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RESEARCH ARTICLE

Perinatal *Listeria monocytogenes* susceptibility despite preconceptual priming and maintenance of pathogen-specific CD8$^+$ T cells during pregnancy

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***Listeria monocytogenes* (Lm) is an intracellular bacterium with unique predisposition for systemic maternal infection during pregnancy and morbid consequences for the developing fetus. Given the high mortality associated with prenatal Lm infection, strategies for augmenting protective immunity during the exceedingly vulnerable period of pregnancy are urgently needed. Herein, protection conferred by attenuated Lm administered before pregnancy against subsequent virulent Lm prenatal infection was evaluated. We show that protection against secondary Lm infection in non-pregnant mice is sharply moderated during allogeneic pregnancy because significantly more bacteria are recovered from maternal tissues, despite the numerical and functional preservation of pathogen-specific CD8$^+$ T cells. More importantly, preconceptual priming does not protect against *in utero* invasion or fetal wastage because mice inoculated with attenuated Lm prior to pregnancy and naive pregnant controls each showed near complete fetal resorption and pathogen recovery from individual concepti after Lm infection during pregnancy. Remarkably, the lack of protection against prenatal Lm infection with preconceptual priming in allogeneic pregnancy is restored during syngeneic pregnancy. Thus, maternal–fetal antigen discordance dictates the ineffectiveness of preconceptual vaccination against fetal complications after prenatal Lm infection, despite the numerical and functional preservation of pathogen-specific CD8$^+$ T cells.**
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INTRODUCTION

Listeria monocytogenes (Lm) is a ubiquitous, Gram-positive, intracellular bacterium that causes opportunistic invasive infection among individuals with naturally occurring defects in host defense, which includes newborn infants, the elderly and pregnant women.[1,2] Among these susceptible individuals, pregnancy confers unique vulnerability, with 10- to 20-fold increased rates of invasive systemic infection.[3–5] Furthermore, maternal infection often triggers morbid fetal complications, including spontaneous abortion, stillbirth and neonatal sepsis. In the largest published series containing 178 cases of maternal infection where pregnancy outcomes were available, 20% of pregnancies terminated in spontaneous abortion or stillbirth, whereas 68% of the remaining live offspring were infected.[6] Among infected neonates, the mortality rate ranges from 25% to 35%, despite the judicious use of antimicrobials with potent Lm bactericidal activity.[6–8] This predisposition for maternal Lm infection during pregnancy and fetal wastage is not unique to humans but is widely recapitulated across mammalian species, including non-human primates, ruminants and rodents.[9–14] Given this exceptionally high susceptibility with ensuing negative consequences on perinatal health, improved strategies for protecting pregnant women against Lm infection are urgently needed.

One approach with proven efficacy in protecting pregnant women and babies against infection is preconceptual vaccination. The widespread implementation of vaccines against rubella have nearly eradicated the once-prevalent pregnancy complications and birth defects associated with this congenital viral pathogen in the United States and other developed countries.[15,16] Vaccination before pregnancy is also highly effective in preventing maternal and neonatal tetanus caused by the

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ubiquitous toxigenic bacterium *Clostridium tetani*.[17,18] Similarly, the ongoing evaluation of inactivated or live attenuated vaccine formulations shows promising protection against fetal invasion and mortality in animal models of prenatal cytomegalovirus, *Salmonella enterica* or *Toxoplasma gondii* infection.[19–23] For cytomegalovirus and *T. gondii*, these findings with vaccine-induced immunity parallel the protective benefits of primary infection prior to pregnancy against congenital infection.[24–26] Thus, preconceptual vaccination represents a promising universal strategy for bolstering immunity against pathogens that cause prenatal infection and pregnancy complications.

Notably, in sharp contrast to the protection against the aforementioned prenatal pathogens, an initial evaluation reported that preconceptual priming with attenuated Lm does not protect against the fetal wastage induced by virulent Lm infection among outbred mice.[27] An interesting distinction between Lm and other pathogens for which preconceptual vaccination confers protection is the adaptive mediators of protective immunity. In particular, though protection against rubella, tetanus and cytomegalovirus is associated with the production of high-titer, pathogen-specific antibodies, these immune components play only a modest or no protective role against Lm, which primarily resides within infected host cells.[15–18,20,24,28–31] Instead, protective immunity against Lm infection is conferred by $CD8^+$ T cells with Lm specificity.[32,33] The importance of $CD8^+$ T cells in protection against Lm infection is highlighted by the widespread use of attenuated strains for prime-boost followed by a secondary virulent Lm challenge to probe the bacterial virulence determinants along with the molecular and cellular immune components essential for priming activation, expansion and memory features of pathogen-specific $CD8^+$ T cells.[34–37] An emerging consensus is that Lm entry into the host cell cytoplasm is required for priming protective $CD8^+$ T cells.[38–40] Thus, investigating the use of preconceptual vaccination for protection against prenatal Lm infection also addresses more fundamental questions regarding how pregnancy-associated physiological shifts impact the numerical and functional retention of antigen-experienced $CD8^+$ T cells and their protective capacity.

This study evaluates the immune cell biological mechanisms that determine why prenatal Lm infection is distinctively resistant to preconceptual priming. The primary focus was on how pregnancy impacts the quantitative retention, effector cytokine production and cytolytic properties of the pathogen-specific $CD8^+$ T cells primed by immunogenic Lm that retains cytoplasmic entry, but is attenuated due to a targeted deletion in ActA required for intercellular spreading and productive infection.[34,41] To further investigate the immune tolerance pathways uniquely activated by maternal–fetal antigen discordance, the effectiveness of preconceptual Lm ΔactA priming on protection against virulent Lm prenatal infection was evaluated with regard to maternal susceptibility, pregnancy outcomes and fetal invasion, with a comparison made between allogeneic and syngeneic pregnancies. Together, data highlighting maternal–fetal antigen heterogeneity in mitigating the protective properties of antigen-experienced $CD8^+$ T cells during pregnancy are provided.

MATERIALS AND METHODS

Mice

C57BL/6 (H-2^b), Balb/c (H-2^d) and B6.PL-*Thy1*^{a} (H-2^b, CD90.1^+) mice were purchased from The National Cancer Institute or Jackson Laboratory (Bar Harbor, ME, USA). After mating, the timing of pregnancy was evaluated by visualization of a copulation plug, and the mice were used 12 days thereafter (embryonic day 12.5 (E12.5)). For infection, recombinant Lm-OVA and the isogenic ΔactA mutant, Lm-OVA ΔactA, were each grown in brain heart infusion medium at 37 °C, back-diluted to early log-phase (OD_{600} 0.1), washed and suspended in sterile saline, and then injected intravenously through the lateral tail vein at the following dosages: Lm-OVA ΔactA, 10^6 colony forming units (CFUs); Lm-OVA, 10^4 or 10^5 CFUs.[42,43] Recoverable Lm-OVA were enumerated by plating serial dilutions of the homogenate for each maternal tissue or conceptus (placental–fetal unit) onto agar plates as described.[36,37] Pregnancy outcomes were evaluated 2 or 3 days after Lm-OVA prenatal infection in E12.5 pregnant mice. All experiments were performed under Cincinnati Children's Hospital Institutional Animal Care and Use Committee approved protocols.

Tissue harvest, cell staining and stimulation

Single-cell splenocytes were prepared by tissue dissociation between frosted glass slides and red blood cell lysis in ammonium chloride buffer. Decidual cells were isolated from each uterine horn by dissection in ice-cold Hanks' balanced salt solution and peeling away the decidua from each conceptus. Thereafter, the decidual cells were harvested by dissociation between frosted glass slides and filtration through a 70-μm cell strainer. Fluorophore-conjugated antibodies for cell surface proteins and intracellular staining were purchased from eBioscience (San Diego, CA, USA) or BD Biosciences (San Jose, CA, USA). $CD8^+$ T cells with $OVA_{257–264}$ specificity were identified by staining splenocytes or decidual cells with H-$2K^b$ dimer X loaded with $OVA_{257–264}$ peptide or fluorophore-conjugated H-$2K^b$ $OVA_{257–264}$ tetramer as described.[36,44] For cytokine production, splenocytes were stimulated *ex vivo* with $OVA_{257–264}$ peptide (1 μM) for 5 h at 37 °C in medium (DMEM plus 10% fetal bovine serum) supplemented with Brefeldin A.[36,37,43]

In vivo cytotoxicity

To measure *in vivo* cytolytic activity, splenocytes from CD90.1^+ congenic mice were stained with different (50 nM or 1 μM) concentrations of carboxyfluorescein succinimidyl ester (CFSE) for 10 min at room temperature, pulsed with $OVA_{257–264}$ peptide ($CFSE^{low}$) or $M38_{316–323}$ control peptide ($CFSE^{high}$) and combined at a 1 : 1 ratio before intravenous transfer into recipient CD90.2^+ mice.[37,45] Twenty-four hours after transfer, the ratio of $OVA_{257–264}$ ($CFSE^{low}$) compared with $M38_{316–323}$ control peptide-pulsed cells ($CFSE^{high}$) was enumerated. Percent killing was calculated as 100−[(percent $OVA_{257–264}$ peptide-pulsed cells recovered/percent $M38_{316–323}$

peptide-pulsed cells recovered)/(percent $OVA_{257–264}$ peptide-pulsed cells transferred/percent $M38_{316–323}$ peptide-pulsed cells transferred)]×100, as described.[37,45,46]

Statistical analysis

The percentage, cell number, $\log_{10}$ recoverable bacterial CFUs and frequency of fetal resorption were first analyzed and found to be normally distributed. Thereafter, differences between groups were evaluated using the unpaired Student's *t*-test (Prism; GraphPad Software, La Jolla, CA, USA), with $P<0.05$ taken as indicating statistical significance.

RESULTS

$CD8^+$ T cells activated by preconceptual priming are retained during pregnancy

Given the necessity for $CD8^+$ T cells in protection against Lm infection, maintenance of these pathogen-specific immune components during pregnancy was evaluated. These experiments utilized the well-characterized attenuated ΔactA mutant of recombinant Lm engineered to stably express ovalbumin (Lm-OVA ΔactA), transforming this protein into a surrogate Lm-specific antigen.[42,43] In turn, endogenous Lm-OVA-specific $CD8^+$ T cells are identified based on the MHC class I H-$2K^b$-restricted $OVA_{257–264}$ peptide in C57BL/6 mice during allogeneic pregnancy.[47] Consistent with our prior studies,[36,44] $CD8^+$ T cells with $OVA_{257–264}$ specificity among the splenocytes that bind cognate peptide-loaded H-$2K^b$ multimers and downregulate CD62L expand sharply within the first week after Lm-OVA ΔactA inoculation in non-pregnant mice (Figure 1a). Thereafter, activated Lm-OVA specific $CD8^+$ T cells contract in percentage and absolute numbers to ~5% of the peak expansion levels by days 14 through 42 post-infection (Figure 1a).

To investigate how pregnancy impacts the retention of $CD8^+$ T cells primed by preconceptual vaccination, $OVA_{257–264}$-specific $CD8^+$ T cells were evaluated in pregnant mice inoculated with Lm-OVA ΔactA prior to mating. Thirty days after Lm-OVA ΔactA inoculation in C57BL/6 female mice, mating with Balb/c males was initiated, followed by the analysis of maternal OVA-specific $CD8^+$ T cells embryonic day 12.5 (E12.5) compared with virgin control mice. Using this approach that does not noticeably impact fertility,[48] the majority of the mice became plugged within 5 days after mating, allowing for a direct comparison between pregnant and non-pregnant mice 42–47 days after Lm-OVA ΔactA priming. We found that whereas a modest ~50% reduction in the percent of $CD8^+$ T cells with $OVA_{257–264}$ specificity in pregnant compared with

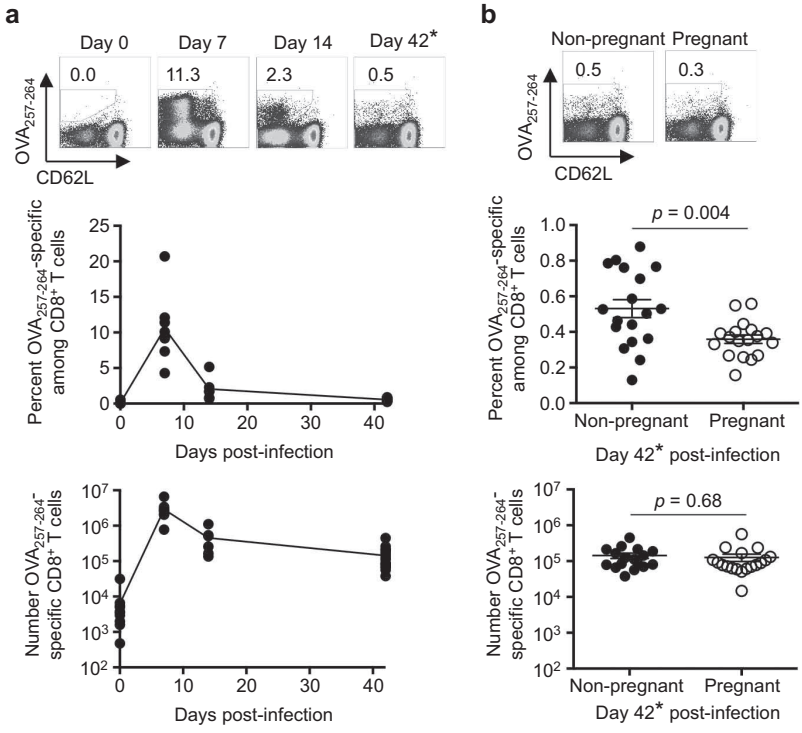

Figure 1 $CD8^+$ T cells primed by preconceptual attenuated Lm are retained during pregnancy. (**a**) Representative plots gated on $CD8^+$ T cells and composite analysis showing the percent and absolute number of OVA-specific $CD8^+$ T cells among splenocytes at each time point following primary infection with Lm-OVA ΔactA (10^6 CFUs), as identified by $OVA_{257–264}$ peptide-loaded H-$2K^b$ dimer staining. (**b**) Representative plots gated on $CD8^+$ T cells and composite analysis showing the percent and absolute number of OVA-specific $CD8^+$ T cells among splenocytes in virgin non-pregnant and pregnant (E12.5) mice at 42–47 days after Lm-OVA ΔactA priming. Thirty days after primary infection with Lm-OVA ΔactA (10^6 CFUs), female C57BL/6 mice were mated with Balb/c males, and the majority became pregnant within 5 days after mating. Data shown are from day 42*, encompassing the combined data for mice harvested 42–47 days after Lm-OVA ΔactA priming. Each data point represents the results from an individual mouse, representative of four independent experiments with similar results. Bar, mean±one standard deviation. CFU, colony forming unit; Lm, *Listeria monocytogenes*.

virgin control mice was consistently observed, the number of $OVA_{257–264}$-specific $CD8^+$ T cells among splenocytes was not significantly diminished (Figure 1b). This discordance between pregnancy-associated shifts in percent compared with absolute cell numbers may reflect the expansion of $CD8^+$ T cells with paternal alloantigen specificity,[49] which likely dilutes, but does not reduce, the overall number of cells with non-fetal Lm-OVA specificity. Thus, pregnancy does not appreciably impact the retention of antigen-specific $CD8^+$ T cells primed by preconceptual vaccination.

Diminished IFN-γ and TNF-α production by $CD8^+$ T cells retained during pregnancy

Additional studies using this model of preconceptual Lm-OVA ΔactA priming addressed whether pregnancy regulates the functional properties of $CD8^+$ T cells by investigating their capacity to produce the canonical effector cytokines IFN-γ and TNF-α, which are essential for protection against prenatal Lm infection.[50,51] Following *ex vivo* stimulation with $OVA_{257–264}$ peptide, cytokine production by $CD8^+$ T cells was evaluated by intracellular cytokine staining. In contrast to tracking cells of the same specificity using peptide-loaded MHC multimers, the percent and absolute number of IFN-γ producing $CD8^+$ T cells were significantly reduced among splenocytes isolated from pregnant compared with virgin control mice, each inoculated with Lm-OVA ΔactA 42–47 days prior (Figure 2a and b). In particular, while the IFN-γ production remained consistently above the background levels in cells from naive mice or each group of Lm-OVA ΔactA-primed mice without peptide stimulation, the percent and number of IFN-γ producing $CD8^+$ T cells were both reduced by ~50% during pregnancy (Figure 2b). Furthermore, despite the diminished overall levels of TNF-α compared with IFN-γ cytokine production, similar magnitude reductions in the percent and number of TNF-α producing $CD8^+$ splenocytes following $OVA_{257–264}$ peptide stimulation were found in pregnant compared with non-pregnant mice (Figure 2c). Taken together, these results demonstrate that antigen-specific $CD8^+$ T cells primed by preconceptual Lm-OVA ΔactA have diminished capacity for effector cytokine production during pregnancy.

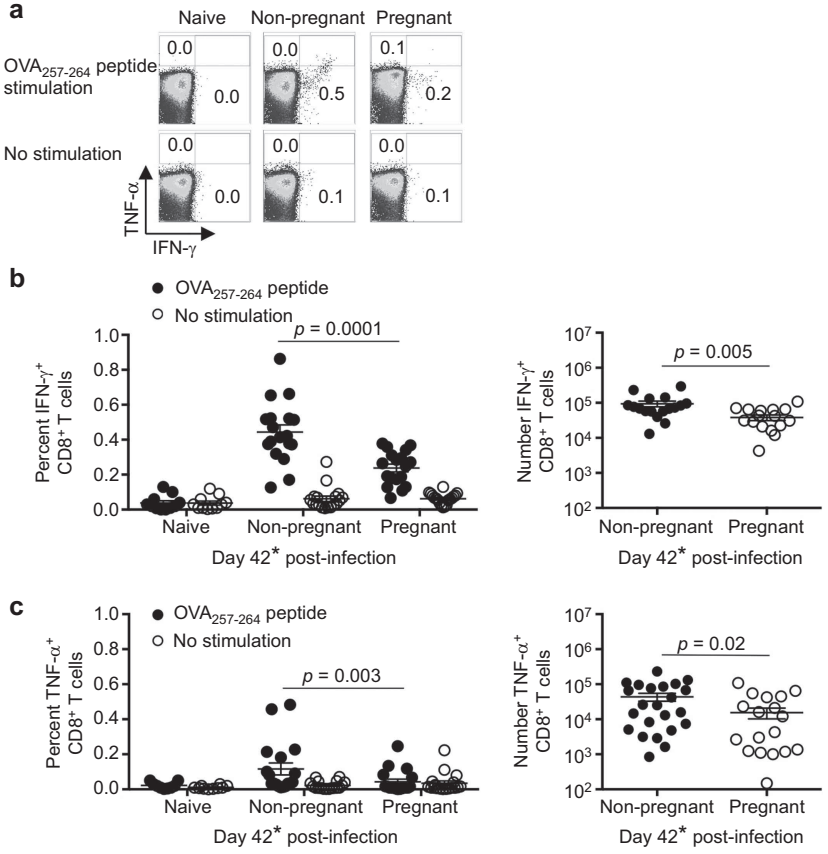

Figure 2 Diminished IFN-γ and TNF-α production by antigen-specific $CD8^+$ T cells during pregnancy. (**a**) Representative plots showing IFN-γ and TNF-α production by $CD8^+$ T cells after $OVA_{257–264}$ peptide stimulation compared with unstimulated controls among naive (no prior Lm-OVA ΔactA), virgin non-pregnant or pregnant E12.5 mice at 42–47 days after primary infection with Lm-OVA ΔactA (10^6 CFUs). (**b**) The percent and absolute number of IFN-γ-producing $CD8^+$ splenocytes for the mice described in **a**. (**c**) The percent and absolute number of TNF-α-producing $CD8^+$ splenocytes for the mice described in **a**. Data shown are from day 42*, encompassing the combined data for mice harvested 42–47 days after Lm-OVA ΔactA priming. Each data point represents the results from an individual mouse, representative of four independent experiments with similar results. Bar, mean±one standard deviation. CFU, colony forming unit; Lm, *Listeria monocytogenes*.

Pregnancy moderates protection against secondary infection primed by attenuated Lm

Given the importance of IFN-γ and TNF-α in placental immunity against Lm infection,[51] pregnancy-associated reductions in the production of these cytokines may cause the reported susceptibility to secondary Lm infection during pregnancy.[27] To investigate this hypothesis and further address whether preconceptual priming protects against prenatal Lm infection-induced fetal complications, susceptibility to virulent Lm-OVA was evaluated in pregnant and non-pregnant mice that were each primed with Lm-OVA ΔactA 42–47 days prior compared with naive control mice. Here, a dosage of 10^5 virulent Lm-OVA representing the minimum inoculum that triggers consistent fetal complications after infection in naive mice during allogeneic pregnancy was utilized (fetal resorption=28% (range: 0–100%), n=10 after infection with 10^4 Lm-OVA). As expected, non-pregnant mice previously primed with Lm-OVA ΔactA were protected against secondary infection, with near complete elimination of bacteria in the spleen and 1000-fold reduced Lm recovery in the liver compared with naive control mice (Figure 3a). Comparatively, pregnancy significantly diminished the level of protection of mice previously inoculated with Lm-OVA ΔactA; recoverable bacteria in these maternal tissues were increased 40- to 100-fold compared with virgin control mice (Figure 3a). However, protection was not completely eliminated during pregnancy because Lm recovery from the maternal spleen and liver remained significantly reduced compared with naive control mice without prior Lm-OVA ΔactA priming (Figure 3a). Notably, with regard to pregnancy outcomes and *in utero* fetal invasion, preconceptual priming conferred no appreciable protective benefits, as the frequency of fetal resorption, loss of live pups and pathogen recovery from the individual concepti each approached 100% and were identical after prenatal Lm OVA infection, regardless of prior Lm-OVA ΔactA inoculation (Figure 3b). To investigate whether the diminished protection against virulent Lm-OVA prenatal infection in the maternal tissues becomes permanently imprinted by pregnancy, postpartum susceptibility for mice primed with Lm-OVA ΔactA prior to pregnancy was evaluated. The analysis showed that recoverable bacteria in

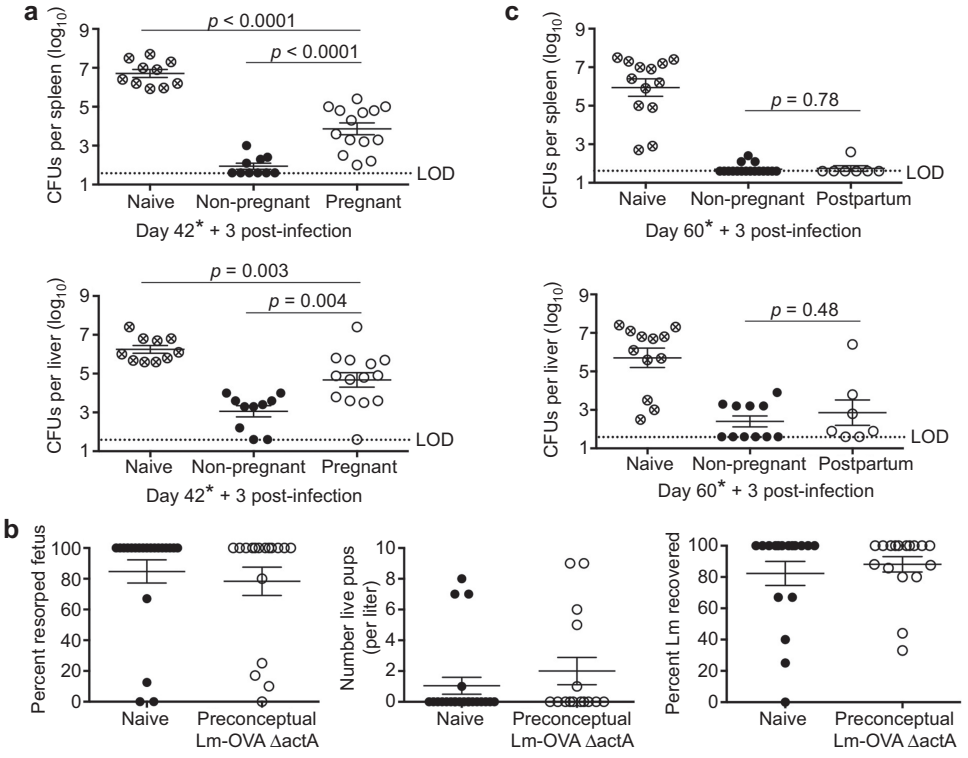

Figure 3 Protection against secondary virulent Lm infection is modulated during allogeneic pregnancy. (**a**) The number of recoverable Lm in the spleen and liver 3 days after virulent Lm-OVA (10^5 CFUs) infection in naive (no prior Lm-OVA ΔactA) or secondary infection among virgin non-pregnant or pregnant E12.5 mice at 42–47 days after primary infection with Lm-OVA ΔactA. Data shown are from day 42*+3, encompassing the combined data 3 days after secondary Lm-OVA challenge in mice primed 42–47 days prior with Lm-OVA ΔactA. (**b**) The percent fetal resorption, number of live pups, and Lm recovery from each placental fetal unit 3 days after Lm-OVA (10^5 CFUs) infection in pregnant naive mice (no prior Lm-OVA ΔactA) or pregnant mice primed 42–47 days prior with Lm-OVA ΔactA. (**c**) The number of recoverable Lm in the spleen and liver 3 days after virulent Lm-OVA (10^5 CFUs) infection in naive (no prior Lm-OVA ΔactA) or secondary infection in virgin non-pregnant or postpartum day 10 mice each primed with Lm-OVA ΔactA 60–65 days prior. The data shown are from day 60*+3, encompassing the combined data 3 days after secondary Lm-OVA challenge in mice primed 60–65 days prior with Lm-OVA ΔactA. Each data point represents the results from an individual mouse, representative of six independent experiments with similar results. Bar, mean±one standard deviation. CFU, colony forming unit; Lm, *Listeria monocytogenes*; LOD, limit of detection.

the maternal spleen and liver were eliminated to near completion similarly in postpartum day 10 and virgin control mice that were each inoculated with Lm-OVA ΔactA 60–65 days prior to rechallenge (Figure 3c). Thus, the protective benefits of Lm-OVA ΔactA priming are distinctively modulated during pregnancy, with only partial reductions in pathogen recovery from the maternal tissues and no discernable protection against *in utero* fetal invasion and wastage. In turn, sterilizing immunity returns postpartum, indicating that the diminished protection is transient and unique to the physiological changes that occur during pregnancy.

$CD8^+$ T-cell secondary expansion and cytolytic activity are not diminished during pregnancy

The retained susceptibility to prenatal Lm infection despite a comparable accumulation of pathogen-specific $CD8^+$ T cells may also reflect pregnancy-associated alterations in immune cell function.[52,53] With regard to preconceptual Lm-OVA ΔactA priming, this notion is supported by reduced $CD8^+$ T-cell production of IFN-γ and TNF-α, which are cytokines essential for placental Lm immunity (Figure 2).[51] To further investigate this hypothesis, the capacity for secondary expansion that represents a hallmark feature of functional memory immune cells was evaluated. We found that the percentage and number of $OVA_{257–264}$-specific $CD8^+$ splenocytes re-expanding at 3 days after secondary Lm-OVA infection in mice previously primed with Lm-OVA ΔactA was similar between pregnant and non-pregnant mice (Figure 4a). Similarly, the number of IFN-γ- and TNF-α-producing $CD8^+$ splenocytes re-expanded to comparable levels after secondary Lm-OVA infection in Lm-OVA ΔactA-primed pregnant and virgin control mice (Figure 4b). Notably, OVA-specific $CD8^+$ T-cell accumulation was not restricted only for the systemic immune cells among the splenocytes, but was apparent within the decidua among mice previously primed with Lm-OVA ΔactA prior to and shortly after secondary virulent Lm-OVA infection (Figure 4c). For this analysis, decidual cells were harvested 48 h after Lm-OVA prenatal infection. At this time point, viable

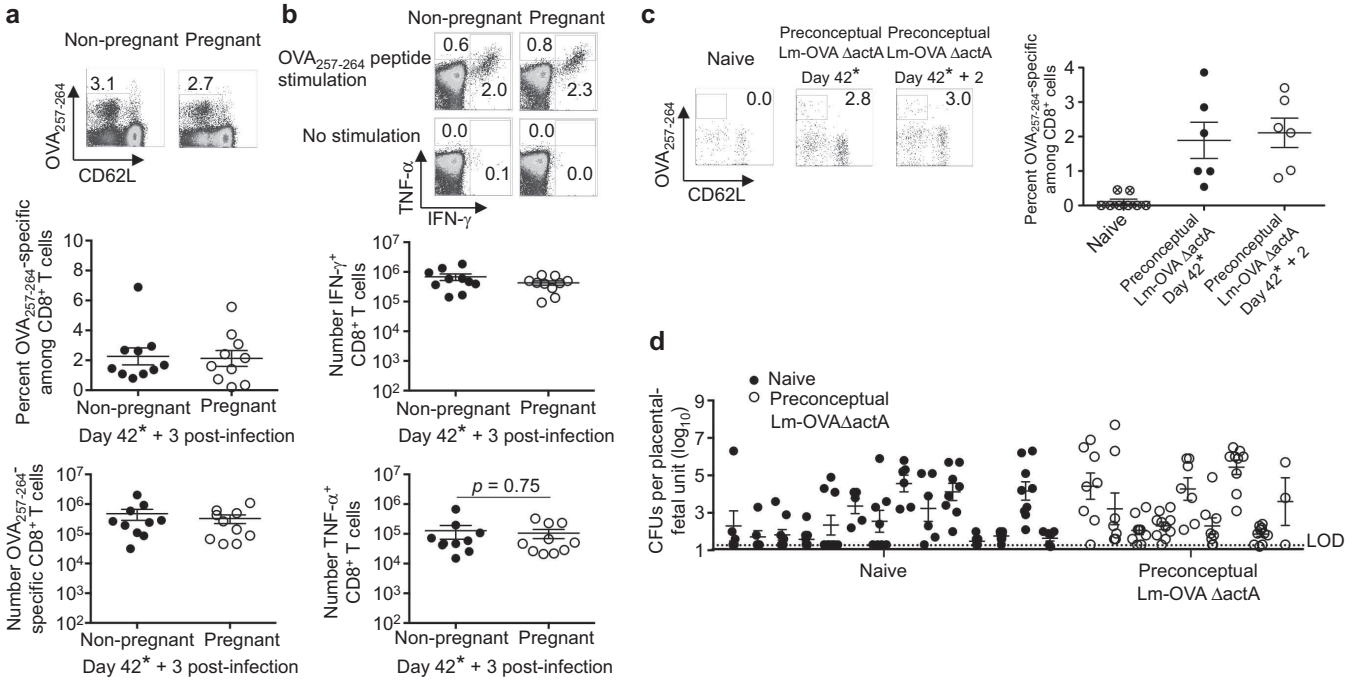

Figure 4 Secondary expansion and decidual accumulation of antigen-specific memory $CD8^+$ T cells during pregnancy. (**a**) Representative plots gated on $CD8^+$ T cells and composite analysis showing the percent and absolute number of OVA-specific $CD8^+$ T cells among splenocytes, as identified by $OVA_{257–264}$ peptide-loaded H-$2K^b$ dimer staining day 3 after Lm-OVA (10^5 CFUs) secondary infection in virgin non-pregnant or pregnant E12.5 mice primed with Lm-OVA ΔactA 42–47 days prior. (**b**) Representative plots gated on $CD8^+$ T cells and composite analysis showing the absolute number of IFN-γ- and TNF-α-producing $CD8^+$ splenocytes 3 days after Lm-OVA (10^5 CFUs) secondary challenge in virgin non-pregnant or pregnant E12.5 mice primed with Lm-OVA ΔactA 42–47 days prior. Data shown are from day 42*+3, encompassing the combined data 3 days after secondary Lm-OVA challenge in mice primed 42–47 days prior with Lm-OVA ΔactA. (**c**) Representative plots gated on decidual $CD8^+$ T cells and composite analysis showing the percent of $CD8^+$ T cells with OVA specificity, as identified by H-$2K^b$ tetramer staining among decidual cells from E12.5 mice at 2 days after Lm-OVA infection (naive), compared with E12.5 pregnant mice primed with Lm-OVA ΔactA 42–47 days prior either before or at 2 days after secondary Lm-OVA infection. (**d**) The number of recoverable Lm from each placental-fetal unit 2 days after virulent Lm-OVA infection from individual pregnant mice without prior Lm-OVA ΔactA infection (naive), or mice previously infected with Lm-OVA ΔactA 42–47 days prior. Data shown are from day 42*+2, encompassing the combined data 2 days after secondary Lm-OVA challenge in mice primed 42–47 days prior with Lm-OVA ΔactA. Each data point represents the results from an individual mouse (**a**–**c**), or an individual placental–fetal unit (**d**), representative of six independent experiments with similar results. Bar, mean±one standard deviation. CFU, colony forming unit; Lm, *Listeria monocytogenes*; LOD, limit of detection.

bacteria are consistently recovered from each placental-fetal unit, and fetal resorption is ongoing, bypassing the inconsistent recovery of decidual tissue associated with fetal resorption by 72 h post-infection (Figure 4d). Together, these data indicate that retained susceptibility to prenatal Lm infection cannot be explained by an inadequate number of pathogen-specific $CD8^+$ T cells, their capacity for production of protective cytokines, or their ability to infiltrate the maternal-fetal interface. In fact, considering that IFN-γ- and TNF-α-producing $CD8^+$ T cells are each significantly diminished among pregnant mice prior to secondary infection (Figure 2), the swift normalization of these cell numbers within three days after secondary prenatal Lm-OVA infection could represent a more exaggerated re-expansion among pregnant compared with virgin control mice.

However, a potential caveat in the interpretation of these results is that the significantly higher Lm-OVA pathogen burden in pregnant mice after secondary infection may provide additional antigen for *in vivo* stimulation (Figure 3a). To circumvent this limitation and further investigate how pregnancy impacts the functional capacity of retained $CD8^+$ T cells with OVA specificity, a complementary approach of evaluating the antigen-specific *in vivo* cytolytic activity that more directly correlates with protection against Lm infection was utilized.[54,55] Splenocytes from isogenic C57BL/6 mice that express the CD90.1 congenic marker were labeled with various concentrations of CFSE and loaded with either $OVA_{257–264}$ ($CFSE^{low}$) or $M38_{316–323}$ control ($CFSE^{high}$) peptides that allow for discrimination by flow cytometry (Figure 5a). Thereafter, these cells were combined at a 1 : 1 ratio and intravenously injected into pregnant or non-pregnant mice each day from days 42–47 after Lm-OVA ΔactA priming, along with naive control mice. In this assay, a reduction in the starting ratio of $OVA_{257–264}$-pulsed ($CFSE^{low}$) compared with $M38_{316–323}$ control peptide ($CFSE^{high}$)-loaded $CD90.1^+$ cells indicates selective *in vivo* cytolytic activity by cells with $OVA_{257–264}$ specificity. As expected, $CD90.1^+$ cells recovered from naive mice remained at an ~1 : 1 ratio of $CFSE^{low}/CFSE^{high}$, illustrating only a negligible background $OVA_{257–264}$-specific cytolytic activity. Comparatively, $OVA_{257–264}$ peptide-loaded ($CFSE^{low}$) cells were selectively eliminated to near completion from Lm-OVA ΔactA-primed non-pregnant and pregnant mice with comparable efficiency, demonstrating that OVA-specific $CD8^+$ T-cell cytolytic properties are retained during pregnancy (Figure 5b). Thus, resistance to Lm prenatal infection is not observed, despite the numerical and functional preservation of pathogen-specific cytolytic $CD8^+$ T cells.

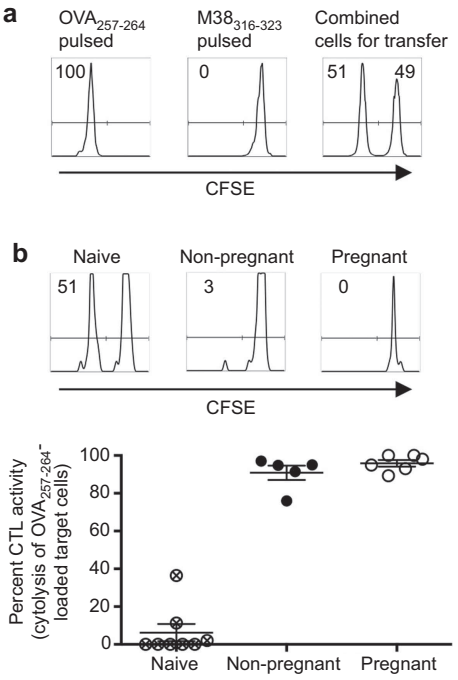

Figure 5 Antigen-specific memory $CD8^+$ T-cell cytolytic properties are not diminished during pregnancy. (**a**) Representative plots show staining with various CFSE concentrations, allowing $OVA_{257–264}$ and $M38_{316–323}$ peptide-loaded $CD90.1^+$ target cells to be discriminated by flow cytometry. (**b**) Representative plots and composite analysis showing the recovery of each $CD90.1^+$ donor cell subset 24 h after adoptive transfer into naive (no prior Lm-OVA ΔactA), compared with virgin non-pregnant or pregnant E12.5 mice at 42–47 days after primary infection with Lm-OVA ΔactA. Each data point represents the results from an individual mouse, representative of two independent experiments with similar results. Bar, mean±one standard deviation. CFSE, carboxyfluorescein succinimidyl ester; CFU, colony forming unit.

Diminished protection by preconceptual priming during allogeneic pregnancy is restored in syngeneic pregnancy

Given that susceptibility to secondary Lm-OVA infection during allogeneic pregnancy cannot be explained by either diminished retention of cytokine-producing, pathogen-specific memory $CD8^+$ T cells or their cytolytic functional properties, related experiments more broadly evaluated the contribution of the immunological shifts in place to mitigate maternal–fetal conflict. In particular, we reasoned that if the diminished protection reflects the activation of immune-modifying pathways such as $Foxp3^+$ regulatory T cells (Tregs), tryptophan catabolism by IDO, galectin-1, or ligands for programmed cell death protein 1 or inducible T-cell costimulator, each of which was previously shown to be essential for maintaining fetal tolerance in allogeneic pregnancy,[56–61] the protection gained by preconceptual Lm-OVA ΔactA priming would be restored during syngeneic pregnancy, when the necessity for these immunosuppressive pathways is moderated. To test this hypothesis, C57BL/6 males were substituted for Balb/c males for breeding with C57BL/6 females, and Lm-OVA susceptibility was re-evaluated among pregnant compared with non-pregnant mice previously primed with Lm-OVA ΔactA mice. After secondary infection during syngeneic pregnancy sired by C57BL/6 males, the number of recoverable bacteria in the spleen and liver of Lm-OVA ΔactA-primed pregnant and non-pregnant control mice was indistinguishably low, and each was markedly reduced compared with naive mice without prior Lm-OVA ΔactA inoculation (Figure 6a). In turn, protection against fetal wastage and *in utero* invasion with preconceptual Lm-OVA ΔactA priming became markedly improved during syngeneic pregnancy compared with the pregnancy outcomes after secondary

Lm-OVA infection in mice bearing allogeneic pregnancy (Figure 6b). Notably, restored protection against prenatal Lm infection among syngeneic compared with allogeneic pregnancies paralleled increased pathogen-specific $CD8^+$ T-cell accumulation in the decidua early (day 2) after secondary Lm-OVA infection (Figure 6c). Taken together, these results illustrate that maternal–fetal antigen discordance dictates the ineffectiveness of preconceptual priming for protection against Lm prenatal infection.

DISCUSSION

Preconceptual vaccination provides unambiguous protection to mother and fetus against prenatal pathogens of ongoing global importance, including rubella and tetanus.[15–18] These protective benefits primed by natural infection or experimental vaccination extend to many other human pathogens that cause perinatal complications, including cytomegalovirus, influenza, pertussis, pneumococcus, *Salmonella* and *Toxoplasma* sp. in epidemiological studies and preclinical analysis.[19–24,62,63] Accordingly, we investigated whether this strategy could be applied to bolstering maternal immunity and protection against the fetal complications triggered by Lm prenatal infection. In sharp contrast to the protective benefits previously described for other prenatal pathogens, our data show that preconceptual priming with immunogenic attenuated Lm that protects against virulent Lm challenge in non-pregnant mice does not protect against fetal wastage during allogeneic pregnancy (Figure 3). Along with near complete fetal invasion and resorption, the Lm pathogen burden was significantly increased in the maternal tissues (e.g., spleen and liver) compared with non-pregnant controls, illustrating that the accumulation of normally protective pathogen-specific adaptive immune components does not circumvent host defense defects that cause innate Lm susceptibility during pregnancy.[13,14,45] In

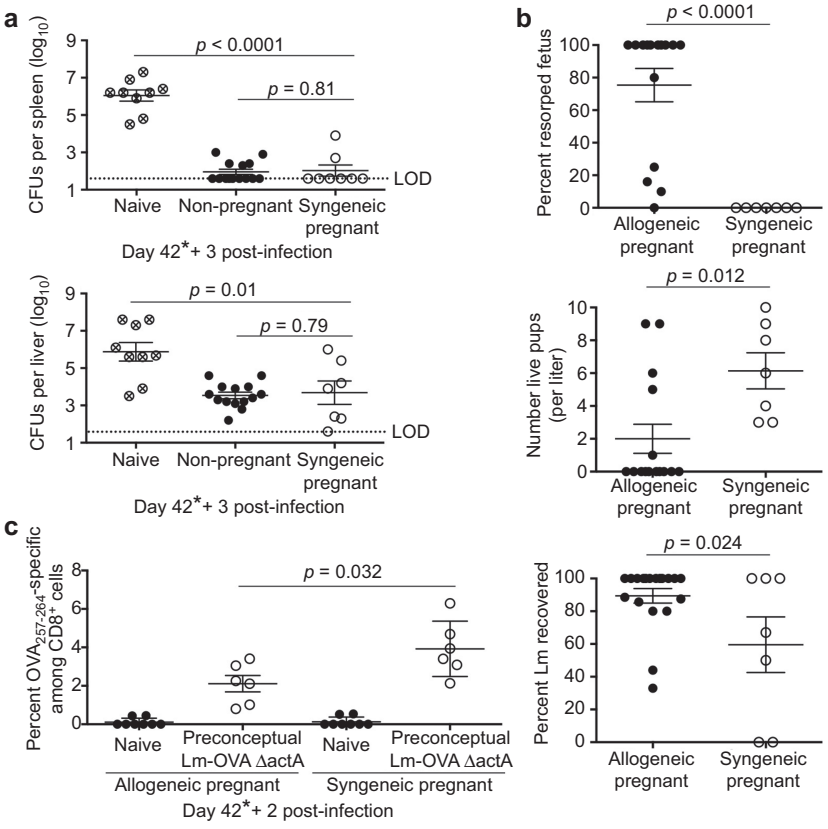

Figure 6 Diminished protection against secondary virulent Lm infection during allogeneic pregnancy is restored with syngeneic pregnancy. (**a**) The number of recoverable Lm in the spleen and liver at 3 days after virulent Lm-OVA (10^5 CFUs) infection in naive (no prior Lm-OVA ΔactA), or secondary infection in virgin non-pregnant compared with E12.5 mice bearing syngeneic pregnancy at 42–47 days after primary infection with Lm-OVA ΔactA. Data shown are from day 42*+3, encompassing the combined data 3 days after secondary Lm-OVA challenge in mice primed 42–47 days prior with Lm-OVA ΔactA. (**b**) The percent fetal resorption, number of live pups and Lm recovery from each placental fetal unit at 3 days after Lm-OVA (10^5 CFUs) infection in mice bearing allogeneic compared with syngeneic pregnancy primed 42–47 days prior with Lm-OVA ΔactA. (**c**) The percent of OVA-specific cells among $CD8^+$ T cells, as identified by H-$2K^b$ tetramer staining among decidual cells at 2 days after Lm-OVA (10^5 CFUs) infection from mice bearing allogeneic compared with syngeneic pregnancy without prior Lm-OVA ΔactA infection (naive), or primed with Lm-OVA ΔactA 42–47 days prior. Data shown are from day 42*+2, encompassing the combined data 2 days after secondary Lm-OVA challenge in mice primed 42–47 days prior with Lm-OVA ΔactA. Each data point represents the results from an individual mouse, representative of six independent experiments with similar results. Bar, mean±one standard deviation. CFU, colony forming unit; Lm, *Listeria monocytogenes*; LOD, limit of detection.

turn, sterilizing immunity was restored following parturition among mice primed with attenuated Lm prior to pregnancy. These results, indicating that transient impairment in protective immunity against Lm infection during pregnancy, are consistent with previous reports demonstrating that Lm inoculation prior to or during early pregnancy does not protect against fetal wastage or placental–fetal invasion, along with restored Lm-specific resistance postpartum in mice primed by primary intrapartum infection.[14,27,64] Although these findings are somewhat disappointing with regard to extending the application of preconceptual vaccination to augment protection against prenatal Lm infection, they also uncover exciting new clues and reinforce existing concepts surrounding how unique physiological shifts during pregnancy regulate immune responsiveness.

For example, diminished protection against prenatal Lm infection cannot be explained by either quantitative or qualitative reductions in pathogen-specific T cells. During pregnancy, $CD8^+$ T cells with specificity to Lm-expressed OVA were found in equivalent numbers, maintained cytolytic activity, and re-expanded comparably after secondary infection (Figures 1, 4 and 5). Similarly, only modest (~50%) reductions in IFN-γ- and TNF-α-producing Lm-specific $CD8^+$ T cells were identified during pregnancy (Figure 2). Although each of these cytokines is essential for placental immunity within the first 48 hours after primary Lm infection,[51] the actual contribution of $CD8^+$ T cell-produced cytokines is uncertain, considering that attenuated ΔactA Lm priming confers near complete sterilizing immunity against secondary infection, even in mice deficient in IFN-γ, TNF-α or the receptors for each cytokine.[65–67] Instead, the protective immunity against Lm infection observed in non-pregnant mice more consistently reflects $CD8^+$ T cell-intrinsic, perforin-mediated cytotoxicity, particularly for bacterial eradication in the spleen compared with the liver.[54,55] Given the potential for organ-specific discordance in effector molecules utilized by $CD8^+$ T cells, it is intriguing to consider the mechanisms whereby ~50% reductions in IFN-γ and TNF-α production by pathogen-specific memory $CD8^+$ T cells may contribute to diminished protection during pregnancy, especially in placental trophoblast cells, representing an important site for Lm invasion, replication and systemic dissemination after prenatal infection.[13,68–70]

Furthermore, because chemokine expression silencing among decidual stromal cells protects the immunologically foreign fetus from 'rejection' by maternal Tc1/Th1 T cells with fetal specificity,[71] we considered whether pathogen-specific $CD8^+$ T cells primed by preconceptual activation infiltrate the decidua. Adaptions unique to Lm for trophoblast cell invasion, intracellular survival and replication may allow even a small number of bacteria to find refuge and replicate within the placenta, then traffic back to the maternal spleen and liver.[14,64,68,70,72] However, our finding that Lm-specific $CD8^+$ T cells accumulate in higher proportions in the decidua compared with splenocytes after preconceptual priming (Figures 4c and 1b), and remain at expanded levels 2 days after secondary Lm-OVA infection, suggest that the fortification against T-cell recruitment to the maternal–fetal interface is incomplete, with leakiness later in gestation or for T cells primed by infection.[71] In other words, the incomplete protection primed by preconceptual vaccination during allogeneic pregnancy cannot be solely explained by T-cell restriction from the maternal–fetal interface. Nonetheless, there were also interesting parallels between the enhanced protection primed by preconceptual vaccination that occurs during syngeneic compared with allogeneic pregnancy and the significantly increased decidual accumulation of pathogen-specific $CD8^+$ T cells (Figure 6). Thus, more comprehensive investigation is required to determine how T-cell accumulation within the decidua protects against pathogens with defined decidual tropism and how the immune modulatory pathways that are selectively activated during allogeneic pregnancy control T-cell recruitment to the maternal–fetal interface.

In this regard, an increasingly wide assortment of immune modulatory cells and molecules have been shown to play essential roles in sustaining fetal tolerance during allogeneic pregnancy. This includes the expansion of maternal $Foxp3^+$ Tregs, tryptophan catabolism through indoleamine 2,3-dioxygenase, expression of galectin-1 and ligands for the costimulatory molecules programmed cell death protein 1 or inducible T-cell costimulator.[56–61] Our recent studies show that the sustained accumulation of immune suppressive Tregs that occurs naturally during allogeneic pregnancy or artificially in transgenic mice confers susceptibility to primary Lm infection.[45] By extension, our present results show that the diminished protection against secondary Lm infection during allogeneic pregnancy is restored when maternal Treg expansion is moderated during syngeneic pregnancy. These results suggest that at least some of the immunological shifts required for sustaining fetal tolerance also play dominant roles in overriding the protective benefits of preconceptual T-cell priming. This notion is supported by the restored protection against prenatal Lm infection during syngeneic compared with allogeneic pregnancy conferred by preconceptual Lm-OVA ΔactA priming (Figure 6). These data are consistent with more substantial protection against natural Lm infection conferred by attenuated Lm in newborn lambs among highly inbred sheep herds[11] and the Treg-mediated suppression of antigen-experienced effector memory T cells after infection with other pathogens or in models of tumor and allograft immunity.[73–75] Conversely, given these potent physiological immunological alterations during allogeneic pregnancy, it is perhaps more remarkable to consider the many specific instances with other prenatal pathogens where preconceptual priming, either by vaccine-induced immunity or natural infection, is sufficient to protect against disseminated infection, despite the naturally occurring defects in host defense during pregnancy or the early newborn period.[53,76] Overall, the previously recognized holes in innate host defense and the functional defects in pathogen-specific adaptive immunity we describe here create exciting new opportunities for using prenatal Lm infection to further dissect the intricate co-evolution between mammalian hosts and

microbial pathogens with a unique predisposition for infection during the reproductive process.

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