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Th1 Cytokines Are Essential for Placental Immunity to *Listeria monocytogenes*

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The fetal allograft poses an immunological challenge: how is it protected while immunity to pathogens, particularly those that replicate in the placenta, is maintained? Several theories have been proposed to explain this fetal protection, including a pregnancy-based bias towards a Th2 rather than Th1 cytokine profile in order to avoid generating cytotoxic T cells that could threaten the fetus. *Listeria monocytogenes* preferentially replicates in the placenta and systemically requires a Th1 response for sterile eradication. In the placenta, the Th1 cytokines tumor necrosis factor alpha (TNF- α) and gamma interferon (IFN- γ) are also synthesized in response to this pathogen, without fetal loss. Here we show, by using mice homozygous for null mutations in either the cytokine or cytokine receptor genes, a requirement for both TNF- α and IFN- γ signaling for an effective placental immune response to *L. monocytogenes*. However, T cells were not recruited to the placenta. Genetic studies in which the fetal component of the placenta was genetically different from the mother indicated that both the production of and response to these cytokines were maternal. Despite the requirement for these cytokines, the early recruitment of neutrophils to the placenta was normal. Consequently, the bacterium appeared to be delayed in its colonization of this organ and did not fully gain hold until 72 h postinfection. These data show a requirement for Th1 cytokines during pregnancy for effective immunity and indicate that a bias away from Th1 cytokine synthesis is not a necessary prerequisite of pregnancy.

The fact that viviparous animals carry a fetus that is an allograft poses an interesting immunological question: how does the mother tolerate this situation while at the same time not losing immune protection from pathogens that can replicate in the placental bed? The uteroplacental unit is an immunoprivileged site since fetal tissue is rejected if transplanted elsewhere in the body (8). Thus, there must be immune regulation at this site that prevents fetal rejection. Many theories have been proposed to explain this phenomenon, ranging from immune evasion to active suppression (33). However, several recent incisive experiments have indicated that a considerable portion of the explanation for fetal survival is that cytotoxic T cells are excluded from the placenta and/or their activity is suppressed (33). Thus, the CD4⁺ CD25⁺ regulatory T-cell population is expanded during pregnancy, and these cells are required to prevent a cytotoxic T-cell response to an allogeneic fetus (52, 55). Similarly, indoleamine-2,3-dioxygenase (IDO), the rate-limiting enzyme in tryptophan catabolism, suppresses T-cell proliferation, and inhibition of this enzyme by chemical inhibitors during pregnancy results in the rejection of allogeneic but not syngeneic pregnancies (36).

It has also been proposed that the cytokine microenvironment is biased towards the synthesis of Th2-type cytokines and away from Th1-type cytokines, both systemically and in the placenta, such that cytotoxic T-cell responses are not developed (61). There are reports that the Th2 cytokines interleu-

kin-10 (IL-10) and IL-4 are found abundantly in the placenta, while IL-12 is not (23, 29, 42, 43, 46). These data are contradicted by other data, including some from our own laboratory showing little IL-10 but significant levels of IL-12, IL-18, and tumor necrosis factor alpha (TNF- α) (21). In other studies, gamma interferon (IFN- γ) was shown to be synthesized in the placenta throughout pregnancy, as it is required for the proper development of the placental vasculature (3, 21). Despite these differences, what is clear from all studies is that the T-cell mitogen IL-2 is never found (44). This fact, together with the extreme paucity of T cells in the murine placenta, suggests that the cytokine and immunoregulatory cellular environment is not conducive to the activation or proliferation of these potentially dangerous T cells.

The above studies have focused on the problem of immunological tolerance to the fetus, but they beg the question of how an effective immune response can be mounted in the placenta, particularly against those organisms that require an acquired immune response characterized by systemic cytotoxic T cells for clearance. To explore this question, we have used infection with the gram-positive intracellular bacterium *Listeria monocytogenes*. We chose this bacterium for two principal reasons. Firstly, immunity against *Listeria* has been extensively studied with nonpregnant mice and involves an early innate response that limits its initial spread, allowing the development of an adaptive sterilizing immunity characterized by a Th1 response (28, 35, 59). Secondly, *L. monocytogenes* has a predilection for replication at the maternal-fetal interface (1, 22, 30, 31, 49) and is a common cause of placental infection in humans in situations where public health preventative measures are not strictly enforced (31). This placental listerial infection substan-

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tially increases the risk of abortion and neonatal morbidity and mortality (54).

Studies with mice have shown that *L. monocytogenes* infection is restricted to decidual cells at the uteroplacental interface (48, 49). In murids, macrophages are excluded from these sites of infection, and instead, the major cellular defense is through neutrophils that are recruited, at least in the placenta, in response to the synthesis of the murine IL-8 homologues KC and MIP-2 early in the infection (21, 48, 49). It is also important that vaccination produced immunity to systemic infection upon subsequent infection without altering the infection in the placenta (48). These studies suggest that in the placenta the adaptive immune response to this organism is either not functional or severely attenuated. Despite this fact, in our studies of listerial infection we observed that the Th1 cytokines IL-12, IL-18, and TNF- α were synthesized in the placenta in elevated amounts in response to the bacterium early in the infection, followed by the synthesis of IFN- γ (21). However, even though there was synthesis of these Th1 cytokines, provided that the mice were immunocompetent, there was minimal fetal loss (21). Thus, at least in this context, Th1 cytokines can be synthesized in the placenta without the induction of abortion. In this paper, we show that these Th1 cytokines are required for an effective response against *L. monocytogenes* in the placenta, but without T-cell recruitment.

MATERIALS AND METHODS

Mice. All procedures involving mice were conducted in accordance with the National Institutes of Health regulations concerning the use and care of experimental animals. The Albert Einstein College of Medicine's Animal Use Committee approved this study. The following strains were all obtained from Jackson Laboratories, Bar Harbor, Maine: C57BL/6J, C57BL/6-Tnfrsf1a^{tm1Mak}, C57BL/6^{Jngm1Ts}, 129S3/SvImJ, 129/Sv-Ifngr^{tm1}, B6129SF2/J, and B6129S-Tnf^{tm1Gkl} (15, 25, 39, 41). These mice were maintained and mated under pathogen-free conditions in a barrier facility at the Albert Einstein College of Medicine. Pregnant female mice were used for all studies. The day of the vaginal plug was considered day 1 of pregnancy.

Female mice at 2 to 5 months of age were mated to male mice of the same strain, except for experiments where particular genetic crosses are described. To determine the genotype of each individual placenta from each of these crosses, genomic DNA was isolated from a small piece of the corresponding embryo using DNeasy purification columns (QIAGEN, Valencia, CA). For genotyping of the *Tnfrsf1a*^{tm1Mak} (*TNFR-1*) mutation, three primers were used, with one specific for the neomycin (*neo*) selectable marker (HSV-TK [ATT CGC CAA TGA CAA GAC GCT GG]), one near to and 3' of the site of insertion (p55TNFR636 [GGC TGC AGT CCA CGC ACT GG]), and one specific for a site 5' of p55TNFR636, but after the insertion site and too far away for a successful PCR product to be generated (p55TNFR515 [AGA AAT GTC CCA GGT GGA GAT CTC]). These primers allowed the detection of all three possible genotypes (homozygous null, heterozygous, and homozygous wild type) with just one PCR, and the product for the null allele was specific only for a *neo* insertion into the *TNFR-1* locus. The PCR program was as follows: 5 min at 94°C, 10 min at 80°C, 30 cycles of 1 min at 94°C, 30 s at 61°C, and 1.5 min at 72°C, and a hold at 4°C.

For the other three targeted mutations, PCR required four primers, two for the *neo* cassette and two for the intact gene. This also allowed the detection of all genotypes with one reaction, but the *neo*-specific primers would generate a product from *neo* inserted into any locus. For genotyping of the 129S-Tnf^{tm1Gkl} (TNF- α) mutation, the following primers were used: NEO1 (CTT GGG TGG AGA GGC TAT TC), NEO2 (AGG TGA GAT GAC AGG AGA TC), TNF- α P1 (GCA CAG AAA GCA TGA TCC), and TNF α P2 (TAG ACA GAA GAG CGT GGT GG). Due to the different melting temperatures of the primers, this PCR assay utilized "touchdown cycling," in which the annealing temperature was reduced 0.5°C per cycle for the first 12 cycles. The PCR program was as follows: 3 min at 94°C, 12 cycles of 20 s at 94°C, 30 s at 64°C (-0.5°C per cycle), and 35 s at 72°C, 25 cycles of 20 s at 94°C, 30 s at 58°C, and 35 s at 72°C, a 5-min extension at 72°C, and a hold at 4°C.

For genotyping of the C57BL/6^{Jngm1Ts} (IFN- γ) mutation, the following primers were used: GKO1 (AGA AGT AAG TGG AAG GGC CCA GAA G), GKO2 (AGG GAA ACT GGG AGA GGA GAA ATA T), NEO4 (TCA GCG CAG GGG CGC CCG GTT CTT T), and NEO5 (ATC GAC AAG ACC GGC TTC CAT CCG A). The PCR program was as follows: 6 min at 94°C, 35 cycles of 1 min at 94°C, 30 s at 55°C, and 1.5 min at 72°C, 5 min at 72°C, and a hold at 4°C.

For genotyping of the 129/Sv-Ifngr^{tm1} (IFN- γ R) mutation, the following primers were used: IFgrP3 (CCC ATT TAG ATC CTA CAT ACG AAA CAT ACG G), IFgrP4 (TTT CTG TCA TCA TGG AAA GGA GGG ATA CAG), NEO1, and NEO2 (the last two were also used for the TNF- α mutation). For PCR, the same program as that used for TNF- α genotyping was used for IFN- γ receptor (IFN- γ R) analysis.

Bacteria and infections of mice. *L. monocytogenes* strain EGD was grown to log phase in tryptic phosphate broth (Difco Laboratories, Detroit, MI) and stored in aliquots at -80°C. Serial dilutions were plated on tryptic phosphate agar plates in duplicate for bacterial quantification. Colonies were counted 24 h after growth at 37°C, and the number of CFU was determined (21). The virulence of the strain was maintained by routine passaging in mice.

Mice were infected intravenously on day 14 of gestation via the lateral tail vein with 10⁴ CFU per mouse. In some cases, mice were also infected on day 10 of gestation with 10⁴ CFU or on day 14 with 10³ CFU. The mice were sacrificed at 24, 48, and 72 h postinfection, and their livers, spleens and placentas were removed separately using sterile technique as described before (21). To isolate the uteroplacental unit, the embryo and its membranes were cut away, and the uterine wall around the placenta was cut; thus, all uterine layers just above the decidua basalis were included in the uteroplacental unit. The organs were homogenized individually in sterile phosphate-buffered saline (PBS) on ice. Homogenates were plated onto tryptic phosphate agar plates as described above to determine the number of CFU per organ (average wet weight, ~100 mg/placenta). Blood was also collected in some cases from the tail vein, and cell-associated and free bacteria were determined by plating as described above.

To determine the limit of detection for bacteria from mouse placentas, uninfective placental homogenates were spiked with known amounts of *L. monocytogenes* and assayed by serial dilution. Titers as low as 40 CFU/ml could be consistently recovered. Therefore, any titer above this was considered positive.

For the statistical analysis of placental titers, the placentas were not considered individual events but were grouped by mother. The ratios for the mice in the experimental group, defined as the number of titer-positive placentas over the total for each pregnancy, were compared using the Mann-Whitney rank sum test as described previously (5). Bacterial titers between groups were also compared by Mann-Whitney U tests.

Histological analysis. Organs were fixed immediately after dissection by incubation in 10% buffered formalin overnight at 4°C. Tissues were washed several times with and stored in 70% ethanol at 4°C. After at least 24 h in 70% ethanol, tissues were dehydrated through graded ethanol solutions into HistoClear (National Diagnostics, Atlanta, GA), embedded in paraffin, and then cut into 5- μ m sections that were floated onto charged glass slides.

On tissue sections, neutrophils were detected using a clone 7/4 biotinylated monoclonal rat antibody from Caltag (Burlingame, CA), and T cells were detected with anti-CD3- ϵ antibody (Ab) as described before (20, 21). Briefly, tissue sections were deparaffinized in HistoClear (three times for 5 min each time) and hydrated in a series of ethanol from 95 to 70% (3 min each) and in distilled water (dH₂O). To unmask the antigen (Ag) and increase the sensitivity of immunodetection, the tissue sections were put through a treatment of boiling for 15 min in 1 liter of buffer containing 50 mM glycine and 0.01% EDTA at pH 3.5. After the slides were cooled to room temperature and washed in dH₂O, endogenous peroxidase activity was quenched by two 8-min incubations in 15% H₂O₂-50% methanol in dH₂O. The tissue was blocked with 10% normal rabbit serum in PBS for 1 h at room temperature and then incubated with 8 mg/ml of antibody in 1.5% normal rabbit serum-PBS for 2 h at room temperature. Biotinylated clone 7/4 antibody was detected using an avidin-biotin-peroxidase kit (Vector Laboratories Inc., Burlingame, CA) as described in the manufacturer's protocol. Enhanced DAB (Pierce, Rockford, IL) was used as the substrate. All sections were counterstained with Gill's no. 3 hematoxylin solution (Sigma Diagnostics, St. Louis, MO) before being dehydrated in HistoClear and mounted with a glass coverslip using Permount (Fisher Scientific, Pittsburgh, PA).

The gram-positive bacterium *L. monocytogenes* was detected in tissue sections using Gram's stain (Sigma Diagnostics, St. Louis, MO) (21). Hydrated slides were covered with crystal violet solution for 1 min, rinsed with tap water, and covered with Gram's iodine solution for 5 min to mordant the crystal violet. Excess crystal violet was removed by applying a few drops of decolorizing solution (75% isopropyl alcohol, 25% acetone). The tissue was counterstained with

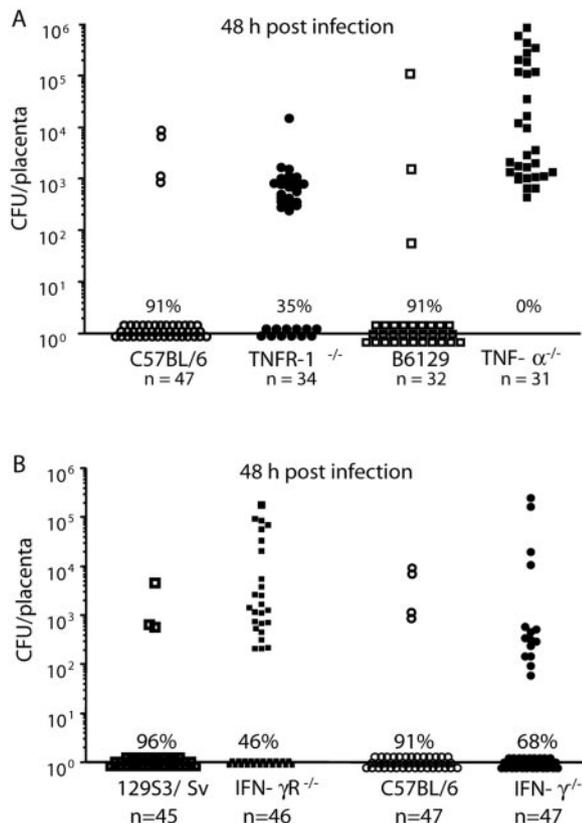


FIG. 1. TNF- α and IFN- γ signaling is required for resolution of placental *L. monocytogenes* infection. (A) Pregnant mice that were the wild type (C57BL/6) (\circ), the F1 generation from a C57BL/6 \times 129 mating (\square), or nullizygous for the TNF- α receptor (\bullet) or its ligand (\blacksquare), as indicated on the x axis, were infected intravenously on day 14 of pregnancy with 10^4 CFU of *L. monocytogenes*. Forty-eight hours later, individual placentas isolated from the numbers of mice shown were assayed for their bacterial titer as described in Materials and Methods. Each point is the titer (y axis) for one placenta, with the number assayed given below the strain designation. The percentages represent the percentages of placentas that had titers below the level of detection (>40 CFU/placenta). (B) Identical experiment to that described for panel A, except that mice nullizygous for IFN- γ (\bullet) or its receptor (\blacksquare) and appropriate matched control mice (129S3/Sv [\square] or C57BL/6 [\circ]) were used. Placental titers are significantly different from the appropriate control group, C57BL/6 (eight mice), 129S3/Sv (six mice), or F1 offspring from C57BL/6 \times 129 crosses (six mice), as follows: TNFR-1^{-/-} (six mice) and IFN- γ R^{-/-} (eight mice), $P < 0.01$; TNF- α ^{-/-} (six mice) and IFN- γ ^{-/-} (eight mice), $P = 0.03$.

Safarin O solution for 30 to 60 seconds, rinsed, and then dipped in tartrazine solution for 5 to 10 seconds. After being rinsed in dH₂O for 5 min, slides were dehydrated and mounted with a glass coverslip as described above.

RESULTS

IFN- γ and TNF- α are required for an effective placental immune response to *Listeria*. Our previous observations have shown an elevation in the placental TNF- α concentration within 24 h of infection with *L. monocytogenes* that was followed by an increase in IFN- γ 24 h later (21). We therefore wished to determine whether these cytokines were required for an effective immune response in the placenta against this bacterium. Figure 1A shows that at 48 h postinfection (p.i.), there

was a significantly elevated level of listerial infection in the placenta for both TNF- α receptor 1 (TNFR-1) and TNF- α ligand null mutant mice compared to that in either of the respective control wild-type strains, C57BL/6 and C57BL/6.129 F1. Similar results were found for IFN- γ R and IFN- γ null mice, which also displayed significantly elevated levels of infection at 48 h p.i. compared to their 129S3/Sv and C57BL/6 wild-type strains (Fig. 1B), although they were slightly lower than those observed in the absence of TNF- α signaling.

For these experiments, every placenta was harvested from each of the mice, and placentas were individually processed and assayed. Each placental titer is shown as an individual symbol in Fig. 1. It was notable that 48 h after infection the bacterial titers seemed to conform to a binomial distribution in which some placentas had titers below the level of detection while other had titers ranging from 10^2 to 10^6 CFU (Fig. 1A and B). The limit of detection represents a titer of <40 CFU, and the data points below this limit are shown as zero points arranged along the x axis, with the percentage of placentas with titers below the limit of detection displayed immediately above. A detailed analysis of the data showed that there was no obvious discernible pattern in the distribution of placental infections, since the placentas derived from an individual mouse could show the same range of listerial titers as that observed between mice. Thus, even in the same mouse, some placentas had high titers while others were below the detection limit, and these placentas could be adjacent to each other. For statistical reasons, the difference between mice was determined by the percent infections of individual placental units and was shown to be highly significant (Fig. 1 and 2). This is because individual placentas cannot be regarded as independent variables, and only the variation between mice of different genotypes is relevant. However, the bacterial titers can also be averaged for each mouse (aggregate titer divided by the number of placentas). In this analysis, the effect of the ablation of either TNF- α or IFN- γ signaling was highly significant at the 48-h time point ($P < 0.0001$ and 0.03 , respectively; Mann-Whitney test). Thus, not only the number of placentas infected but also the overall bacterial titers were significantly higher in the absence of TNF- α or IFN- γ signaling. It can therefore be concluded that these cytokines are required for an adequate immune response to *L. monocytogenes* infection in the placenta.

The binomial distribution of infectious titers in the placenta could be due to either a suppression of the early infection followed by recovery of the bacteria or an initial poor seeding of the placenta followed by reinfection. The former would predict a steady increase in the number of placentas infected and a logarithmic increase in bacterial titer. Consequently, we performed time courses of infection in TNFR-1 and IFN- γ nullizygous mice, both of which are in a C57BL/6 background (Fig. 2). For both mutant strains at 24 h p.i., only a few placentas exhibited a titratable infection, whereas the vast majority were below the limits of detection, and this extent of infection was not significantly different from that of the control strains. As discussed above, at 48 h there were significant increases in the numbers of placentas infected and listerial titers in both cytokine-deficient mouse strains. By 72 h p.i., there was a further increase in the numbers of infected placentas in the mutant strains until essentially all of the placental units showed infection (100% for TNFR-1 and 98% for IFN- γ ;

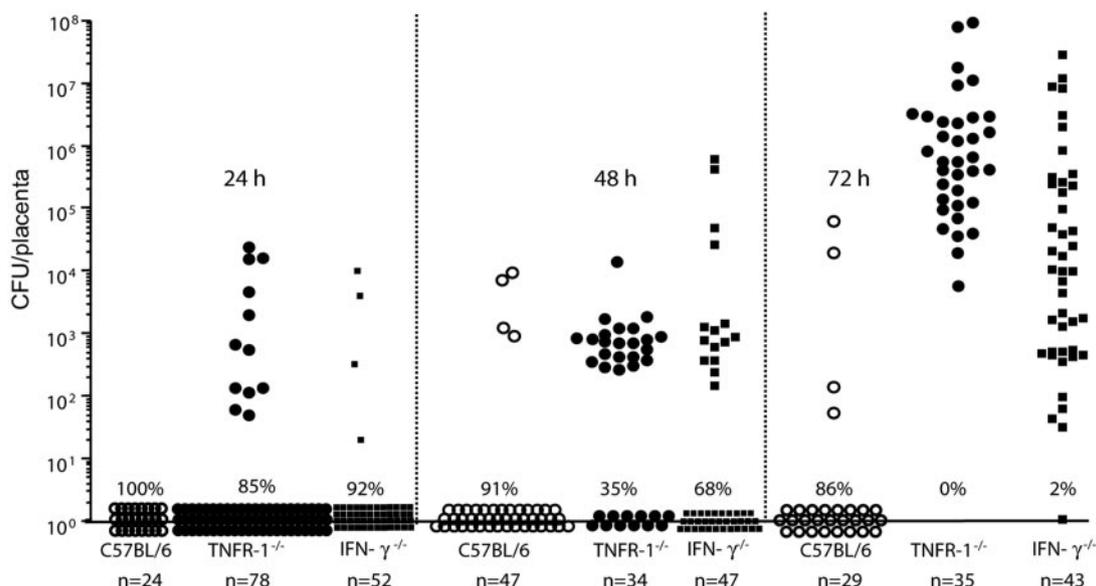


FIG. 2. Time course of *L. monocytogenes* infection in TNF- α and IFN- γ null mutant mice reveals an impairment of the placental immune response after 48 h. The experiments were performed and the data are presented as described in the legend to Fig. 1, with either control wild-type, C57BL/6, or homozygous null mice with mutations in the TNFR-1 (●) or IFN- γ (■) gene being killed at 24, 48, and 72 h postinfection. At 24 h, there were no significant differences between C57BL/6 (4 mice) and TNFR-1^{-/-} (13 mice) or IFN- γ (6 mice) mice. At 48 h and 72 h, both TNFR-1^{-/-} and IFN- γ ^{-/-} mice had significantly greater infections than the control C57BL/6 mice (eight and four mice for 48 and 72 h, respectively); for TNFR-1^{-/-} mice at 48 h (six mice), $P < 0.01$; for the same mice at 72 h (four mice), $P < 0.001$; for IFN- γ ^{-/-} mice at 48 h (eight mice), $P = 0.03$; for the same mice at 72 h (six mice), $P = 0.01$.

Fig. 2). Furthermore, the titers increased logarithmically in these mutant placentas over the time course of infection ($P < 0.001$ for both TNFR-1^{-/-} and IFN- γ ^{-/-} mice compared to wild-type controls). In contrast, in the C57BL/6 control mice, even at 72 h of infection, only an occasional placental unit showed an infectious titer. These results demonstrated highly significant differences both in numbers of infected placentas and in bacterial titers between control mice and TNFR-1^{-/-} and IFN- γ ^{-/-} mice (Fig. 2). In other experiments, mice were infected on day 10 of pregnancy with 10⁴ CFU. Statistically identical titers to those shown in Fig. 2 were found in the placentas harvested on day 12 (48 h p.i.) for all three strains of mice (data not shown). This indicates that once the placenta has formed, the timing of infection does not materially alter the immune response to this bacterium.

To confirm the degree of infection and to determine the cell types infected, Gram staining was performed on sagittal sections of the placentas. Bacteria were only occasionally detected in the sections examined from C57BL/6 mice, although in these experiments, given the occasional nature of infection, a highly infected C57BL/6 placenta was not examined (Fig. 3A). However, in all the placentas from the TNFR-1 null mice at 72 h p.i., gram-positive rods were abundantly observed (Fig. 3B). Indeed, in these null mutant mice, bacteria were not only found in decidual cells, the previously documented area of infection (49), but also were found in trophoblastic giant cells and spongiotrophoblasts (Fig. 3B), representing spread from the decidua basalis into the fetal layers. An analysis of embryos showed that none of them were infected in the C57BL/6 mice (0/3 mice; 23 embryos analyzed), whereas there was significant infection in 2/6 (33 embryos analyzed) and 4/6 (38 embryos

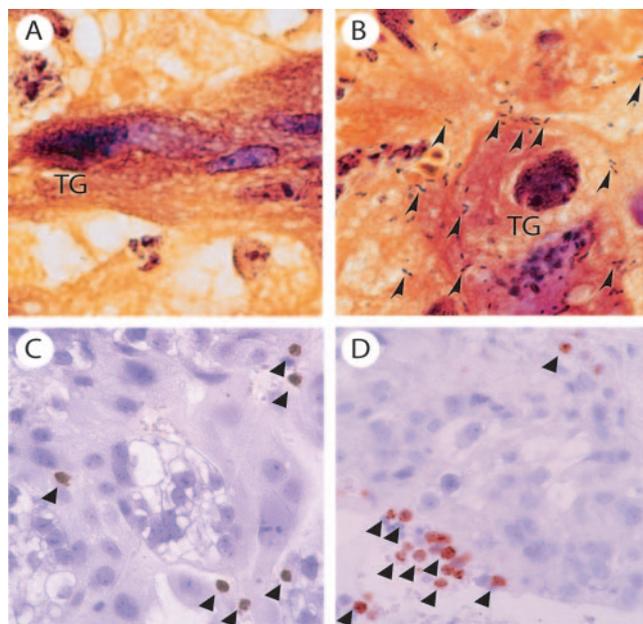


FIG. 3. Loss of TNFR-1 resulted in abundant bacterial infection even with normal neutrophil recruitment. (A and B) Gram staining of sagittal sections of uteroplacental units on day 17 of pregnancy and at 72 h p.i. with *L. monocytogenes* showing the trophoblastic-decidual interface of C57BL/6 (A) and TNFR-1^{-/-} (B) mice. Abundant gram-positive organisms (purple rods) in decidual as well as trophoblastic giant cells (TG) (Magnification, $\times 100$) were found only in the TNFR-1^{-/-} (B) mice. (C and D) Anti-Gr1 antibody staining for neutrophils (red-brown stain) at the uteroplacental interface on day 15 of pregnancy and at 24 h p.i. with *L. monocytogenes* in C57BL/6 (C) and TNFR-1^{-/-} (D) mice shows neutrophil invasion into the decidua basalis in both mouse strains.

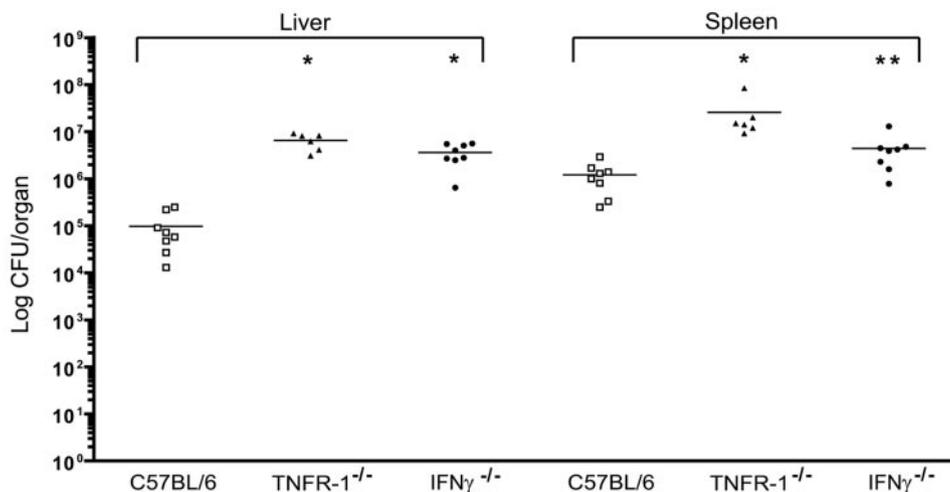


FIG. 4. Increased liver and spleen titers during pregnancy in TNFR-1^{-/-} and IFN-γ^{-/-} mice compared to those in C57BL/6 mice. The y axis shows the titers of *L. monocytogenes* in livers and spleens at 48 h p.i. for C57BL/6 (□), TNFR-1^{-/-} (▲), and IFN-γ^{-/-} (●) mice, as indicated on the x axis. Each symbol is the value for an individual pregnant mouse, and the horizontal bars are the mean titers. The loss of TNF-α and IFN-γ cytokine signaling results in significantly higher titers than those in the wild-type C57BL/6 strain. *, $P < 0.001$; **, $P = 0.007$ (Mann-Whitney test).

analyzed) TNFR-1^{-/-} and IFN-γ^{-/-} pregnant mice, respectively, at 72 h p.i. This suggests that the infection passes into the embryo through the placenta since, at least for the TNFR-1^{-/-} mice, all the placentas had high bacterial titers at this time.

It has also been suggested that during pregnancy there is a bias in the systemic cytokine profile from one that drives a Th1-type response to one that favors Th2 responses. To examine this, we also measured titers of *Listeria monocytogenes* in the liver and spleen at all time points examined, concurrent with the analysis of infection in the placenta. Shown in Fig. 4 are the data from 48 h p.i. for TNFR-1 and IFN-γ knockout mice in a C57BL/6 background. These data show a 1- to 2-log increase in infectious titer at this time point for the mice lacking cytokine signaling compared to the control strain (Fig. 4). Similar differences between cytokine null mutant mice and wild-type mice were observed at 24 and 72 h p.i. and also for these strains of mice assayed 48 h after infection on day 10 of pregnancy (data not shown). These differences are similar to those observed during the systemic infection of nonpregnant mice (25, 51), and they show that a Th1 cytokine-driven response is also required for an adequate immune response during pregnancy.

It can be concluded, therefore, that in the mutant strains it takes 48 to 72 h for the infection to truly gain hold in the placenta. Although previous experiments had shown that *L. monocytogenes* infection in the placenta progressed independently of the infection in the rest of the body (48), it was possible for the mutant strains that the infection was secondary to the rampant systemic infection. To ensure that this was not the case, we measured the blood titers of *L. monocytogenes* in the mutant and wild-type mice. In the latter, blood-borne bacteria could not be identified at any time point (four, two, and two mice at 24, 48, and 72 h, respectively). In the TNFR-1 null mutants, there were detectable bacteria at 48 h, but only 10³ PFU per ml of blood ($n = 7$; 61% cell associated), which increased to ~10⁴/ml ($n = 3$) at 72 h. At this time, the placenta

could have titers up to 10⁸/placenta. To determine if this could explain the subsequent infection in the placenta, we infected day 14 TNFR1^{-/-} pregnant mice ($n = 6$) intravenously with a titer of 10³ CFU. Forty-eight hours later, only 1 of 36 placentas showed any infection, and the remainder were below the level of detection, suggesting that a blood titer of 10³ CFU/ml was insufficient to give the observed placental titers. In the IFN-γ null mice infected with 10⁴ CFU, no bacteria were detected in the blood at 48 h ($n = 4$), even though the infection was often well established in the placenta, and only ~7 × 10³/ml of blood ($n = 2$; 51% cell associated) was detected at 72 h, while placental titers ranged up to 10⁷. Considering these data, it seems unlikely that blood-borne bacteria can explain the progress of the placental infection.

Neutrophils are recruited normally in response to listerial infection in the absence of TNF-α and IFN-γ signaling, but T cells are never found in the placenta. It is striking that in the above experiments listerial infections were almost undetectable in both the TNFR-1 and IFN-γ ligand knockout mouse placentas until 48 h postinfection and that even at this stage, 30 to 40% of the placentas did not contain titratable bacteria. This is in stark contrast to the case for the loss of colony-stimulating factor 1 (CSF-1) signaling in the placenta, when all the placental units were infected with considerable titers even at 24 h postinfection (21; our unpublished data). We have previously demonstrated that this sensitivity to the loss of CSF-1 is caused by the failure of early neutrophil recruitment, which is responsible for the clearance of ~95% of the initial bacterial infection (21). Therefore, in CSF-1 null mutant mice, there are essentially no neutrophils in the decidua basalis postinfection, in contrast to an abundant infiltrate in wild-type mice. Thus, we investigated whether neutrophils were recruited to the decidua basalis in both TNFR-1 and IFN-γ null mice at 24 h postinfection. In wild-type (Fig. 3C) and TNFR-1 (Fig. 3D) and IFN-γ null mutant mice (data not shown), there was an abundant neutrophil invasion at 24 h. These cells were seen associated with the blood vessels and throughout the decidua in

patterns that were similar between the mouse strains. Thus, the early responses of neutrophil recruitment in the cytokine null mutant mice were intact, consistent with the time course of infection in TNF- α and IFN- γ ligand- or receptor-deficient mice that showed that infection required 48 h to become firmly established, a time when neutrophils have already begun to disappear from the tissue.

To determine whether T cells were found in the placenta postinfection, immunostaining with anti-CD3- ϵ Ab specific for T cells was used. This antibody showed extensive staining in the splenic control sections but was negative in the placenta at all times of infection (data not shown) for all strains, including the fully responsive wild-type mice, regardless of whether the pregnancies were syngeneic or allogeneic, as we and others have previously reported (21, 32, 49). Thus, T cells are not recruited to the placenta in significant numbers in the face of a listerial infection and therefore cannot be required locally.

TNF- α and IFN- γ production and responses are maternal. Published data have shown that trophoblastic giant cells in the uninfected placenta are positive for TNF- α (26, 27) and that these cells also express the IFN- γ R (13). Thus, we hypothesized that in a pattern similar to that found systemically, the IL-12 and TNF- α produced by these fetal cells caused the maternal uterine NK-cell population in the decidua to produce IFN- γ that in turn was targeted to the fetal trophoblastic giant cells to respond to a listerial infection by phagocytosis and bactericidal activities. To test this hypothesis, we used genetic means to remove the ligand or receptor from the maternal or fetal cells as described in Fig. 5E. Thus, in a null mutant mother crossed with a +/+ father, all the trophoblastic cells will be +/-, and in a +/- mother crossed with a -/- father, 50% of the placentas will derive from +/- embryos and 50% will derive from -/- embryos. The latter can be distinguished by PCR from embryonic tissue. Using these crosses, we infected pregnant mice on day 14 with *L. monocytogenes* and harvested placentas 48 h later for determinations of the listerial titer. Interestingly, and in contrast to our hypothesis, those placentas whose fetal cells were TNFR-1^{-/-} had little to no overt infection, as opposed to the abundant infection in the placentas when the fetal contribution was heterozygous but the maternal contribution was null (Fig. 5A). This can be seen by the number of placentas infected and by the bacterial titer (compare Fig. 5A to Fig. 1A). A similar result was found when the maternal or fetal requirement for TNF- α was explored (Fig. 5B). In this case, only when the mother was TNF- α positive could a placental infection be resolved (Fig. 5B). Thus, we concluded from these data that the production of and response to TNF- α are maternal.

We next performed similar experiments with IFN- γ and IFN- γ R null mice. These data were the mirror image of those for TNF- α and its receptor, in that both the production and response to IFN- γ are maternal (Fig. 5C and D). Thus, in contrast to our hypothesis, trophoblastic cells do not appear to play a significant part in the Th1 cytokine-induced placental immune response to listerial infection.

DISCUSSION

Listeria monocytogenes infection has been an important tool for probing the innate immune response and the transition to

a Th1 type of acquired response that generates the cytotoxic anti-*Listeria* T cells required for sterile eradication (28, 53). It has also been important for studies of pregnancy that have delineated the differences between the systemic response and the regional immune responses in the placenta, where the bacterium preferentially replicates (21, 47, 49, 50). Systemically, the primary site of *L. monocytogenes* replication is in macrophages, and thus the spleen and liver are principal sites of infection (53, 58, 59). Initially, in these organs, there is a rapid neutrophil recruitment in response to chemokine synthesis by macrophages (17, 20). These neutrophils are responsible for the elimination of much (~95%) of the infection (6, 58, 59). During this early phase, IL-12 and TNF- α are also synthesized, and these induce the production of IFN- γ (24, 57). This IFN- γ synthesis is thought to be mostly performed by NK cells (58, 59). The IFN- γ directs CD4-naive Th0 cells to become Th1 cells that in turn produce IFN- γ , which together with the initially synthesized IFN- γ , activates the newly recruited CSF-1-regulated macrophages to become bactericidal, and together with dendritic cells (18, 37), to present Ags to the newly formed *L. monocytogenes*-specific cytotoxic T lymphocytes (19, 20). The need for each of these cytokines in the systemic response of nonpregnant mice has been established using genetic ablation of each cytokine gene in mice in the face of an *L. monocytogenes* challenge (14, 15, 20, 25, 39–41, 60). Similarly, the need for CSF-1-regulated macrophages (20), dendritic cells (18, 37), neutrophils, and T cells (35) in this response is also well established. However, the central role for NK cells in the transmission of signals from macrophages to T cells is now in doubt since the absence of these cells results in protection from listerial infection and not increased susceptibility, as would be predicted if they were required (5). This may be because of the ability of CD8⁺ T cells to produce IFN- γ during the innate stage of the infection (9). Moreover, the system is clearly more complex than the brief review presented above, as shown by a recently defined negative role for IFN- α , whose actions seem to enhance the severity of listerial infection (4, 12, 38).

In pregnant mice, in addition to replication in the macrophage-rich organs, *L. monocytogenes* also replicates in decidual cells immediately after implantation and in the decidua basalis once the placenta has been formed (49). There is considerable evidence that the regional placental response is also different from the systemic one. For example, vaccination protects against a secondary systemic infection but does not protect against the placental one, implying that the adaptive immune response is not engaged in this organ and that the local innate response proceeds independently (48, 49). Furthermore, in murids, macrophages are excluded from the maternal-fetal interface (45), suggesting that these cells are not involved in the immune response in this organ. However, the CSF-1 receptor is expressed in both macrophages and trophoblastic cells (2, 10). An analysis of mice homozygous for a null mutation in the CSF-1 gene has indicated that CSF-1 receptor signaling is required in both macrophages and trophoblasts for effective immune responses to listerial infection. However, CSF-1 signaling is not required for neutrophil recruitment in the liver and spleen, but it is required in trophoblastic cells in order for neutrophils to be recruited to the decidua basalis (20, 21), further emphasizing the uniqueness of the placental re-

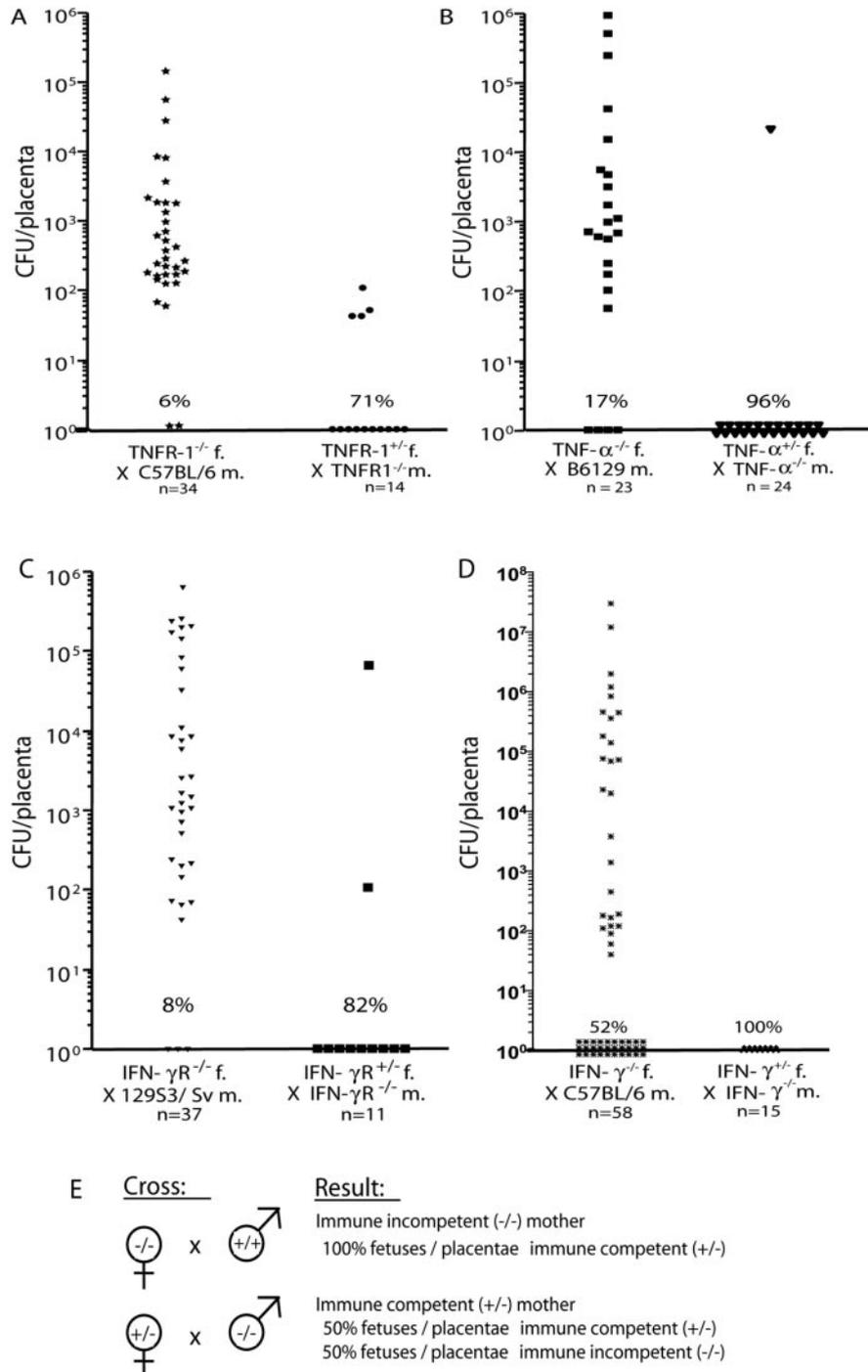


FIG. 5. TNF- α and IFN- γ responses and production in the placenta are maternal. (A to D) Responses to *L. monocytogenes* infection of the different maternal (m) and paternal (f) genotypes (x axis) obtained as described in panel E. Infection strategies and representations of the infectious titers (y axis) as CFU per placenta are as described in the legend to Fig. 1. Mice were killed at 48 h p.i. The combination of the TNF- α ligand and receptor pair of null mice (A and B) or the IFN- γ and receptor pair of null mice (C and D) is represented, with the number (n) of placentas assayed indicated under the mating strategy. In each case, the loss of cytokine or cytokine signaling in the maternal compartment was significantly different from the corresponding loss in the fetal compartment but not from the total amount of cytokine, as shown in Fig. 1. Placental titers from TNFR-1^{-/-} mice (six mice) crossed to C57BL/6 males are significantly different from those of C57BL/6 mice ($P = 0.0007$), but not from those of TNFR-1^{-/-} mice ($P = 0.4$). Titers of homozygous null placentas from TNFR-1^{+/-} females (eight mice) crossed to TNFR-1^{-/-} males are comparable to those of C57BL/6 mice ($P = 0.32$). Titers from TNF- α ^{-/-} female mice (four mice) crossed to BL6129 males are comparable to those of C57BL/6 mice ($P = 0.02$) but not to those of TNF- α ^{-/-} mice ($P = 0.13$). Titers of homozygous null placentas from TNF- α ^{+/-} females (five mice) crossed to TNF- α ^{-/-} males are comparable to those of B6129 mice ($P = 0.72$). Titers from IFN- γ R^{-/-} females (six mice) crossed to 129S3/Sv males are significantly different from those of 129S3/Sv mice ($P = 0.009$) but not from those of IFN- γ R^{-/-} mice ($P = 0.99$). Titers of homozygous null placentas from IFN- γ R^{+/-} females (four mice) crossed to IFN- γ R^{-/-} males are comparable to those of 129S3/Sv mice. Titers from IFN- γ ^{-/-} females (six mice) crossed to C57BL/6 males are significantly different from those of C57BL/6 mice ($P = 0.001$) but not from those of IFN- γ ^{-/-} females ($P = 0.1$). Titers of homozygous null placentas from IFN- γ ^{+/-} females (four mice) crossed to IFN- γ ^{-/-} males are comparable to those of C57BL/6 mice ($P = 0.32$). (E) Genetic strategy used to determine the maternal and fetal contributions to cytokine signaling pathways during *L. monocytogenes* infection in the placenta. The crosses were performed using mice harboring null mutations in TNF- α , IFN- γ , and their receptor genes with mice from corresponding wild-type strains.

gional immune response. In addition, in the classical studies of Redline and Lu (48, 49) it was observed that T cells were not recruited to the decidua, which has been confirmed by the studies reported in this paper and our previous publication (21).

Nevertheless, despite the absence of T cells in the placenta, we found that Th1 cytokines were synthesized in this organ following infection, in a pattern similar to that observed systemically, with the early induction of IL-12, IL-18, and TNF- α and then of IFN- γ (21). The present studies using gene-targeted mice have established that maternally synthesized TNF- α and IFN- γ and maternal responses are required for adequate systemic and placental immune responses to *Listeria*. This also indicates that Th1 cytokines are needed for successful pregnancies in the face of bacterial challenge. It also shows that there is not an obligate bias towards an immune response characterized by Th2 cytokines during pregnancy.

In the cytokine-deficient mutant mice, it was notable that listerial infection took significantly longer to take hold than was observed with CSF1 null mutant mice, and it was 3 days before all placental units were infected at detectable levels in either the TNFR-1 or IFN- γ null mutant mice. We interpret this as a consequence of the suppression of the early bacterial infection by neutrophils that are still recruited even in the absence of TNF- α and IFN- γ , in contrast to the absence of these cells in the CSF-1 null mutant mice. Thus, in CSF-1 null mutants the bacterial infection is well established in all placentas by 24 h and becomes rampant and transmitted through trophoblastic cells to all fetuses by 72 h (21). Neutrophils,

however, are short-lived cells and rapidly disappear from the tissue after 48 h p.i. (49; our unpublished data). Consequently, we speculate that in the TNF- α and IFN- γ null mice, the few residual bacteria that survive the neutrophil attack take time to recover and to grow to detectable levels. However, in the absence of the TNF- α - and IFN- γ -induced responses thereafter, they are able to continue to replicate unrestrainedly until all the placentas have high titers, in contrast to the restrained infection in wild-type mice.

We propose that this early but transient action of neutrophils recruited to the placenta in the first 24 h postinfection is the explanation of the binomial nature of the early infection in TNF- α and IFN- γ null mice. Thus, in some cases the bacteria recover more quickly and, due to their exponential growth, are easily detectable, while in other uteroplacental units they lag and still have not reached a detectable level, even within the same mouse. An alternative explanation would be that the initial seeding of bacteria is inefficient, with some placentas being infected and others not. Infection would then occur at later stages either from bacteria circulating in the blood or from placenta-to-placenta transmission. However, this hypothesis appears to be inconsistent with previous data from immunization studies showing that placental infection continues in the absence of a systemic one (48) and with the observation in CSF-1 null mutant mice that all the placentas show significant titers at 24 h p.i. (data not shown). Furthermore, it is unlikely because listerial titers in the blood were undetectable for 48 h postinfection in IFN- γ null mutant mice and only at very modest levels at 72 h, although at each time point the titer of

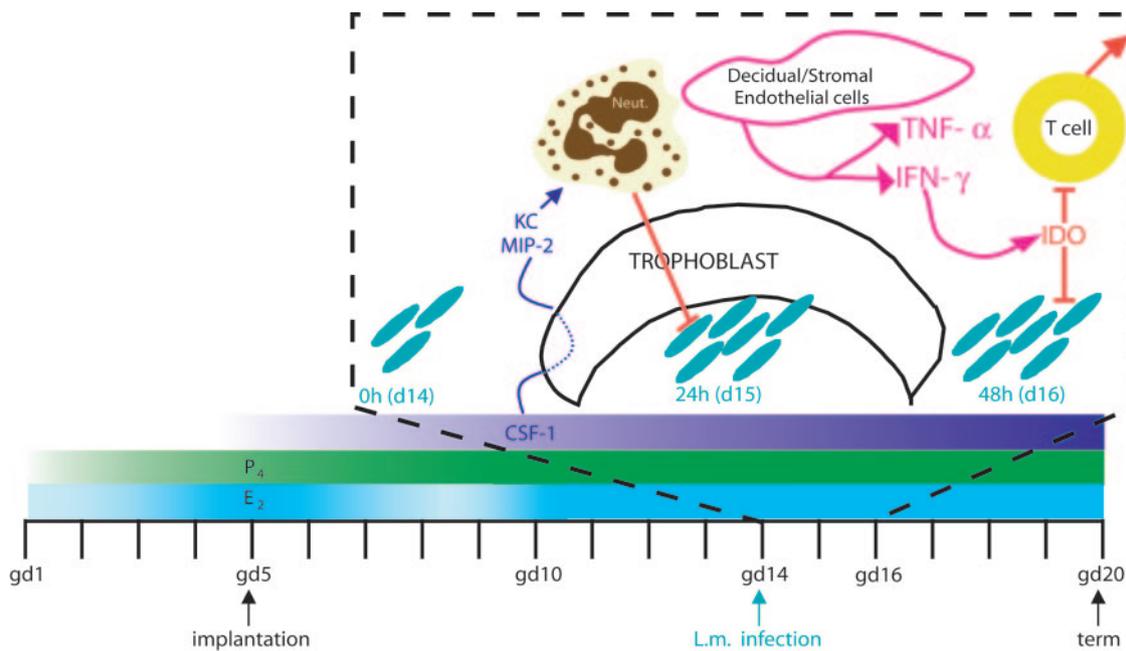


FIG. 6. Schematic of placental immune response to *L. monocytogenes*. The figure shows a timeline in days of gestation (gd) on the x axis. Progesterone (P₄, green) and estradiol-17 β (E₂, blue) regulate uterine synthesis of CSF-1 (mauve), whose concentration elevates dramatically through gestation. The color density represents the concentration, with the darkest being the highest. CSF-1 is targeted to receptor-bearing trophoblastic cells that, upon *L. monocytogenes* infection at gd14, synthesize the neutrophil chemoattractants KC and MIP-2. These chemokines recruit neutrophils at 24 p.i. that are responsible for the eradication of most of the listerial infection. At 24 h p.i., placental TNF- α is also elevated, followed by IFN- γ at 48 h. These cytokines are made in the maternal decidual and stromal cells and are required for suppression of the infection between 48 and 72 h p.i. IFN- γ induces IDO, which we postulate is part of the innate response against *Listeria* and which also blocks T cells from proliferating in the placenta, hence protecting the fetus from their cytotoxic activities.

placental infection was already several orders of magnitude higher. Furthermore, the usual route of listerial trafficking is in mononuclear phagocytes (16), and these are excluded from the murid placenta (21, 45).

In light of the current and previous observations, we propose the following chronology of the immune response in the placenta (Fig. 6). Initially, estrogen and progesterone cause maternal CSF-1 synthesis that primes the placenta to rapidly respond to the listerial infection with synthesis of the IL-8 homologs, KC and macrophage inflammatory protein 2. These chemokines recruit neutrophils within 24 h postchallenge, and these cells resolve most, but not all, of the infection. At 24 h p.i., TNF- α , IL-12, and IL-18 cytokine synthesis peaks and in turn leads to IFN- γ production within 48 h of infection. TNF- α and IFN- γ are required for the placental immune response at 48 and 72 h p.i. It is unclear how TNF- α and IFN- γ function, since T cells are absent from the placenta. We have previously shown that IFN- γ , but not TNF- α , induces IDO in decidual stromal and endothelial cells in response to *L. monocytogenes* infection (32). Elegant studies using inhibitors of IDO have revealed that this rate-limiting enzyme in tryptophan catabolism is required for the suppression of T-cell responses to the allogeneic fetus and that its inhibition results in T-cell-mediated killing of genetically disparate fetuses (34). IDO has other well-documented roles as part of the innate response to intracellular pathogens, killing organisms such as *Chlamydia* through tryptophan deprivation or via the production of toxic metabolites (7, 11, 56). We suggest that in the context of a listerial infection, the placenta uses this ancient means of defense to kill the bacterium by toxic metabolites (*Listeria* is not a tryptophan auxotroph) while at the same time preventing the cytotoxic T cells required for sterile eradication systemically from entering the placenta and posing a threat to the fetus. This cannot be the only method of T-cell exclusion, however, since cytotoxic T lymphocytes are generated even in IFN- γ null mice and yet do not accumulate in the placentas of these null mutant mice, even in the absence of IDO induction, although basal levels of IDO are still present.

We also suggest that this T-cell exclusion from the placenta has its costs, since *L. monocytogenes* is never completely eradicated from the tissue but instead is kept restrained at a minimal level. Normally, this is of little consequence for the mother because she has sterilizing immunity systemically, and as long as the fetus remains sufficiently protected to be born, the minimally infected organ, the placenta, is discarded upon birth without posing a further threat. The downside of this strategy is that occasionally the organism escapes the immune response in the placenta and replicates profusely. This may be the reason that *L. monocytogenes* infection remains a significant pathogen for pregnant women and is a major cause of fetal morbidity and mortality (54).

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