



Interleukin-10 Delays Viral Clearance in the Placenta and Uterus of Mice With Acute Lymphocytic Choriomeningitis Virus Infection During Pregnancy

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Pregnant mice infected with Lymphocytic Choriomeningitis Virus (Armstrong) (LCMV-Arm) experience high viral loads in the placenta and uterine tissue by 5–8 days post-infection, a time when the virus is nearly completely cleared from the spleen and blood. Interleukin 10 (IL-10) plays a crucial role in T cell responses associated with systemic viral clearance. Using the LCMV-arm model of infection, we examined first, whether IL-10 is involved in viral clearance in the placenta and uterine tissue and secondly, the potential mechanisms underlying this phenomenon. C57BL/6 (WT) and mice deficient in IL-10 (IL-10 KO) were infected with LCMV-Arm on day 10 of pregnancy. Placenta and uterine tissue, collected 2 and 8 days later, were analyzed using real time RT-PCR, plaque assays for viral load, and flow cytometry. In WT mice placenta and uterine tissue expression of IL-10 was elevated with LCMV-Arm infection. Fetus resorption was elevated in WT on days 2 and 8 post-infection as compared to IL-10 KO, and by day 19 of gestation delivery was greater. Viral loads in the placenta and uterine tissue were resolved early in IL-10 KO mice, but persistent in tissues of WT mice. Levels of NRF2 and FAS were equivalent, but BCL2L11 was higher in IL-10 KO uterus. IL-6, Interferon- β (IFN- β), CCL2, and IL-17 levels were also equivalent. IL-10 KO tissues tended toward higher expression of interferon- γ (IFN- γ) and had significantly lower expression of Transforming growth factor beta (TGF- β). The proportion of placenta and uterine tissue CD8T cells expressing the activation markers CD44^{hi} and PD1 were elevated in IL-10 KO mice. These data suggest that high IL-10 expression at the fetal-maternal interface following LCMV-Arm infection prevents clearance of viral load by impairing CD8 T cell activation and poses a significant threat to successful pregnancy outcome. The ability to modulate IL-10 expression at the maternal-fetal interface may help overcome negative pregnancy outcomes arising during acute LCMV and other viral infections in humans.

Keywords: CD8 T cells, cytokines, pregnancy, lymphocytic choriomeningitis (LCMV), mouse

INTRODUCTION

Emerging infections with such agents as Zika virus and Coronavirus, and more common agents such as Influenza and Cytomegalovirus continue to serve as reminders about our deficient understanding of maternal immunity. This is especially true with regard to the maternal fetal interface. In spite of potentially relevant conflicting data [reviewed elsewhere (1)], there still exist models of maternal tolerance which rely on mechanisms of inherent suppression of maternal immunity. These mechanisms are thought to act systemically or at the maternal-fetal interface, and at the tissue or cellular level. The study of infections during pregnancy offers an opportunity to examine and determine the relevance of some of these mechanisms. In addition, it presents a means to uncover possible subtle elements and regulatory circuits in the maternal immune system. These may support fetal tolerance, but at the same time protect fetus and mother against overwhelming disease. Accumulating evidence suggests that pregnancy drives the expansion of unique cellular phenotypes particularly at the maternal fetal interface (2–4) which may specifically express mechanisms which can both support immune surveillance and minimize harmful anti-fetal tissue responses (5, 6).

Lymphocytic choriomeningitis virus (LCMV) is an ambisense single stranded RNA virus belonging to the family *Arenaviridae*, which can cause human disease (7, 8). Generally, infection occurs through contact with aerosolized mouse feces and urine, causing a self-limited febrile illness (9). However, the virus can be transmitted vertically in mice (10) and humans (11). In humans, transmission vertically results in congenital neurologic abnormalities (12, 13) while death is an outcome if viral transmission occurs *via* transplantation of infected organs (14, 15) in immune suppressed patients. A recent estimate is that ~4% of pregnant women may be seropositive with LCMV specific IgG (16).

Laboratory strains of this virus have long been used to study mechanistic aspects of the elicited immune response in mice. Infection with the LCMV-Armstrong strain results in an acute infection with a peak systemic viral load about 3 days after inoculation. This is followed by a rise in CD8 virus-specific effector T cells that peaks between 5 and 8 days post-infection (17) and which contributes to the resulting clearance of virus. By day 15 of infection, the CD8 T cell population consolidates and forms a memory T cell pool (18). In contrast, infection with Clone 13 leads to persistent infection and is associated with dysregulation of T cell function (19).

We have previously observed that infection with the LCMV-Arm on day 8–10 of pregnancy results in systemic (spleen, peripheral blood) immunity, including specific CD8 T cell expansion, interferon- γ (IFN- γ) production, cytotoxicity and viral clearance that is equivalent to that found in non-pregnant mice infected at the same time. However, by 8 days after infection, when the systemic circulation is cleared of virus, the placenta and uterus remain infected (20) until birth.

Interleukin-10 (IL-10) is an important driver of “anti-inflammatory” and regulatory immune functioning in subsets of T cells (21), B cells (22) “alternative” macrophages (23), and

dendritic cells (24). It is a complex moderator of autoimmunity (25) and anti-tumor immunity (26). Particularly, the activity of IL-10 is thought to be a driver of pathogen persistence and chronicity in parasitic (27) and viral infections (28–31), including infection with LCMV Clone 13 in non-pregnant mice. This activity is thought to be related to its role in inhibiting T cell production of IFN- γ and other immunomodulatory mechanisms (28, 31, 32). IL-10 may also have pleiotropic functions as it may control angiogenesis (33) vascular function (34) and apoptosis (35). In pregnancy, IL-10 is expressed by trophoblast (36) and decidua (37). It is also thought to be important in early pregnancy development, as relative deficiency is a contributor to early pregnancy loss in both mouse models (38, 39) and in human recurrent early pregnancy loss (40). In addition, mid-gestation deficiency in IL-10 has been linked to the inflammatory response leading to lipopolysaccharide-induced preterm birth in mouse models (41–43). However, early higher expression of IL-10 (44) or a group of factors that include IL-10 (45) in humans identifies women who subsequently have preterm birth, and is elevated in chronic placental infection [e.g., malaria (46)] in humans. This suggests a complex relationship between viral infection, immunity, IL-10 and homeostasis in pregnancy-related tissues. To begin to understand this relationship, we returned to our finding of viral persistence in mice infected with LCMV-Arm, which does not cause systemic viral persistence, but does cause prolonged infection in the placenta with mid-gestation infection (20). We hypothesized that IL-10 modulates the local immune response to LCMV-Arm. We report that deficiency in IL-10 led to a significantly lower viral load in the placenta and uterine tissue 8 days post mid-gestation infection. This was correlated with alterations of cytokine expression in these tissues, and an increase in activated CD8 T cells, but a decrease in the extent of fetal resorption. These data point to a complex interaction between maternal immunity, acute LCMV infection and the fetal-placental unit, and support an important role for IL-10 in aberrant pregnancy outcome following acute infection with LCMV.

MATERIALS AND METHODS

Mice and Infection

Adult male and female C57BL/6 (B6, or “Wild Type” WT stock # 00664) and IL-10 KO mice of the same genetic background (Stock # 002251) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained under specific pathogen-free conditions and used in procedures approved by the Institutional Animal Care and Use Committee at the University of Vermont and in accordance with The Association for Assessment and Accreditation of Laboratory Animal Care. Females were mated with same-strain males and the males were removed 24 h later which was taken as ~day 0 of pregnancy. On day 10 of gestation, pregnant B6 and IL-10 KO females were infected with LCMV-Arm (2×10^5 PFU/mouse) by intraperitoneal (IP) injection in 100 μ l PBS or left uninfected and unmanipulated (20). At days 2 and 8 post-infection (i.e., ~days 12 and 18 of gestation) mice were euthanized by CO₂ inhalation. Fresh samples from the spleen,

uterine tissue (wall and decidua), placenta and uterine draining lymph nodes were harvested and either used immediately for flow cytometry or stored in RPMI (Sigma, St. Louis, MO) or frozen at -80°C until further use for either plaque assay or RNA expression. Analysis elaborated here include one representative, healthy-appearing (e.g., not necrotic) placental sample or one uterine sample (2–3 implantation sites and underlying nonplacental, e.g., decidua/uterine/myometrium, tissue plus intervening non-implantation uterus) per mouse. All infection-related experiments were carried out in an ABSL2+ facility at the University of Vermont.

Flow Cytometry

The spleen, uterine tissue, placenta and uterine draining lymph nodes of pregnant B6 and IL-10 KO mice were placed in “Ghost special” medium [Iscove’s Modified Dubeco’s Medium, IMDM, without bicarbonate (Gibco, Carlsbad, CA)], supplemented with 1% fetal bovine serum (FBS; Gibco). From spleen and uterine draining nodes, single cell suspensions were generated through mechanical dissociation using 70 μm nylon mesh. Uterine tissue and placenta were washed once in Ghost medium, cut into small pieces and subjected to enzymatic digestion by incubation in Hanks Balanced Salt Solution (HBSS, Gibco/Invitrogen, Grand Island, NY) containing 200 U/ml hyaluronidase (Sigma), 0.2 mg/ml DNase I (Sigma) and 28 U/ml Liberase Blendzyme 3 (Roche, Indianapolis, IN) for 20 min in a 37°C warm water bath. After digestion, samples were pressed through 70- μm mesh. The resulting cell suspension was washed with phosphate buffered saline (27) (Mediatech, Manassas, VA) supplemented with bovine serum albumin (0.1%; PBS-BSA; Sigma). Approximately 1×10^6 cells were incubated in a 1:50 dilution of 2.4G2 (BD Biosciences, San Jose, CA) in order to block non-specific antibody staining due to Fc receptors, and then incubated for 1 h at room temperature in a specific antibody mix, washed twice with PBS-BSA and then fixed with PBS-BSA+1% paraformaldehyde (Fisher Scientific, Fair Lawn, New Jersey). The antibodies used in these studies included CD45.2 (clone 104, PerCP-CyTM 5.5), PD1 (Clone J43 PE), from e-Bioscience (San Diego, CA); CD8 (Clone 5H10-PE-Texas Red), Invitrogen, and CD44 (clone IM7 APC BD-Biosciences, San Jose, CA). Samples were processed with a LSRII flow cytometer (BD Biosciences) and the results of $\sim 10\text{K}$ cells were analyzed using FLOWJO software (Version 8.8, Tree Star, Inc, Ashland, OR). The gating scheme used was previously described (47).

Plaque Assay

Placenta and uterine tissue were dissected, weighed and kept in 200 μl of RPMI (Sigma) supplemented with 1% FBS at -80°C until they were homogenized using a Mini-BeadBeater setup (BioSpec, Bartlesville, OK). Plaque assays were then performed on homogenates as previously described (20). Briefly, serial dilutions of virus-infected homogenates were placed on monolayers of VeroE6 cells and incubated for 1 h at 37°C . A warm mix of $1 \times \text{M199}$ media (Sigma) and 0.5% agarose was overlaid and allowed to solidify for 20 mins. Plates were then incubated for another 6 days, fixed with 25% paraformaldehyde (Fisher Scientific) and stained with 0.1% crystal violet. Excess

stain was washed with water and then the plaques were visually counted. Data are expressed as PFU (number of plaques $\times 10^6$) / gm of tissue used in the assay).

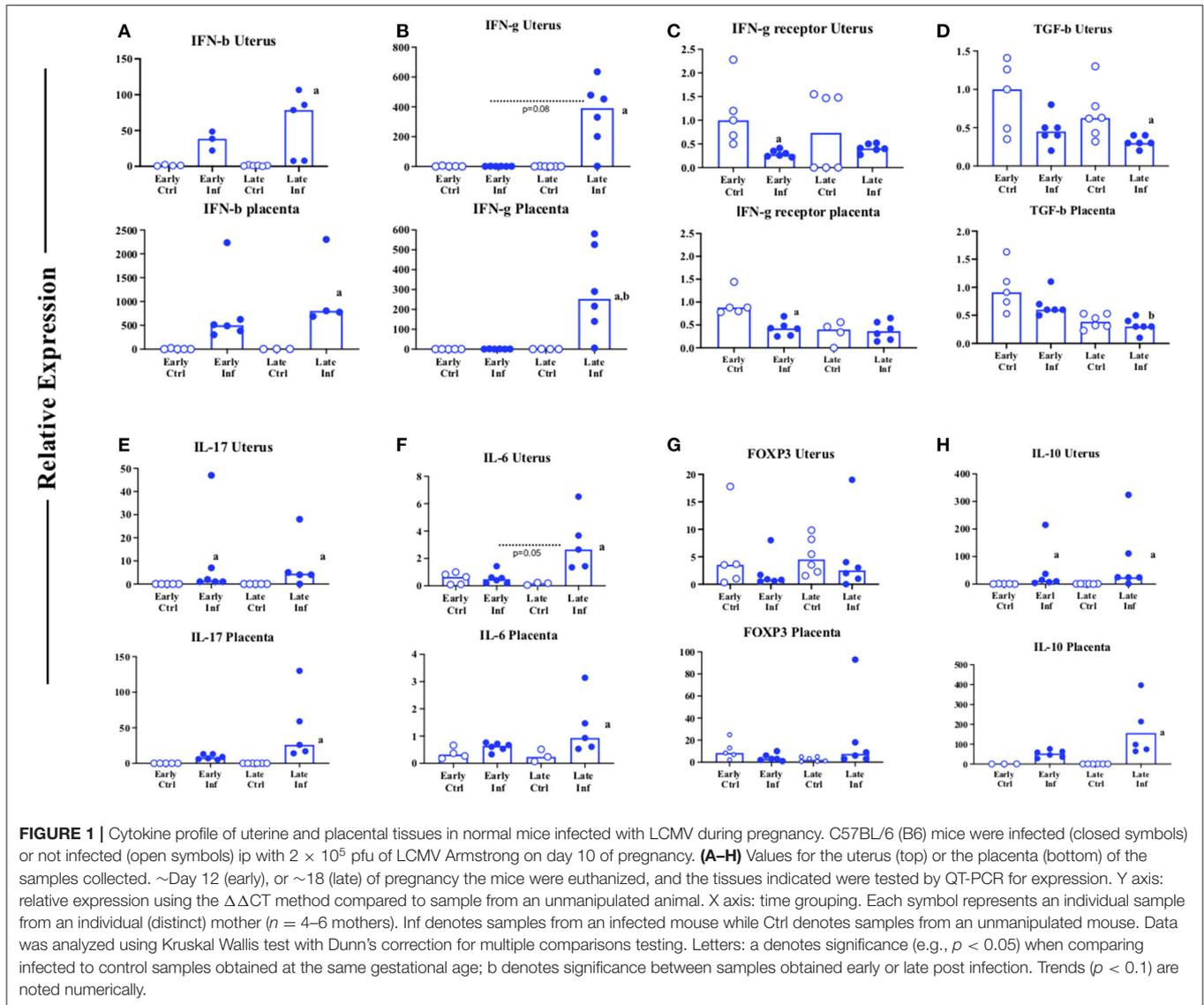
QRT-PCR

Placenta (one representative healthy-appearing per mouse) and samples of uterine tissue (2–3 implantation sites with intervening tissue) were dissected, immediately frozen and stored at -80°C until ready for processing. Total RNA was extracted from 0.5 to 1 mg of tissue using the PrepEase RNA spin kit from USB [The iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA)] was used to synthesize cDNA from 250 ng of RNA template using a mix of random hexamers and oligo dTs. From each sample 1 μl cDNA was used to amplify the genes of interest. QRT-PCR was performed on an ABI Prism 7000 (Applied biosystems-CA) using Power Sybrgreen master mix. Each sample was run in triplicate and the CTs were averaged. The following primers (forward and reverse, each in 5’-3’ orientation) were used for amplification:

Beta-2 microglobulin ($\beta 2\text{m}$) ATGCTATCCAGAAAACCCCTCAA and CAGTTCAGTATGTTCCGCTTCCC; **Fas**, AACAAAGTCCCAGAAATCGCCTATG and TCCTGTCTCCTTTCCAGCACTT; **Forkhead box protein P3 (Foxp3)**, AATGGGTGCCAGGGAGC and TGGCAGTGCTTGAGAACTC; **Transforming growth factor beta ($\text{Tgf-}\beta$)**, CGCAACAACGCCATCTATGAG and TGCTCCACACTTGATTTAATCTCTGC; **Interferon gamma ($\text{Ifn-}\gamma$)**, CCTCATGGCTGTTTCTGGCTGTTA and CATTGAATGCTGGCGCTGGACC; **Ifn- γ receptor**, CAGGTAAGGTGTATTTCGGGTTCC and CCAGGCAGATACATCAGGATACATAAT; **Interleukin-10 (IL-10)**, TTACTGACTGGCATGAGGATCA and GAAAGAAAGTCTTCACCTGGCTGA; **IL-17**, GACTCTCCACCGCAATGAAGACC and CCCACACCCACCAGCATCT; **IL-6**, AGAAAGACAAAGCCAGAGTCCTTCAG and GTCCTTAGCCACTCCTTCTGTGACT; **Bcl-2-like protein 11 (Bcl2L11)** GACGGAAGATAAAGCGTAACAGTTGT and TCCATACGACAGTCTCAGGAGGAA; **Nuclear factor ($\text{Erythroid-derived 2}$)-like Nrf2** ATGATGGACTTGAGATTGCCA and GTCATAGTCCTTCTGTGCGC; **Interferon beta ($\text{Ifn-}\beta$)**)TGTCCTCAACTGCTCTCCAC and CCTGCAACCACCACTCATTC; **CCL2 (monocyte chemoattractant protein-1, MCP-1)**, TGATCCCAATGAGTAGGCTGGAG and ATGTCTGGACCCATTCTCTTG. Relative expression was determined using the $\Delta\Delta\text{CT}$ method with an uninfected uterine sample as a comparator, and the results of PCR with $\beta 2\text{m}$ as a “loading control.”

Statistics

Data was graphed, visualized for trends and analyzed using GraphPad Prism 7 (GraphPad Software, San Diego, CA). Data points are displayed individually with lines delineating means or the tops of columns delineating medians. Data passing at least one of three normality tests were compared using parametric

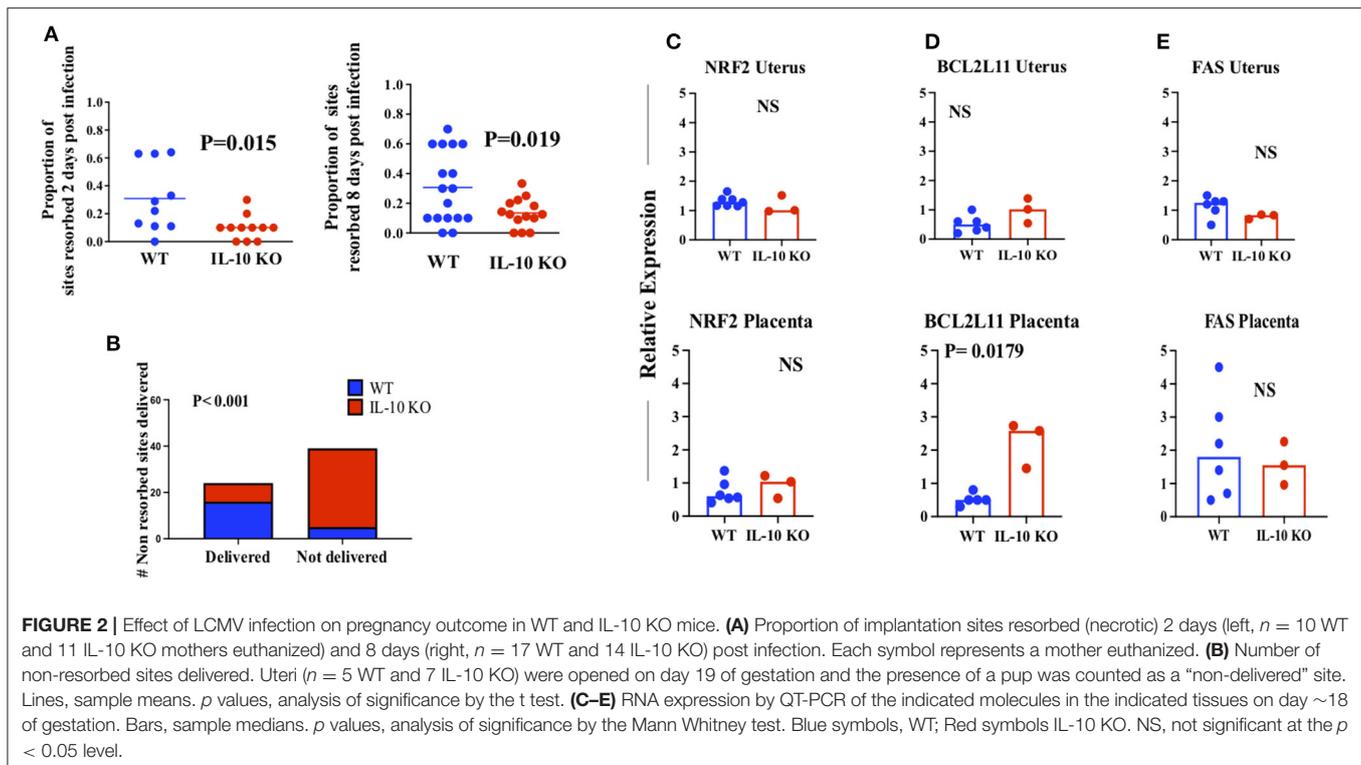


analysis. Two groups were compared with the t test while more-than-two group analysis used ANOVA with post-test correction for multiple-comparison testing. For data not passing normality, two group comparisons utilized the Mann-Whitney test, while more than two groups utilized Kruskal Wallis testing, and adjusted using the Dunn's multiple comparisons test. Significance was set at $p < 0.05$, although some trends are noted.

RESULTS

Previous studies of LCMV infection in the mouse placenta and decidua showed that 8 days after infection with an intraperitoneal dose of LCMV (20), the systemic circulation developed an increase in peripheral blood and spleen LCMV-specific CD8 cells and activated CD4 cells as occurs in non-pregnant mice. However, while the spleen is cleared of LCMV by 8 days post infection, the decidua (uterus) and placenta is not. One

possible explanation for this difference is enhanced viral uptake, as subsequent studies revealed the increased expression of a potential receptor for the virus in mid gestation placenta (48). However, one potential rationale for prolonged viral infection in the relevant tissues are the adaptive and innate immune responses generated in those tissues, as deficiency in tissue specific immunity is a potential contributor to viral persistence in other models of infection. To begin to assess the local immune and inflammatory response to LCMV infection, we examined the expression of a variety of cytokines relevant to innate and adaptive immunity in uterine and placental tissues of C57BL/6 (Wild Type, WT) mice. Adult mice were mated to same-strain males and infected on ~day 10 of pregnancy, since previous studies suggested a very high rate of resorption with infection earlier in gestation (20). **Figure 1** shows a comparison to the tissues in uninfected mice. Two timepoints are examined. "Early" refers to 2 days post infection, ~ 12 days of gestation, while



“late” refers to 8 days post infection, ~ 18 days of gestation. While expression of molecules such as $\text{IFN}\gamma\text{-R}$ (**Figure 1C**) and FOXp3 (**Figure 1G**) are not significantly changed over time and infection, three other patterns of RNA expression are discernable. In one pattern (e.g., **Figure 1B**, $\text{IFN}\gamma$) expression does not appear to be different from uninfected tissues until late in infection. In another (e.g., **Figure 1D**, $\text{TGF}\beta$) infection over time is associated with decreased expression. In a third, (e.g., **Figure 1E**, IL-17, uterus) expression is elevated soon after infection and continues to be elevated late in infection, raising the speculation of pleiotropic functions in the context of infection.

One cytokine with this pattern raised this same speculation: IL-10 (**Figure 1H**). Because of our finding, and the existing evidence in the literature, we examined pregnancy outcome in infected WT mice and in mice deficient in Interleukin-10 (IL-10 KO, **Figure 2**). Because we suspected a high level of resorption in the IL-10 KO mice based on a mouse model of early pregnancy loss (38), we examined the uteri of WT and IL-10 KO mice 2 and 8 days after infection. When we reviewed the proportion of resorption sites with necrotic fetal-placental units consistent with resorption, WT mice had a significantly greater proportion at both 2 (**Figure 2A**, left) and 8 days (**Figure 2A**, right) after infection as compared to IL-10 KO mice who experienced a level roughly similar to that found in uninfected WT mice (49). Because IL-10 deficient mice are more susceptible to preterm birth in response to toll like receptor activation with agents such as LPS (42), we expected that deficiency in IL-10 in the face of LCMV infection would lead to earlier delivery. Observations suggest that mice on this

genetic (e.g., C57BL/6) background have a gestational length of ~ 19.5 days (50). Because of this we undertook an investigation the number of non-resorbed implantation sites did or did not have an attached fetus (non-delivered vs. delivered **Figure 2B**). Unexpectedly, analysis of the calculated proportions suggested that proportionately more WT pups of infected mothers had delivered by day 19 of gestation (**Figure 2B**). Parturition is the outcome of a complex developmental process [reviewed in (51)] that likely is influenced by infection. To begin to probe the potential factors that might mediate oxidative stress (which could also lead to pathways such as senescence), cell death, and tissue dysregulation in infected WT and IL-10 infected tissues at day ~ 18 of gestation, we examined tissue RNA expression of NRF2 (52), BIM/BCL2L11 (53, 54) and FAS/CD95 (35, 54) (**Figure 2**). Although uterine NRF2 is decreased by infection in WT mice (**Supplementary Figure 1**), we observed that both NRF (**Figure 2C**), and FAS (**Figure 2E**) were similarly expressed in tissues of IL-10 KO mice as compared to WT. In addition, RNA expression of the pro-apoptotic mitochondrial molecule BCL2L11 was increased only in the placenta in IL-10 KO mice (**Figure 2D**).

Differences in reproductive outcome in WT and IL-10 KO mice could be due to viral load over time after infection in reproductive tissues, even if virus is cleared from systemic lymphoid tissues (20) (**Supplementary Figure 2**). We thus examined uterus and placenta of WT and IL-10 KO mice using a plaque assay (**Figure 3**). We infected mice on \sim day 10 of gestation and harvested tissues 2 (early) and 8 days later. We found that viral loads appeared equivalent and low early post

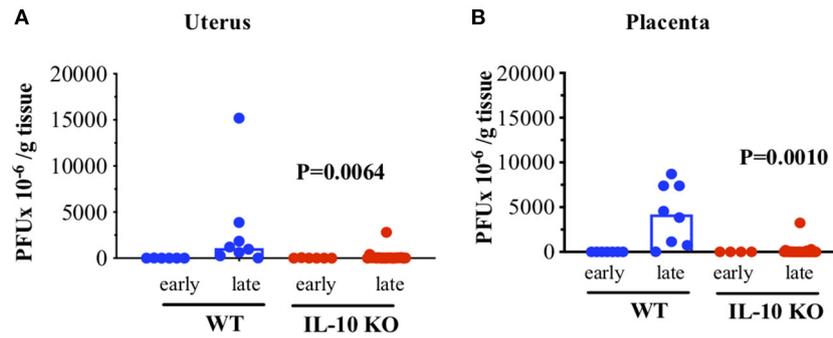


FIGURE 3 | Viral load in WT versus IL-10 KO mice infected with LCMV. WT (C57BL/6) females or IL-10 KO females were mated to same-strain males and on day ~10 of gestation were infected as in **Figure 1** and euthanized on days ~12 and ~18 of gestation. Placentas were homogenized and then tested for viral load by plaque assay. **(A)** Uterine tissues ($n = \sim 10\text{--}20$ /strain/time). **(B)** Placental tissues ($n = \sim 10\text{--}20$ /strain/time). Y axis: PFU $\times 10^{-6}$ virus per gram tissue as assessed by plaque assay. X axis: early (2 days post infection or ~12 of gestation) or late (8 days post infection or day ~18 of pregnancy). Each symbol is a unique tissue representing a distinct mother. Blue, WT tissues; Red, IL-10 KO tissues. Bars indicate median for the sample group. p values generated by Kruskal Wallis testing with Dunn's multiple comparisons test to compare WT vs IL-10 KO late values. Significance set at the $p < 0.05$ level.

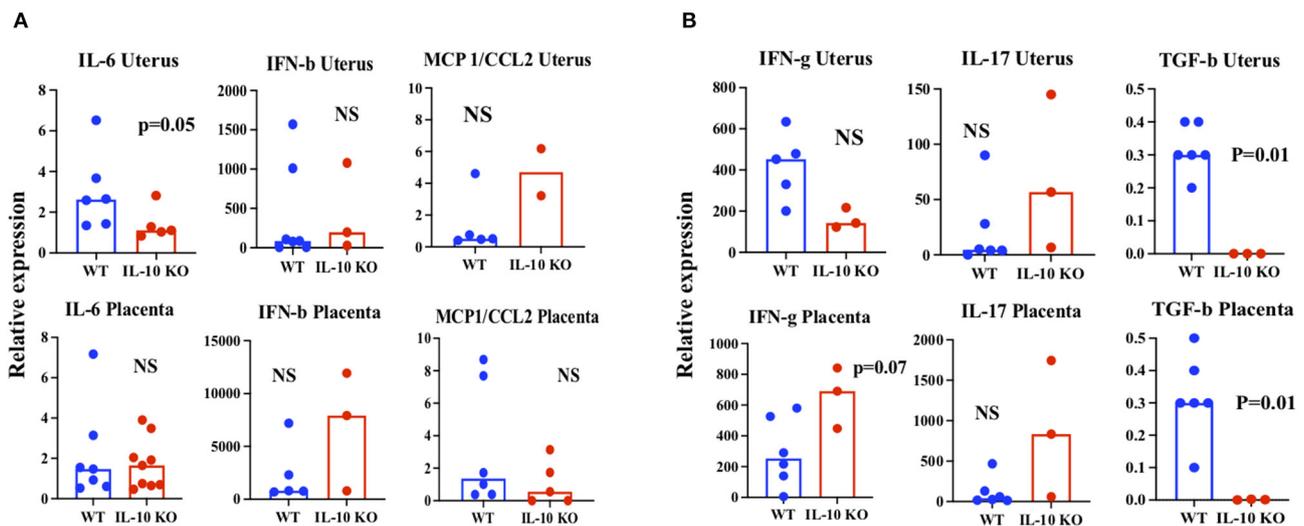
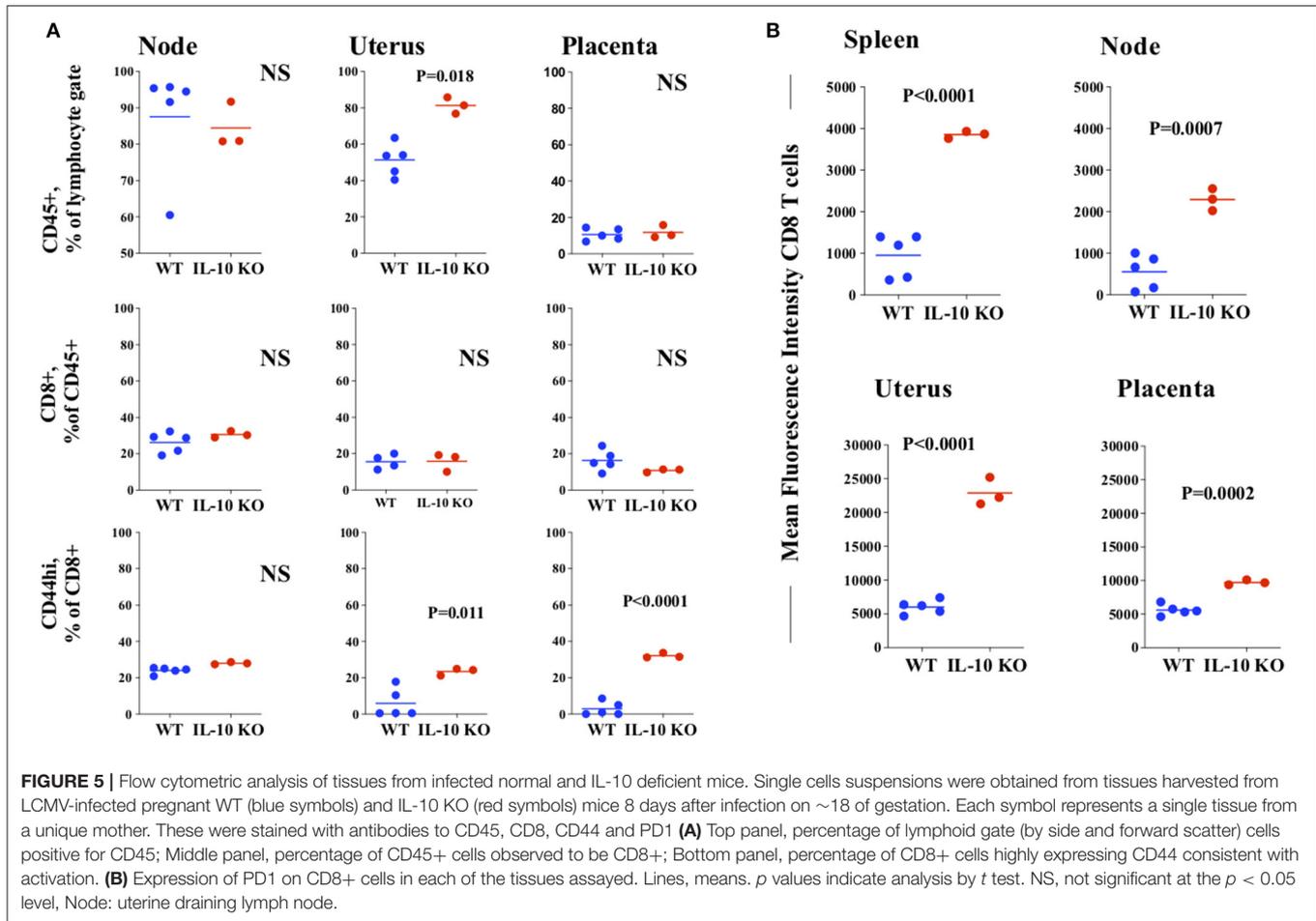


FIGURE 4 | RNA expression of cytokines in WT vs. IL-10 KO tissues. Tissues from WT (blue) or IL-10 KO (red) mice who were infected on ~day 10 of pregnancy and harvested 8 days later were harvested and RNA extracted to determine expression by QT-PCR as in **Figure 1**. Each symbol denotes a single tissue from a unique mother. **(A)** “Innate immunity” cytokines IL-6 (left), Interferon- β (middle) and CCL2 (right). **(B)** “Adaptive immunity” cytokines Interferon- γ , IL-17, Transforming growth factor- β . Upper panels, uterus. Lower panels, placenta. Bars—medians. p values analysis of significance utilized the Mann Whitney test. NS, not significant at the $p < 0.05$ level. Trends ($p < 0.1$) are noted.

infection in both uterus (**Figure 3A**) and placenta (**Figure 3B**) from WT and IL-10 KO mice. However, late post infection, the viral load was statistically greater in both tissues from WT as compared to IL-10 KO mice.

A potential connection between late pregnancy loss (or shortened gestation) and viral load could be related to innate immunity, especially as this connection has been documented in other infections (55). In contrast, the driver of our observations could be related to adaptive immunity. To begin to examine this potential dichotomy, we examined the expression of molecules

related to innate vs. adaptive immunity in tissues from WT or IL-10 KO mice. Tissue samples from WT and IL-10 KO mice taken 8 days after infection e.g., day ~18 of gestation were harvested and examined for RNA expression of IL-6 (56, 57), IFN- β (58), and CCL2 (59) which are part of the innate response to viral infection (**Figure 4A**). Further, we examined IFN- γ (60), IL-17 (61) and TGF- β (60, 62) (**Figure 4B**) which are considered important for adaptive immunity. At this time point, there was no difference in expression of innate immune mediators (**Figure 4A**). However, we observed a trend toward increase in IFN- γ in the placenta of



IL-10 KO mice and lower TGF β in both tissues of IL-10 KO as compared to WT mice (Figure 4B).

This finding allowed speculation that the local CD8 T cell response might be different in the tissues of WT and IL-10 KO mice late in infection. To test this, we harvested tissues for examination by flow cytometry. We first analyzed single cell suspensions of tissues to estimate the proportion of relevant cells (Figure 5A and see Supplementary Figure 4A). CD45+ cells were increased in proportion in IL-10 KO uterus as compared to WT (Figure 5A, top row, middle panel), while the proportion of CD8+ cells was equivalent regardless of tissue (Figure 5A, middle row). However, the proportion of CD8+ cells that were positive for the activation marker CD44 was higher in both the uterus and placenta of IL-10 KO mice as compared to WT (Figure 5A, bottom row, middle and right panels and see Supplementary Figure 4B, left panel). This suggested that either there was increased trafficking and/or local activation of CD8+ cells in IL-10-infected vs. WT-infected utero-placental tissues (Figure 5A).

The molecule programmed death-1, PD1/CD279 (22, 63) is expressed on T cells and is associated with regulation of T cell homeostasis, activation and tolerance. Expression is upregulated on activation (64) and expression is associated with modulation

of the response to viral infection. LCMV infection with less virulent strains leads to transient upregulation of PD1 and retained functional capacity, while chronic infection with more virulent strains leads to retained PD1 expression as part of the “exhausted” T cell phenotype (65). Based on previous findings of infection with a more virulent strain (28), we expected to see a decreased level of PD1 on CD8 T cells from IL-10 KO mice. Although the size of the systemic CD8 T cell pool is similar in infected WT and IL-10 deficient mice (Figure 5A, middle row, left panel), population-based PD1 expression was increased in IL-10 KO CD8 T cells of both the spleen and uterine draining lymph nodes (Figure 5B, top row, and see Supplementary Figure 4B, right panel) as compared to WT. In addition, the expression of PD1 in both uterine and placental CD8 T cells was elevated in IL-10 deficient tissues compared to WT (Figure 5B, bottom row). Taken together, this data suggests tissue specific regulation of the CD8 T cell pool in the face of viral infection.

DISCUSSION

These studies are driven by an earlier finding that infection with LCMV leads to persistent infection of the uterus and placenta,

but not the fetus, despite the fact that the strain used is deemed less virulent and does not usually lead to persistent infection in adult animals (66). We were interested in the role of IL-10 because of its potential role in modulation of both innate and adaptive immunity.

The key findings presented here are the decreased viral load late in infection in IL-10 KO as compared to WT mice, and that IL-10 deficiency in the face of infection leads to comparatively *less* pregnancy loss and potentially prolonged gestation. Based on previous data in IL-10 deficient mice, and the increased expression of proapoptotic proteins such as BCL2L11 (**Figure 2C**) the later finding is somewhat counterintuitive. The trend for increased expression of genes such as IL-6 (**Figure 4A**) is however consistent with developmental dysregulation (57) in the WT pregnancies examined. Although representative of distinct pregnancies, the relatively small sample size in our expression studies demands cautious interpretation. Despite the likelihood that more significant differences might be revealed with a much larger, potentially challenging number of mice, we were able to detect differences consistent with the idea that our findings may be related to differential activity of tissue-localized CD8 T cells.

In studies of neonatal male mice, intracerebral infection with LCMV-Arm, the strain used in these studies, leads to persistent infection as adults, with viral nucleic acid present in several tissues (67). However, in adult mice, infection with LCMV-Arm leads to systemic tissue clearance which occurs in ~ 8 days. This clearance is associated with long-term LCMV-specific immunity and protection against more virulent strains. Key mechanisms of viral persistence in neonatally infected mice include central tolerance, as the virus is persistent in the thymus as well as other tissues and adoptive transfer of T cells from immune mice leads to viral clearance (68).

Viral persistence can be achieved in adult mice through administration of certain clones of LCMV (e.g., clone 13) which, interestingly enough, were isolated from the spleen of mice infected neonatally (66). Viral persistence was initially related to lack of functional cytotoxic T cells. Recently it has been observed that strains of LCMV which cause either limited or chronic LCMV infection systemically, alter male urinary scent proteins (69). The effect on urinary scent proteins appears to last longer than it takes for systemic clearance, and this leads to the speculation that the reproductive tract may harbor “non-virulent” virus for a longer than expected (as compared to systemic circulation) time in males as well as pregnant females. This may support extended examination of different viral strains and LCMV specific immunity in the non-pregnant reproductive track of females (70).

The fact that injection of LCMV-Arm may generate different clones of varying immunity (66) raises the possibility that uteroplacental tissues may generate sub-strains with enhanced tissue specific binding or tropism (48). This may lead to clones which cause persistent infection and would thus be termed “virulent.” Further studies are needed to examine the potential presence of altered virus genetic

elements, such as those relevant to the L protein (71) in placental tissues.

The existing data may support a model whereby there are three different biologies of LCMV infection during pregnancy. First, infection (more likely early) in pregnancy tends to embryonic loss (20), observed in our studies as “resorption” (**Figure 2**). Second, infection leads to utero-placental prolonged viral presence at a time during enhanced expression of a putative viral receptor (48). This may lead to labor complications [e.g., dystocia (20)] and increased perinatal mortality. The third, perhaps with infection in late gestation exists along the same continuum as infection in the brain within the first day of life. During which the placental status is not clear, but the fetus becomes chronically infected due to infection of the immature thymus. These three “biologies” might be related to inherent elements of anatomy (72), viral tropism (48) and mechanisms of host defense and innate immunity which may differ by strain [e.g., SWR/J and HA/ICR (10) vs. C57BL/6 (our studies)]. This should be examined in future investigations.

Since IL-10 is expressed in trophoblast, one could expect that it plays a developmental role as do cytokines such as IFN- γ (73) as well as a protective immune regulatory role in pregnancy. Indeed, this is how we interpreted early expression of IL-10 in women who would go on to have preterm birth (44). We posited that these women experienced some dysregulatory insult for which IL-10 expression was enhanced in a compensatory manner. Because of the proposed role of IL-10 in “anti-inflammatory responses” IL-10 has been examined in models of recurrent pregnancy loss and preterm birth. In models of recurrent pregnancy loss, utero-placental deficiency in IL-10 leads to increased loss (38). Moreover, in models of Toll-like receptor mediated preterm birth, deficiency is related to increase in innate immune activation [e.g., NK cells (41, 74)] and a more profound phenotype. However, in the context of LCMV infection, where we would expect that deficiency would lead to enhanced inflammation, we did not observe increased expression of inflammatory cytokines (**Figure 4A**), an early-expressed interferon (**Figures 1A, 4A**) or a modulator of innate immune cell trafficking (**Figure 4A; Supplementary Figure 3**). Further, we did not see overall decreased “resiliency” in uteroplacental tissues of IL-10-KO vs. WT tissues (**Figure 2**) as pregnancy outcome was improved, and there was no difference in RNA expression of molecules such as NRF2 which should be defense against oxidative stress and FAS which should mediate apoptosis. Our only finding was increased RNA expression of the mitochondrial proapoptotic molecule BCL2L11. This may support examination of mitochondrial function and overall metabolism in the context of LCMV infection.

Cell death and tissue destruction in LCMV infection is said to be related to T cell activation and effector function since the virus is considered to be non-lytic (75). However, the correlation between viral load and poor pregnancy outcome we observed could be explained by subtle (e.g., nonlytic),

toxic effects of the virus on utero-placental tissues or innate mechanisms not examined. If this would be the case, then viral clearance related to immunity would be expected to be protective. This is consistent with our findings. The ability for pregnant animals to clear systemic LCMV, generate anti LCMV memory, and protect against virulent strains (20) tends to mitigate against both central (e.g., deletion in thymus) and systemic (e.g., global, non-thymic) tolerance as a cause for viral persistence in utero-placental tissues. Moreover, it appears in these studies and those previously published that CD8 T cells can traffic to uteroplacental tissues in response to LCMV infection, although this may be due to dysregulation of mechanisms which limit trafficking to this site (76, 77) in the uninfected case.

Existing data suggests that not only chronic infection, but also homeostatic expansion in response to niche, cytokines such as IL-7, or environmental antigen (78) can lead to T cells with a molecular and phenotypic signature similar to “exhaustion” (78), but that the majority of these cells are cleared *via* FAS/FAS ligand interactions. We have observed that maternal T cells may undergo homeostatic expansion, possibly in response to fetal antigen (54, 79). This is associated with the increased expression of molecules such as IL-7 receptor and, in the uterus, expression of PD1, granzyme and downregulation of CD8 (49, 54). This suggests a “quasi exhausted” phenotype which can be relieved systemically (80) and possibly locally through inflammation. The factors driving this phenotype may explain the relative persistence of virus in uteroplacental as opposed to systemic tissues.

Chronic LCMV infection is associated with increased IL-10 production in antigen presenting cells and decreased T cell function. Further, antibody mediated blockade of the IL-10 receptor restores T cell function and enhances viral clearance (28). Consistent with this, in our studies, deficiency in IL-10 is associated with decreased viral load in utero-placental tissues and with a calculated increase in the proportion of CD8 T cells (uterus, **Figure 5A**). This correlated with increased expression of the bona fide activation marker CD44 (81) on uterine and placental CD8 T cells (**Figure 5A**). We were intrigued by the increased CD8 T cell expression of PD1 in both systemic and local tissues of infected IL-10 KO as compared to WT (**Figure 5B**), when we expected this molecule to be downregulated in IL-10 KO cells. We were further somewhat surprised by the contrast with CD44, which occurred at a relatively higher level in CD8 T cells of only local IL-10 KO tissues (**Figure 5A**). We attribute this to the short time-frame of these experiments, as PD1 may be elevated for a time after antigen is cleared and during the revival of exhausted cells [reviewed in (22)]. In addition, we could speculate that genetic deficiency in IL-10 may developmentally alter (upregulate) PD1 even in the uninfected state. PD1 is actually a complex modulator of immunity (82) *via* interaction with a number of ligands at least one of which, PD-L1 is upregulated by IL-10 (22). PD1 signaling is also closely linked to T cell metabolism (83). As such, the highly metabolically active

state of the placenta may influence the cellular expression and function of PD1 and its ligands.

Our data, taken in this context, is consistent with a model for a unique phenotype in uterine/placental tissues compared to systemic CD8 T cells (3, 84), which should in the future be analyzed with respect to exact anatomic site (85) and fetal sex (43). From our point of view (1, 86), these data suggest that in local tissues this may not be a phenotype of inherent suppression or limitation. This state may be one of heightened early activation, which may lead to a state which is like exhaustion, but which can be “cured” by tissue-derived factors when needed to clear viral infections. The concept of a unique intrauterine CD8 T cell phenotype- one that exhibits elements of the “exhaustion-that-can-be-relieved” phenotype is supported by studies in other animal models (87–89) and in humans (90–92). Our hope is that consideration of this concept can be utilized to develop therapeutic interventions to prevent congenital infection.

DATA AVAILABILITY STATEMENT

Requests to access the datasets should be directed to Elizabeth A. Bonney, elizabeth.bonney@med.uvm.edu.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee University of Vermont.

AUTHOR CONTRIBUTIONS

EB prepared manuscript. All authors participated in experiments and data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fviro.2022.829991/full#supplementary-material>

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