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Normal Establishment of Virus-Specific Memory CD8 T Cell Pool following Primary Infection during Pregnancy

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Suppression of cell-mediated immunity has been proposed as a mechanism that promotes maternal tolerance of the fetus but also contributes to increased occurrence and severity of certain infections during pregnancy. Despite decades of research examining the effect of pregnancy on Ag-specific T cell responses, many questions remain. In particular, quantitative examination of memory CD8 T cell generation following infection during pregnancy remains largely unknown. To examine this issue, we evaluated the generation of protective immunity following infection during pregnancy with a nonpersistent strain of lymphocytic choriomeningitis virus (LCMV) in mice. The CD8 T cell response to LCMV occurred normally in pregnant mice compared with the nonpregnant cohort with rapid viral clearance in all tissues tested except for the placenta. Despite significant infiltration of CD8 T cells to the maternal-fetal interface, virus persisted in the placenta until delivery. Live pups were not infected and generated normal primary immune responses when challenged as adults. Memory CD8 T cell development in mice that were pregnant during primary infection was normal with regards to the proliferative capacity, number of Ag-specific cells, cytokine production upon re-stimulation, and the ability to protect from re-infection. These data suggest that virus-specific adaptive memory is normally generated in mice during pregnancy. *The Journal of Immunology, 2007, 179: 4383–4389.

Viral infections in pregnancy are major causes of morbidity and mortality for both mother and fetus. Although the underlying mechanisms are not clearly elucidated, pregnant women have an increased risk for serious complications with certain viral infections, such as influenza (1, 2), varicella (2), and hepatitis (3, 4). Pregnancy-associated suppression of cell-mediated immunity (CMI) has been proposed as a mechanism promoting maternal tolerance of the fetus and an underlying cause for the clinical observations of the increased occurrence and severity of certain infections. Thus, evaluation of CMI during pregnancy has been an active area of interest for decades.

Functional evaluation of CMI during pregnancy has included assessing proliferative responses to mitogens (5–7) and evaluating the ability to mount a delayed-type hypersensitivity response (8–11). Results from these earlier studies have been conflicting, perhaps due to methodological differences, such as cell separation and subset identification, as well as environmental influences and individual variation.

Studies focusing on the maternal response to vaccines suggest that systemic humoral and cell-mediated responses are sufficiently functional during pregnancy to produce neutralizing Ab, which is the desired outcome of vaccination (12–14). Data concerning the generation of CD8 memory T cells in response to vaccines delivered during pregnancy are limited.

It has been shown that pregnant women generate lasting immune responsiveness to paternal MHC (15) and the male Ag H-Y (16, 17). Similarly, animal studies support the idea that a memory T cell response can be generated in pregnancy (18), yet to our knowledge, no direct comparison of pregnant and nonpregnant animals has been done with more recently developed tools that allow direct ex vivo quantification of virus-specific responses. Thus, many gaps remain in our understanding of how pregnancy affects adaptive immunity, including development of protective immunity. In particular, the quantitative effect of pregnancy upon the generation of a functional memory CD8 T cell pool is largely unknown.

LCMV Armstrong infection of mice is an exhaustively characterized model for studying T cell responses to acute viral infections (19–21). Adult mice infected with LCMV (Armstrong strain) will generate a potent immune response, clear the virus within 8–10 days mediated by CD8 CTL, and establish a long-lasting memory cell pool. The memory cell pool is established by day 30 postinfection and is thought to remain stable for the life of the mouse (22). Numerous methods, discoveries, and reagents make this an ideal system to examine the effects of pregnancy on the in vivo generation of memory CD8 T cells in more detail, including MHC class I restricted epitopes, MHC class I tetramers, viral plaque assays, and cytokine production assays. In this report, we begin a systematic evaluation of viral-specific memory CD8 T cells generated in response to LCMV infection during pregnancy. We found
that such cells are functionally equivalent to those generated during viral infection in nonpregnant animals.

Materials and Methods

Mice and mating

Six- to eight-week-old female C57BL/6 (Ly5.1, CD45.1) mice and sexually mature C57BL/6J (Ly5.2, CD45.2) breeder males were purchased from the National Cancer Institute (Frederick, MD). Mice were mated as described for natural matings (23). Female mice in estrus (determined by visual observation of external genitalia) were placed with one male and left overnight. Females were checked for the presence of a copulation plug early the next morning. The efficiency of using the presence of a plug to predict pregnancy has been reported to result in 80–90% of females becoming pregnant (23). Approval for the studies was obtained from the Institutional Animal Care and Use Committee (IACUC) of Emory University.

Infections

Armstrong and clone 13 strains of LCMV were prepared as described (24). Mice were infected with 2 × 10⁵ PFU of LCMV Armstrong through i.p. injection. Pregnant mice were infected on days 8–10 of gestation. All females that were obviously pregnant by visual inspection and males with the presence of a copulation plug following mating were included in the pregnant cohort. For the second-challenge, mice were infected with 2 × 10⁵ PFU LCMV clone 13 i.v. injection 2–4 mo following LCMV Armstrong infection.

Cell isolation and staining

Lymphocytes were isolated from the spleen, lymph nodes, and blood as previously described (22, 25). For isolation of lymphocytes from the uterus and placenta, mice were perfused with cold PBS, uterus and placenta were removed, cut into 1-cm pieces, treated with 1.3 g/ml collagenase (Invitrogen Life Technologies) in 5% RPMI 1640 medium/2 mM MgCl₂/2 mM CaCl₂ (37°C for 60 min shaking at 200 rpm), then treated with 100 U/ml collagenase, then homogenized through a 100-µm cell strainer (Falcon). Lymphocytes from homogenized digested tissue were purified over a 44–67% Percoll gradient (800 × g at 20°C for 20 min). Remaining RBC in the placenta were lysed following Percoll separation and washing using 0.83% ammonium chloride.

Flow cytometry

Single cell suspensions were surface-stained with anti-CD8, CD44, and H-2D⁰ MHC class I tetramers complexed with peptides (gp33, gp276, or np396) presenting known LCMV CD8 T cell epitopes (22). Intracellular staining for IFN-γ and TNF-α was performed after 5-h stimulation with 0.1 μg/ml peptide using the Cytofix/Cytoperm kit in accordance with manufacturer’s directions (BD Pharmingen). All staining reagents were purchased from BD Pharmingen: PE-conjugated monoclonal rat anti-mouse CD8a Ab (clone 53-6.7), FITC-conjugated monoclonal rat anti-mouse IFN-γ (clone XMG1.2), allophycocyanin-conjugated monoclonal rat anti-mouse TNF-α (clone MP6-XT22), and FITC-conjugated monoclonal rat anti-mouse CD44 Ab (clone IM7). Samples were acquired using a Becton Dickinson FACScalibur flow cytometer and data analyzed using FlowJo software (Tree Star).

Plaque assay

LCMV viral titers were determined on Vero cell monolayers as previously described (24). In brief, tissue samples were homogenized and 200 μl of 10-fold dilutions of sample (tissue or sera) were titrated on confluent Vero cell monolayers after the medium was removed. After absorption for 1 h at 37°C, the cells were overlaid with 4 ml of a 50:50 mixture of 1% agarose (in water) and 2% neutral red (Invitrogen Life Technologies). Plaques were counted following incubation at 37°C. Four days later, cells were overlaid with a 50:50 mixture of 2% neutral red and 1% agarose containing 0.2% neutral red (Invitrogen Life Technologies). Plaques were scored the following day.

Measurement of cytolytic activity

Cytolytic activity was measured using ⁵¹Cr sodium chromate-labeled MC57 cells with or without the addition of 0.2 μg/ml np396 peptide, as previously described (22, 26). Serial dilutions of effector cells (beginning with 2 × 10⁵) were incubated in 96-well flat-bottom microtiter plates with 1 × 10⁴ target cells for 5 h at 37°C. Actual E/T ratio was determined by measuring the frequency of epitope-specific CD8⁺ T cells using flow cytometry and MHC class I tetramers.

Immunohistochemistry/immunofluorescent evaluation of placenta

Animals were euthanized and placentas dissected from the uterine wall. Placentas were immediately placed in optimal cutting temperature compound and snap frozen. Light microscopy staining of acetone-fixed 7-µm sections was conducted using the ABC Vectastain kit (Vector Laboratories) following the manufacturer’s directions. Immunofluorescent staining of acetone-fixed 7-µm spleen sections was done as follows. Sections were blocked with 5% normal mouse serum (NMS) in PBS for 20 min. The primary Abs diluted in PBS with 2% NMS were incubated on the sections for 30 min. The sections were then washed for 10 min in PBS. The secondary Abs diluted in PBS with 2% NMS were incubated on the sections for 30 min in the dark. The sections were washed for 10 min in PBS then mounted using ProLong antifade mounting reagent (Molecular Probes). All incubations were done at room temperature in a humidity chamber. Purified rat anti-mouse CD8 (IHC) (clone 53-6.7), purified rat anti-mouse CD4 (L3T4) (clone RM4-5), biotin anti-mouse H-2Kb/Db (clone 28-8-6) and rat anti-mouse CD8 (IHC) (clone 53-6.7), purified rat anti-mouse CD4 (L3T4) (clone RM4-5), biotin anti-mouse H-2Kb/Db (clone 28-8-6) and biotinylated polyclonal anti-rat were purchased from BD Pharmingen. Polyclonal anti-anti-LCMV sera used to detect LCMV-infected cells were raised in guinea pigs, and the crude gamaglobulins obtained by cold methanol precipitation. Biotin-SP-conjugated affinity pure donkey anti-guinea pig IgG (H & L) was purchased from Jackson ImmunoResearch Laboratories. The rat primary Abs were recognized with goat-anti-rat Alexa-488 and the LCMV with streptavidin-conjugated Alexa-568 anti-guinea pig (clone S11226; Molecular Probes). Sections were visualized using a Zeiss fluorescent microscope.

Statistics

Absolute numbers were determined for percentages of CD8 T cells and statistical analysis was applied to all groups of mice using Student’s t test.
bating lymphocytes for 5 h with 51Cr-labeled NP396 peptide-pulsed gp33, 0.429; gp276, 0.954; noted (group). Absolute numbers were compared with no significant difference noted (p values for the different epitopes are as follows: np396, 0.785; gp33, 0.429; gp276, 0.954); B, cytolytic activity was measured by incubating lymphocytes for 5 h with 51Cr-labeled NP396 peptide-pulsed (closed symbols) or un-pulsed (open symbols) target cells. Pregnant mice (n = 2) represented as circles and nonpregnant (n = 4) mice represented as triangles. E:T ratios were based on the actual number of tetramer-positive CD8 T cells per target cell.

**Results**

Similar expansion and function of Ag-specific CD8 T cells in pregnant and nonpregnant mice during the acute phase of LCMV infection

To compare the development of effector CD8 T cell responses between mice that were pregnant during primary infection (experimental) and those that were not pregnant during primary infection (control), we infected age-matched cohorts with 2 × 10^5 PFU of LCMV (Armstrong strain) i.p. on days 8–10 of gestation in the experimental group. The number of virus-specific cells was determined using MHC class I tetramers of LCMV-specific epitopes, i.e., nuclear protein (NP)396, glycoprotein (GP)33, and GP276. Fig. 1A shows equivalent proportions of activated (CD44^+), Ag-specific CD8 T cells (NP396 and GP33) in spleen, lymph nodes, and blood day 8 post primary infection. Fig. 1B shows equivalent numbers of Ag-specific CD8 splenocytes (NP396 and GP33) for the same time period. Longitudinal analysis of the number of Ag-specific CD8 T cells (NP396 and GP33) in pregnant mice for days 30, 60, 90, 120, and 300 post primary infection showed no significant difference between those who delivered normally and those who had fetal resorptions (data not shown).

We compared the functional ability of effector CD8 T cells between the experimental and control groups through evaluation of anti-viral cytokine production, cytolytic activity, and in vivo viral control. Following 5 h of in vitro stimulation, we compared the ability of effector CD8 T cells to produce anti-viral cytokines (IFN-γ and TNF-α). Fig. 2A shows equivalent proportions of CD8 T cells producing cytokine between groups. We compared cytolytic ability using an ex vivo 51Cr release assay. Fig. 2B shows equivalent cytolytic activity among both groups of NP396 peptide-pulsed target cells by Ag-specific CD8 T lymphocytes 8 days postinfection from harvested spleens.

In vivo viral clearance was compared between the experimental and control groups using plaque assays. LCMV (Armstrong) infection is controlled rapidly in naive mice (27). Fig. 3 shows similar in vivo viral expansion and control in sera and all tissues tested for numerous time points in both groups with the exception of the placenta and uterus in the pregnant mice. Despite rapid control in all other tissues tested, viral titers continued to increase in the placenta and remained elevated until delivery, which occurred ~10–12 days post primary infection.

**Infection of reproductive tissue and pregnancy outcomes**

The fact that virus persisted in the placenta prompted further investigation. Immunohistochemical assays were used to determine the location of viral infection in the placenta. Fig. 4A shows that LCMV-infected cells were localized at the maternal-fetal interface (decidua) with a few foci of infected cells near the fetus (in the labyrinth). Immunofluorescent assays revealed CD8 and CD4 T cells in close proximity with LCMV-infected cells, yet colocalization was not evident (Fig. 4B). Infected placenta indicates that the majority of LCMV-infected cells (red) lacked MHC class I (green) expression. MHC class I expression was only noted at the maternal-fetal interface (Fig. 4B).

Pregnancy outcomes were poor in this model with a high rate of maternal mortality, fetal resorptions, and mortality in the pups (Table I). Mortality in the pregnant mice appeared to be related to uterine inertia. The mice did not appear sick before labor, with no loss of mobility or nesting behavior; yet at term, during labor, they were hunched, immobile, with loss of nesting behavior, and they
had extremely swollen abdomens with retained pups. Upon necropsy, the uterine horns were extremely edematous, but the pups appeared morphologically normal.

There was a high rate of fetal resorptions (46%), which could be underestimated by the fact that early resorptions may have occurred before a pregnancy could be detected in infected mice. Fetuses were not infected before delivery, despite high viral titers in the placenta and uterus 10 days post primary infection (Fig. 4C). A significant proportion (70%) of pups died soon after delivery; however, they appeared morphologically normal upon gross examination at birth. Cannibalism of the pups was frequently noted.

Live born pups were not immediately examined for the presence of LCMV infection and allowed to mature. Adult mice that were delivered to infected mothers during pregnancy lacked LCMV-specific CD8 T cells and exhibited a primary immunologic response when infected with LCMV (Armstrong) (Fig. 4D).

**Similar number and function of Ag-specific memory CD8 T cells**

Viral persistence is well known to affect memory T cell differentiation (28). It was unclear whether persistent viral infection at the placenta would affect generation of the memory CD8 T cell pool. Therefore, we compared the number and function of Ag-specific memory CD8 T cells between the two groups. Fig. 5 shows similar proportions of LCMV-specific memory CD8 T cells 300 days post primary infection with LCMV (Armstrong).

The clone 13 strain of LCMV causes a chronic infection in naive mice; however mice that have been immunized with LCMV Armstrong are protected. Thus, challenging immune mice with LCMV (clone 13) is a useful assay to evaluate the protective
Table I. Pregnancy outcomes of mice infected with LCMV (Armstrong) on days 8–10 of gestation

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of pregnant mice allowed to progress through labor</td>
<td>70</td>
</tr>
<tr>
<td>Number of pregnant mice who delivered normally</td>
<td>18</td>
</tr>
<tr>
<td>Number of pregnant mice who had fetal resorptions (with no pups delivered)</td>
<td>32</td>
</tr>
<tr>
<td>Number of pregnant mice who died during labor (uterine dystocia)—fetuses were well-formed</td>
<td>20</td>
</tr>
<tr>
<td>Number of pups born</td>
<td>83</td>
</tr>
<tr>
<td>Number of pups who died at birth or shortly after birth</td>
<td>58</td>
</tr>
<tr>
<td>Number of pups who survived to adulthood</td>
<td>25</td>
</tr>
</tbody>
</table>

* This model was duplicated in 15 experiments comparing pregnant and nonpregnant mice at different time points over the course of infection and generation of protective immunity.

** Twenty-two additional pregnant mice were sacrificed for studies during the primary infection prior to delivery.

† Results reported in this study included 10 mice who delivered normally.

‡ Results reported in this study included 15 mice who had fetal resorptions.

§ Results reported in this study included 48 pups born.

Results reported in this study included 28 pups that died at birth or shortly afterwards.

Viral infection persisted in the uterus and placenta of pregnant mice, despite persistent infection in the placenta, these data indicate that pregnancy does not alter the generation of a functional memory CD8 T cell pool in response to LCMV infection. Specifically, Ag-specific memory CD8 T cell differentiation in mice that were infected during pregnancy was comparable to control mice in quantity, cytokine production, and the ability to protect from a virulent challenge. Functional capability of the memory CD8 T cells was demonstrated by longitudinal evaluation of expansion and contraction of LCMV-specific effector memory CD8 T cells was similar between cohorts (Fig. 6A). Anti-viral cytokine production (IFN-γ and TNF-α) in response to cognate peptide (NP396, GP33, and GP276) showed no significant difference between the two groups (Fig. 6B). Similar viral control on days 3 and 5 post secondary challenge with LCMV (clone 13) was noted between groups (Fig. 6C).

Discussion

In this study, we examined the development of immunologic memory during a primary infection in pregnancy by evaluating CD8 T cell responses to LCMV infection in C57BL/6 mice. Despite persistent infection in the placenta, these data indicate that pregnancy does not alter the generation of a functional memory CD8 T cell pool in response to LCMV infection. Specifically, Ag-specific memory CD8 T cell differentiation in mice that were infected during pregnancy was comparable to control mice in quantity, cytokine production, and the ability to protect from a virulent challenge. Functional capability of the memory CD8 T cells was demonstrated by longitudinal evaluation of expansion and contraction of LCMV-specific effector memory cells in peripheral blood, cytokine production in splenocytes upon stimulation with LCMV-specific peptides, and in vivo viral control upon a secondary challenge with a more virulent strain of LCMV (clone 13).

Viral infection persisted in the uterus and placenta of pregnant mice, despite significant expansion of Ag-specific CD8+ T cells and systemic clearance of the virus in every other tissue assayed. In addition, fetuses were not infected, despite persistent infection in the placenta. Preliminary studies were initiated to explore potential explanations for these findings, including examination of placentas to determine the location of infected cells, the presence or absence of lymphocytes, and MHC class I expression. LCMV-infected cells were located almost exclusively at the maternal-fetal interface with a few foci of infected cells close to the fetus, in the labyrinth of the placenta. We noted significant infiltration of CD8 and CD4 T cells at the maternal-fetal interface in close proximity to LCMV-infected cells; however, colocalization was not evident. MHC class I expression was noted at the maternal-fetal interface, although LCMV-infected cells in the placenta lacked MHC class I expression. Thus, potential explanations for our observations that the fetuses were not infected and failure to clear virus from the placenta include that viral-infected cells were not in close proximity to the fetus, and clearance was partially inhibited due to lack of MHC class I expression. We did not rule out alternative mechanisms at this time and further investigation is warranted.

Pregnancy outcomes in this model were poor compared with general observations for uninfected C57BL/6 mice (23). Cohorts of uninfected pregnant mice were not included in the study, which would have greatly contributed to analysis of pregnancy outcomes. The primary objective of this work was to compare immunologic responses between pregnant and nonpregnant mice in the generation of immunologic memory to an acute viral infection, thus all pregnant mice were infected.

LCMV is not a lytic virus and damage to infected tissue usually occurs as a consequence of cytotoxic T cell responses. Because we observed fetal resorption in 46% of pregnancies, it could be argued that the presence of activated CD8+ T cells in the placenta altered the local cytokine environment, which in turn mediated an inflammatory response at the site resulting in resorption, or uterine inertia. Murine fetal resorptions have been noted with altered levels of IFN-γ and TNF-α (30, 31). It is also possible that innate mechanisms mediated pregnancy loss in our model (32–36), which may have occurred with or without suppression of the local adaptive immune response.

Live-born pups were not infected with LCMV, despite high viral titers in the placenta before birth. Surviving pups were allowed to mature and demonstrated normal primary immune responses to LCMV (Armstrong) infection as adults.

Transplacental viral infections contribute to significant mortality and morbidity; however, the mechanisms enhancing or protecting against congenital infection are not fully understood. In our model, fetuses were not infected. Earlier studies involving LCMV infection during pregnancy have shown a variety of outcomes for the pups. In 1938, Traub (37) noted that pregnant carrier mice gave birth to infected pups. In 1968, Mims (38) noted that pregnancies in carrier mice commonly resulted in fetal resorption and neonatal death. In 1969, Mims (39) investigated the pathogenesis of LCMV in pregnancy using different strains, doses, and routes of infection. The WE3 strain of LCMV was used for the majority of experiments, which is a more virulent strain than Armstrong. He noted that fetal infection occurred, but only after the infection had spread throughout the placenta. He also noted more fetal resorptions in the infected group compared with the noninfected group using WE3.
When he infected pregnant mice with $10^{5.5} \text{LD}_{50}$ Armstrong strain on day 7–8 of pregnancy, however, he observed a few small foci of infection in the placenta by 11 days post infection, and the fetuses were not infected. These results more closely reflect the pregnancy outcomes we observed in our study.

It is well established that clinical outcomes during the prenatal period depend on the timing, route of infection, immune status of the mother, and other particular mechanisms involving host/pathogen interactions (40). In human pregnancy, for example, higher rates of maternal mortality with frequent abortions, stillbirths, and neonatal deaths occur during infections with hepatitis E, mainly when the primary infection occurs in the second or third trimester (3). Congenital infection with hepatitis B rarely occurs unless the primary infection happens during the third trimester (3). Maternal mortality is highest when influenza infections occur during the third trimester, particularly if complicated by pneumonia (1). Murine models have also shown that fetal outcomes are influenced by maternal immune status and timing of the infection (41, 42). For example, transplacental transmission of lactate dehydrogenase-eliciting virus is highly efficient unless the mother is immune, or if the initial infection occurs before 13 days of gestation in nonimmune mice. Virus is quantitated per gram of tissue or per milliliter of serum. The dotted line represents limit of detection. Note that each circle represents one mouse.

FIGURE 6. Immune mice demonstrate similar recall response and function of Ag-specific memory CD8 T cells to a secondary challenge with LCMV (clone 13). A. Mice that were pregnant during primary infection (●) ($n = 9$) and mice that were not pregnant during primary infection (□) ($n = 8$) were challenged with LCMV clone 13, 4 mo after primary infection. H-2Db/NP396-specific CD8 T cell response was monitored in blood via MHC class I tetramers (no significant difference between groups was noted with $p$ values for days 5, 8, and 12 as follows: 0.673, 0.606, and 0.888 respectively). B, Immune mice were challenged with LCMV (clone 13) ~80 days after primary infection, and splenocytes were harvested 5 days post secondary challenge. There was no significant difference in cytokine production (IFN-γ and TNF-α) upon 5 h stimulation with the indicated peptides (NP396, GP33, and Gp276) between the mice that were pregnant during primary infection (●) and those that were not pregnant during primary infection (□) ($p$ values ranged from 0.310 to 0.937 for all epitopes). C and D, Plaque assays of sera and various tissues days 3 and 5 post secondary challenge with LCMV (clone 13) among mice that were pregnant during primary infection (●) and those that were not pregnant during primary infection (□). Naive mice (●) show viral load for nonimmune mice. Virus is quantitated per gram of tissue or per milliliter of serum. The dotted line represents limit of detection. Note that each circle represents one mouse.
maternal-fetal interface, and develop strategies that optimize maternal immune responses to specific pathogens to provide protection against pathogens in the perinatal period and beyond.

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Disclosures

The authors have no financial conflict of interest.

References


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