum IA, et al. Congenital kala-azar and leishmaniasis in the placenta. *Am J Trop Med Hyg*. 1992;46(1):57–62.
70. Schloesser RL, Schaefer V, Groll AH. Fatal transplacental infection with non-typhoidal Salmonella. *Scand J Infect Dis*. 2004;36(10):773–774.
71. Pejcic-Karapetrovic B, Gurnani K, Russell MS, Finlay BB, Sad S, Krishnan L. Pregnancy impairs the innate immune resistance to Salmonella typhimurium leading to rapid fatal infection. *J Immunol*. 2007;179(9):6088–6096.
72. Gu J, et al. H5N1 infection of the respiratory tract and beyond: a molecular pathology study. *Lancet*. 2007;370(9593):1137–1145.
73. McGregor JA, Burns JC, Levin MJ, Burlington B, Meiklejohn G. Transplacental passage of influenza A/Bangkok (H3N2) mimicking amniotic fluid infection syndrome. *Am J Obstet Gynecol*. 1984;149(8):856–859.
74. Yawn DH, Pyeatte JC, Joseph JM, Eichler SL, Garcia-Bunuel R. Transplacental transfer of influenza virus. *JAMA*. 1971;216(6):1022–1023.
75. Lieberman RW, Bagdasarian N, Thomas D, Van De Ven C. Seasonal influenza A (H1N1) infection in early pregnancy and second trimester fetal demise. *Emerg Infect Dis*. 2011;17(1):107–109.
76. Dulyachai W, et al. Perinatal pandemic (H1N1) 2009 infection, Thailand. *Emerg Infect Dis*. 2010;16(2):343–344.
77. O'Riordan M, Yi CH, Gonzales R, Lee KD, Portnoy DA. Innate recognition of bacteria by a macrophage cytosolic surveillance pathway. *Proc Natl Acad Sci U S A*. 2002;99(21):13861–13866.
78. Palese P, Schulman JL. Mapping of the influenza virus genome: identification of the hemagglutinin and the neuraminidase genes. *Proc Natl Acad Sci U S A*. 1976;73(6):2142–2146.
79. Tobita K. Permanent canine kidney (MDCK) cells for isolation and plaque assay of influenza B viruses. *Med Microbiol Immunol*. 1975;162(1):23–27.