Adoptive Transfer of Treg Cells Counters Adverse Effects of Toxoplasma gondii Infection on Pregnancy

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Acute infection with Toxoplasma gondii (T. gondii) during pregnancy is associated with adverse outcomes. The mechanisms that cause this phenomenon are not clear. Regulatory T cells (Tregs) are involved in maternal tolerance, and here we observed a decrease in the absolute numbers of CTLA-4+ Tregs and PD-1+ Tregs in spleen and at the fetal-maternal interface in T. gondii-infected mice. Our results suggest that T. gondii induces apoptosis of Tregs. Additionally, we found that the expression of CTLA-4 and PD-1 on Tregs at fetal-maternal interface were higher than on spleen cells from normal pregnant mice. Therefore, we adoptively transferred Tregs from fetal-maternal interface or from spleens of normal pregnant mice into infected pregnant mice. Pregnancy outcomes were improved when Tregs were transferred from the fetal-maternal interface but not from the spleen. The mechanism appears to be through up-regulation of the number of CTLA-4+ Tregs and PD-1+ Tregs and correction of the imbalance between tolerant cytokines (IL-10, TGF-β) and inflammatory cytokines (IFN-γ). Our data indicate that Tregs at fetal-maternal interface express high levels of inhibitory molecules that play a vital immuno-protective role during pregnancy.

Keywords. CD4+ Foxp3+ regulatory T cell; Toxoplasma gondii; adoptive transfer; pregnancy.

Toxoplasma gondii (T. gondii), a ubiquitous, obligate intracellular parasite, can infect a range of warm-blooded intermediate hosts [1]. A primary infection with T. gondii during human pregnancy can cause miscarriage, stillbirth, or fetal abnormalities [2, 3]. Unfortunately, there is no effective measure to cure congenital toxoplasmosis.

Under normal circumstances, the maternal immune system does not reject its semiallogenic conceptus, so pregnancy has been thought to be a state of immunological tolerance [4]. Accumulating evidence suggests that regulatory T cells (Tregs) allow the maternal immune system to tolerate the fetal allograft [5, 6]. Tregs were first described as a specialized subpopulation of T cells responsible for the maintenance of immunological self-tolerance and tolerance of allogenic organ grafts through active suppression of self-reactive lymphocytes [7, 8]. Maternal Tregs expand during the pregnancy and appear to prevent maternal rejection of the fetus in mice and human; pregnancy fails in the absence of Tregs [5, 6, 9].

The forkhead box transcription factor Foxp3 is exclusively expressed in Tregs and acts as a master switch during Treg development and activity [10, 11]. Sustained ablation of Foxp3+ cells causes nonspecific T-cell activation and systemic autoimmunity [12, 13]. Tregs mediate their protective function by contact-dependent inhibition through cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) [14] and programmed death 1 (PD-1) [15]. CTLA-4 expressed on Tregs interacts with B7 family proteins and transmits inhibitory signaling. It has been revealed the expression of CTLA-4 on Tregs increases in peripheral blood and deciduas during normal human pregnancy; levels are decreased prior to and during human miscarriage [16]. PD-1 also plays an important role in normal
pregnancy by inhibiting the activation and function of lymphocytes; it binds to PD-L1 expressed on villous syncytiotrophoblasts and cytotrophoblasts [13, 17]. Tregs also can exert their actions by secreting interleukin 10 (IL-10) and transforming growth factor β (TGF-β) [18, 19]. Augmented local levels of IL-10 and TGF-β are important for protective tolerance induced by Tregs during pregnancy [20, 21]. Interferon γ (IFN-γ) is a representative proinflammatory cytokine that is needed for the invasion of trophoblasts, but an excess is associated with pathogenesis of recurrent spontaneous abortions [22]. Tregs inhibit inflammatory responses by secreting IL-10 and TGF-β [23].

Our previous research showed that levels of IL-10 and TGF-β were decreased and those of IFN-γ were increased in mice with T. gondii induced adverse pregnancy outcomes [24, 25]. The aim of this study was to explore whether a functional deficiency of Tregs contributes to the adverse pregnancy outcomes induced by T. gondii, and pregnancy outcomes could be improved by adoptive transfer of Tregs from normal pregnant mice into pregnant mice infected with T. gondii.

MATERIALS AND METHODS

Animals
C57BL/6 mice (6–8-week old females) and BALB/C mice (8–10-week old males) were purchased from Beijing Weitong Lihua Experimental Animal Technical Co, Ltd. Female mice were housed 5 per cage, and male mice were housed 1 per cage at 25°C with air humidity of 50%–60% and a 12-h light/dark cycle. All were adequately supplied with water sterilized by autoclaving and food for SPF mice purchased from Jiangsu Biological Engineering Co, Ltd. After overnight cohabitation with males at a ratio of 2:1, females with a vaginal plug (gestational day (gd) 0) were segregated and randomized into 4 groups with 6 mice each: normal pregnancy group, infected pregnancy group, infected mice adoptively transferred with Tregs from spleen, and infected mice adoptively transferred with Tregs from the fetal-maternal interface. All experiments were performed according to the ethical standards formulated by the Institutional Animal Experimental Ethics Committee of Binzhou Medical University.

Maintenance of T. gondii RH Trophozoites
T. gondii tachyzoite RH strain was maintained in our laboratory. Each Kunming mouse was infected with 2 × 10⁶ T. gondii RH trophozoites via an intraperitoneal injection. Peritoneal fluid was obtained 54 hours after infection. T. gondii tachyzoite were removed under sterile condition and injected into another healthy Kunming mouse at an appropriate concentration.

Infection and Pregnancy Outcome
Pregnant mice were inoculated intraperitoneally with 400 tachyzoites in 200 µL sterile phosphate buffer solution (PBS) on gd 8. The control group was inoculated with 200 µL sterile PBS. The mice were killed at 6 days postinfection (dpi), uteri were removed, and the total numbers of implantations and resorption sites were counted. The resorption sites were identified by their small size and were characterized by necrotic cells and a hemorrhagic appearance compared with normal embryos and placenta. The percentage of abortions was calculated as the ratio of resorption sites to total implantation sites (resorptions plus normal implantation sites).

Adoptive Transfer Experiment
For adoptive transfer studies, CD4⁺CD25⁺ T cells were isolated from spleen cells or placenta and uterine cells from normal pregnant mice on gd 14 using magnetic activated cell sorting (MACS) following the instructions by the manufacturer (Miltenyi Biotech, Germany). Single-cell suspensions were separately prepared from spleens or placental and uterine tissues by cutting the tissues into small pieces and filtering through a 48-µm sterile nylon mesh. The mononuclear cells were isolated by Ficoll density gradient centrifugation. CD4⁺CD25⁺ T cells were negatively selected by staining with a cocktail of biotin-conjugated antibodies (monoclonal anti-mouse antibodies against: CD8α, CD11b, CD45R, CD49b, and Ter-119) and anti-Biotin MicroBeads (Miltenyi Biotech, Germany), followed by 2 times of positive selection by staining with anti-CD25-PE and anti-PE MicroBeads. The purity of the preparations was >95% in all experiments. Purified CD4⁺CD25⁺ T cells were centrifuged for 10 minutes at 300 g and then were labeled with 15 µM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) in 1640 medium without serum in the dark for 15 minutes under growth conditions. Cells were washed twice in media containing 10% fetal bovine serum (FBS), centrifuged for 10 minutes at 300 g, resuspended in PBS, counted, and diluted to 10⁶ cells per 1 mL. Pregnant mice infected with T. gondii at gd 8 were intravenously treated with 2 × 10⁶ freshly isolated CD4⁺CD25⁺ T cells in 200 µL sterile PBS at gd 9. The control mice were injected intravenously with an equal volume of sterile PBS. The abortion rates were analyzed at gd 14.

Flow Cytometry
Single-cell suspensions were separately prepared from spleens and placental and uterine tissues by cutting the tissues into small pieces and filtering through a 48-µm sterile nylon mesh. The mononuclear cells were isolated by Ficoll density gradient centrifugation.

The following fluorophore-conjugated mouse monoclonal antibodies (mAbs) were used in the assays: PerCP-conjugated anti-CD4 mAb, PE-conjugated anti-CTLA-4 mAb, and PE-conjugated anti-PD-1 mAb (all from BD Pharmingen, USA), and APC-conjugated anti-Foxp3 (eBioscience, USA). Isolated mononuclear cells were incubated with anti-CD4 mAb, anti-CTLA-4 mAb, and anti-PD-1 mAb at 4°C in the dark for 30
minutes and then were washed twice. For intracellular staining of Foxp3, the cells were fixed and permeabilized in 1 × Fix/Perm buffer (eBioscience, USA) for 30 minutes according to the manufacturer’s instructions. After washing 3 times with PBS, the cells were resuspended for analysis by flow cytometry. For annexin V and propidium iodide (PI) staining, cells were stained using the Annexin V-FITC/PI Stain Kit from KeyGEN Biotech (China) at 4°C in the dark for 15 minutes. In parallel, the corresponding isotypes were used as controls. Samples were analyzed in a BD FACSARia flow cytometer (Becton Dickinson, USA) by using BD FACSDiva 7.0 software (Becton Dickinson).

Pathology Assessment
Mice were killed at 6 days post-intraperitoneal infection. The placentas of pregnant mice were removed and immediately fixed in 4% paraformaldehyde in PBS overnight. Samples were washed with running water and placed in 70% ethanol until paraffin processing using standard methods. Paraffin embedded sections were cut at 4.5 μm and stained with hematoxylin and eosin (H & E). Sections were examined at 40 × magnification.

Cytokine Assays
Total protein extracts from fresh placental tissues were lysed and centrifuged at 12,000 g at 4°C for 30 minutes. Supernatants were collected and cytokine concentrations were determined using enzyme-linked immunosorbent assay (ELISA) kits for TGF-β, IL-10, and IFN-γ (all from R&D Systems) according to the manufacturer’s protocols. A standard curve was generated using recombinant cytokine for each assay. Each sample was analyzed in triplicate.

Data Analysis and Statistics
All data are presented as means ± SD. Statistical analyses were performed using the SPSS 16.0 statistical software package. Unpaired t-tests were used to compare 2 independent groups. One-way ANOVA was used for comparison of the 3 independent groups. P values of <.05 or <.01 were considered as significant or very significant, respectively.

RESULTS

T. gondii Infection Induces Adverse Pregnancy Outcomes in Mice
In the infected group, the pregnant mice were droopy, shambly, and had erect fur. Most fetuses and placentas were bloodless and smaller in the infected group compared to the uninfected pregnant group. Hemorrhages were obvious in the placentas of the infected group but not placentas from normal mice (Figure 1A and 1B). The abortion rate of the infected group was significantly higher, and the weights of placentas and fetuses from infected mice were significantly less than those from uninfected mice (Figure 1C).

The frequencies of Tregs in total lymphocytes and absolute numbers of Tregs were decreased both at fetal-maternal interfaces and in spleens of infected mice compared with those from the control group (Figure 1D and 1E). Tregs from fetal-maternal interfaces and spleens of infected mice expressed higher amounts of annexin V, an apoptotic marker, than those from the normal controls (Figure 1F and 1G).

CTLA-4 and PD-1 are Down-regulated upon T. gondii Infection
CTLA-4 and PD-1 levels were evaluated on Tregs from fetal-maternal interface and from spleen. As shown in Figure 2, the levels of CTLA-4 and PD-1 on Tregs from fetal-maternal interface were higher than those from spleen in normal pregnant mice (Figure 2A and 2B). The absolute numbers of the CTLA-4+ Tregs, and PD-1+ Tregs at fetal-maternal interface and those of PD-1+ Tregs in spleen were down-regulated in the infected group compared with controls (Figure 2C), although the percentage of CTLA-4+ Tregs and PD-1+ Tregs in total Tregs was up-regulated slightly at fetal-maternal interfaces and spleens in T. gondii-infected vs uninfected mice (Figure 2D).

Cytokine Levels at the Fetal-Maternal Interface Differ in Infected and Uninfected Mice
ELISAs were used to investigate the levels of TGF-β, IL-10, and IFN-γ in placental supernatants. The levels of TGF-β and IL-10 were decreased and levels of IFN-γ were increased in infected group compared to the uninfected group (Figure 3A). The ratios of IL-10/IFN-γ and TGF-β/IFN-γ were also decreased in the infected group compared to the uninfected group (Figure 3B).

Adoptive Transfer of Tregs From Fetal-Maternal Interface Improves Pregnancy Outcomes in Mice Infected With T. gondii
We hypothesized that the reduction of Tregs results in the increased in adverse pregnancy outcomes observed in mice infected with T. gondii. To test this, we isolated Tregs from fetal-maternal interfaces and spleens of normal pregnant mice and transferred them into T. gondii-infected pregnant mice. In infected mice treated with Tregs from the fetal-maternal interface, there was a significant improvement of the mouse behavior, a decrease in placental hemorrhage, and an increase in fetus weights compared with untreated infected mice (Figure 4A and 4C). There were no significant differences in these parameters between infected mice given Tregs from spleens of normal pregnant mice and the untreated infected mice (Figure 4A and 4B). Additionally, Tregs transferred from the fetal-maternal interface reduced the damage to placentas and levels of hemorrhaging induced by T. gondii infection compared to the untreated infected mice (Figure 4A–C), whereas Tregs transferred from spleen did not. The abortion rate decreased, and weights of placentas and fetuses were increased in the group treated with Tregs transferred from fetal-maternal interface compared to the untreated infected group (Figure 4D).
Figure 1. Toxoplasma gondii infection induces adverse pregnancy outcomes in mice. A, Normal pregnant mice were full of vitality and their fetuses and placenta developed normally. B, The infected mice were lethargic and their fetus and placenta were small in size and had poor blood supplies. Representative placenta from an infected mouse stained with H & E showed obvious hemorrhaging (black arrow) compared to the placenta from normal group. C, Abortion rates and the weights of placenta and fetuses were determined at 6 days post infection. The abortion rate was calculated as ratio of resorption sites to the total number of implantation sites. D, Dot plots illustrate CD4 and Foxp3 staining of Tregs from normal pregnant mice and infected pregnant mice at the fetal-maternal interfaces and in the spleens. E, The absolute numbers of CD4⁺Foxp3⁺ Tregs at fetal-maternal interfaces and in spleens of normal pregnant mice and infected pregnant mice. F, Comparative analysis of the apoptosis of CD4⁺Foxp3⁺ Treg cells at the fetal-maternal interface and in spleen during pregnancy with or without T. gondii infection. G, Data shown are means ± SD from groups of 6 mice assayed individually. The asterisks indicate statistically significant differences, as determined by the unpaired t-test (**P<.01). NP indicates normal pregnant mouse, and IP indicates infected pregnant mouse. Abbreviation: SD, standard deviation.
Tregs Transferred From Fetal-Maternal Interface Alter Cytokine Balance of Recipients

To explore whether transferred Tregs from different origins have different distributions, the isolated Tregs were stained with CFSE and the percentage of CFSE<sup>+</sup> Tregs in all Tregs at the fetal-maternal interface and in the spleen were equivalent.

Figure 2. CTLA-4 and PD-1 are down-regulated upon Toxoplasma gondii Infection. A, Histograms represent the percentage of CTLA-4<sup>+</sup> Tregs and PD-1<sup>+</sup> Tregs in total Tregs at fetal-maternal interfaces and in spleens of normal pregnant mice. The expression of CTLA-4 and PD-1 on Tregs is higher at the fetal-maternal interface than in spleen. Numbers in quadrants refer to the percentage of each subset. B, Data are shown as means ± SD (**P < .01). C, The absolute number of CTLA-4<sup>+</sup> Tregs and PD-1<sup>+</sup> Tregs at fetal-maternal interfaces and in spleens of normal pregnant mice and infected pregnant mice. D, Histograms represent the percentage of CTLA-4 and PD-1 expression on Tregs from normal pregnant mice and infected pregnant mice at fetal-maternal interfaces and in spleens. Data are given as means ± SD from groups of 6 mice assayed individually (**P < .01). Abbreviation: SD, standard deviation.

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Levels of TGF-β, IL-10, and IFN-γ at fetal-maternal interface abnormal after *Toxoplasma gondii* infection. A, TGF-β and IL-10 levels were both decreased and IFN-γ levels were increased in infected group compared to the normal group. B, The ratios of TGF-β/IL-10 and IL-10/IFN-γ decreased in the infected group relative to the normal group. ELISAs were performed to quantify cytokine levels in placenta at 6 days post infection. Each sample was assayed in triplicate (**P < .01). Abbreviations: ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon γ; IL-10, interleukin 10; TGF-β, transforming growth factor.

whether the transferred Tregs came from the spleens of fetal-maternal interface of donor mice (Figure 5A).

In order to explore the effect of Tregs transferred from fetal-maternal interface and spleen on recipients, we analyzed the changes of CTLA-4⁺ Tregs and PD-1⁺ Tregs in recipients by flow cytometry. The absolute numbers of CTLA-4⁺ Tregs and PD-1⁺ Tregs were significantly increased at fetal-maternal interface and in spleens of infected mice treated with Tregs from fetal-maternal interface compared to untreated, infected mice. Mice treated with Tregs transferred from spleens had significantly lower increases in numbers of CTLA-4⁺ and PD-1⁺ Tregs than did those given Tregs from fetal-maternal interface (Figure 5B).

In infected mice given Tregs from fetal-maternal interface, the levels of TGF-β and IL-10 were increased significantly relative to levels in untreated, infected mice, but the levels of IFN-γ did not change significantly. In infected mice with Tregs transferred from spleen, TGF-β, IL-10 and IFN-γ levels were all up-regulated significantly relative to infected controls (Figure 5C). Further, we found that the ratio of IL-10 to IFN-γ and the ratio of TGF-β to IFN-γ increased markedly in infected group treated with Tregs from fetal-maternal interface but that these did not differ from infected controls in the infected group treated with Tregs from spleen (Figure 5C).

**DISCUSSION**

Infection with *T. gondii* can cause adverse pregnancy outcomes [26]. Some studies have shown that lymphocytes and secreted cytokines ensure maternal tolerance to the persistence of paternal alloantigens during normal pregnancy [27]. Tregs also play a critical role in the maintenance of fetal-maternal tolerance during pregnancy [5]. *T. gondii*-infected mice have significantly higher levels of abortion, hemorrhage of the placenta, and lower weight fetuses than uninfected controls. The percentages and total numbers of Tregs were significantly decreased at fetal-maternal interface and spleen in pregnant mice infected with *T. gondii* infection compared with uninfected animals in agreement with our previous research [23]. This result suggested that *T. gondii* infection induces a decline in the Treg population that may contribute to abnormal pregnancy outcomes during *T. gondii* infection. In accordance with a previous study [28], we found that there were higher levels of apoptosis in spleens of infected mice than in spleens of controls. Interestingly, we also found that the Treg apoptosis was increased at fetal-maternal interface in infected group relative to levels in uninfected mice. Thus, during *T. gondii* infection, Treg apoptosis is enhanced and the resulting decrease in Treg numbers likely results in the observed up-regulation in cytotoxicity of maternal activated lymphocytes toward fetuses.

Tregs exert their immune-suppressive function through surface-bound inhibitory molecules CTLA-4 and PD-1 and secreted immune-suppressive cytokines TGF-β and IL-10 [11–14]. CTLA-4, PD-1, and the cytokines TGF-β and IL-10 are beneficial during pregnancy; levels of these molecules are abnormally low in women who suffer from recurrent spontaneous abortions [14, 17, 18, 21]. IFN-γ is necessary in the peri-implantation period but is detrimental to the development of placenta and fetus when present at high levels [22, 29]. In this study, we found that the absolute numbers of CTLA-4⁺ Tregs and PD-1⁺ Tregs and the ratios of IL-10/IFN-γ and TGF-β/IFN-γ were decreased in infected mice relative to uninfected controls. These results demonstrated that the down-regulation of CTLA-4 and PD-1 expression on Tregs and the imbalance between tolerant cytokines and inflammatory cytokines all play roles in abnormal pregnancy outcomes induced by *T. gondii* infection.

It has been shown that adoptively transferred Tregs can prevent rejection of skin grafts in mice [30]. Moreover, fetal-maternal tolerance can be adoptively transferred by treating abortion-prone mice with Tregs from healthy pregnant mice [31]. In this study, we showed that pregnancy outcomes of mice infected with *T. gondii* were improved through adoptive transfer of Tregs from the fetal-maternal interface of healthy mice. The abortion rate was decreased, weights of fetuses were increased, and hemorrhaging of the placenta reduced in group treated with Tregs from the fetal-maternal interface compared with these parameters in untreated, infected mice.

The positive effect of Treg transfer may be due to the observed increase in percentages of CTLA-4⁺ Tregs and PD-1⁺ Tregs relative to all Tregs at the fetal-maternal interface from
Figure 4. Adoptive transfer of Tregs from fetal-maternal interface improves pregnancy outcomes in Toxoplasma gondii infected mice. A, Representative infected mice and their fetuses. B, Infected mice treated with Tregs transferred from spleen behaved in a manner similar to untreated infected mice. Their fetuses were small and placentas were hemorrhagic. C, Infected mice treated with Tregs from a fetal-maternal interface behaved normally and had larger fetuses and less hemorrhagic placentas than those of infected mice. Hemorrhaging was obvious by H&E staining of placentas of infected mice. Placentas of infected group treated with Tregs transferred Tregs from spleen appeared similar to those of untreated, infected mice, whereas placentas from mice treated with Tregs from the fetal-maternal interface showed less hemorrhaging than those of untreated infected mice. D, Abortion rates and the weights of placenta and fetuses were determined at 6 days postinfection. Data are expressed as the means ± SD, and each group contained 6 mice (**P < .01). N.P. indicates normal pregnant mouse; I.P. indicates mouse infected with T. gondii; I.P. + Treg from spleen indicates infected mouse treated with Treg cells from spleen of normal pregnant mouse; I.P. + Treg from fetal-maternal interface indicates infected mouse treated with Tregs from fetal-maternal interface of normal pregnant mice. Abbreviation: SD, standard deviation.
normal pregnant mice. The increase in CTLA-4 and PD-1 expression on Tregs is expected to strengthen the ability of these cells to inhibit lymphocyte activation, which will protect the fetus. We also found that the ratios of IL-10/IFN-γ and TGF-β/IFN-γ were significantly higher in infected group given Tregs from the fetal-maternal interface rather than from spleen. Tregs from fetal-maternal interface thus have a unique ability to restore the balance between tolerant cytokines and inflammatory cytokines necessary for fetal development.

The decrease in numbers and reduction in function of Tregs following *T. gondii* infection likely resulted in the adverse outcomes during pregnancy. Adoptive transfer of Tregs from the fetal-maternal interface of normal pregnant mice improved pregnancy outcomes in *T. gondii* infected mice significantly.

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**Figure 5.** Tregs transferred from fetal-maternal interface alter cytokine balance of recipients. A, The percentages of the donor CFSE+ Tregs from the fetal-maternal interface and the spleen both homed to fetal-maternal interface and spleen to approximately the same extent. B, The absolute number of CTLA-4+ Tregs and PD-1+ Tregs were determined at fetal-maternal interfaces and in spleens of hosts. Mice treated with Tregs transferred from fetal-maternal interface had significantly higher increases in numbers of CTLA-4+ and PD-1+ Tregs than did those given Tregs from spleens. C, The levels of TGF-β, IL-10, and IFN-γ in placentas and the ratios of IL-10/IFN-γ and TGF-β/IFN-γ were determined. Each sample was analyzed in triplicate (*P<.05; **P<.01). Data are presented as means ± SD and were analyzed by 1-way ANOVA. Each group contained 6 mice. Abbreviations: ANOVA, analysis of variance; IFN-γ, interferon γ; IL-10, interleukin 10; SD, standard deviation; TGF-β, transforming growth factor.
Our work provides a theoretical basis for improving pregnancy outcomes in women infected with *T. gondii* infection during early pregnancy.

**Notes**

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