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ORIGINAL ARTICLE

The Role of Decidual PD-1$^+$ Treg Cells in Adverse Pregnancy Outcomes due to *Toxoplasma gondii* Infection

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Abstract— *Toxoplasma gondii* infection during pregnancy can result in adverse pregnancy outcomes. Previously, we have reported that these outcomes are associated with the impaired function of decidual Treg cells; however, the detailed mechanisms involved were unclear. It has been reported that the suppressive capacity of Treg cells is dependent on PD-1 expression. The present study explored the role of decidual PD-1$^+$ Treg cell function in adverse pregnancy outcomes due to *T. gondii* infection. *Toxoplasma gondii*–infected pregnant mice were sacrificed on gestational day 14 and their pregnancy outcomes were observed. The expression of PD-1 on decidual Treg cells and expressions of Foxp3, CTLA-4, TGF-β, and IL-10 on decidual PD-1$^+$ and PD-1$^-$ Treg cells were determined using flow cytometry. The results showed that the expression of PD-1 on decidual Treg cells was clearly higher in the *T. gondii*–infected mice than in the normal mice. Meanwhile, the expressions of Foxp3, CTLA-4, TGF-β, and IL-10 on decidual PD-1$^+$ Treg cells were higher in the infected mice than in the normal mice. The expressions were higher in decidual PD1$^+$ Treg cells than in PD-1$^-$ Treg cells in the infected mice. However, these expressions on PD-1$^-$ Treg cells did not significantly differ between the infected and normal mice. Nonetheless, the absolute percentages of decidual PD-1$^+$ Treg cells decreased significantly in the infected mice compared with those in the normal mice. These results suggest that *T. gondii* infection mainly influences the function of decidual PD-1$^+$ Treg cells, which would result in an insufficiently immunotolerant microenvironment and consequently in adverse pregnancy outcomes.

KEY WORDS: decidual PD-1$^+$ Treg cells; *Toxoplasma gondii*; adverse pregnancy outcome; infection.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite, and as one of the TORCH pathogens, its primary infection can have unfavorable pregnancy outcomes [1]. Over the course of gestation, several decidual immune cells (DICs) and molecules are involved in maintaining the maternal–fetal tolerance. DICs include decidual NK cells, decidual regulatory T (Treg) cells, decidual macrophages, and dendritic cells [2]. Decidual Treg cells are one of the most important cell populations that induce

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pregnancy immune tolerance and maintain successful pregnancy [3, 4]. Impairment of Treg cells can result in abnormal pregnancy outcomes due to immunological incompatibility of the fetus in humans and mice [5, 6]. Our group has previously reported that the effect of *T. gondii* infection on decidual Treg cells plays an important role in adverse pregnancy outcomes [7] and that the transfer of decidual Treg cells [8] or supplementation of cytokines (TGF-β or IL-10) [9, 10] clearly ameliorates adverse pregnancy outcomes. It has been reported that the suppressive capacity of Treg cells is dependent on their PD-1 expression [11, 12] and that PD-1$^+$ Treg cells would exert a higher suppressive function than PD-1$^-$ Treg cells [13, 14]. Notably, abnormalities in PD-1$^+$ Treg cells have been found to be associated with pre-eclampsia in pre-eclamptic rats [15]. However, the role of PD-1$^+$ Treg cells in adverse pregnancy outcomes due to *T. gondii* infection remains unclear.

In addition to PD-1, the suppressive capacity of Treg cells also depends on surface CTLA-4 [16, 17] and inhibitory cytokines TGF-β and IL-10 [18, 19]. It has been reported that PD-1$^+$ Treg cells with robust expressions of CTLA-4, IL-10, and TGF-β exhibit higher suppressive function than PD-1$^-$ Treg cells [20]. Endothelial cells can induce PD-1$^+$ Treg cells to secrete higher levels of IL-10 and TGF-β and augment their suppressor capacity in the *in vitro* suppression assays [21]. The specific expression of the transcriptional regulator Forkhead winged helix protein-3 (Foxp3) has been found to be associated with the development and regulatory ability of Treg cells [22]. Reportedly, PD-1 can enhance Treg suppressive activity by promoting the expression of Foxp3 [23]. However, variations in the expressions of Foxp3, CTLA-4, IL-10, and TGF-β on decidual PD-1$^+$ Treg cells in *T. gondii*–infected mice as well as their roles in adverse pregnancy outcomes have not been well-documented.

In the present study, *T. gondii*–infected mice with adverse pregnancy outcomes were established and the expressions of Foxp3, CTLA-4, TGF-β, and IL-10 on decidual PD-1$^+$ Treg and decidual PD-1$^-$ Treg cells were determined to evaluate the role of these cells in adverse pregnancy outcomes.

MATERIALS AND METHODS

Animals

C57BL/6 mice (8–10-week-old females and 10–12-week-old males) were purchased from Centre of Experimental Animals, Slek of Shanghai (Shanghai, P.R. China). Mice were bred with sufficient food and water sterilized by autoclaving, under conditions of controlled temperature (20–24 °C) and humidity (40–60%) under a 12-h light/12-h dark cycle. Female and male mice were allowed to mate in the evening. The next morning, females with vaginal plugs were defined as being at day 0 of gestation (0 gd) and were randomly segregated to the infected or control group. All experiments were performed in accordance with the ethical standards formulated by the Institutional Animal Experimental Ethics Committee of Binzhou Medical University. The animal protocols were approved by the Institutional Animal Care Committee of Binzhou Medical University.

Infection and Pregnancy Outcomes

The *T. gondii* tachyzoite RH strain was maintained in HEp-2 cells in MEM. Tachyzoites for the experiments were prepared by centrifugation of the cell culture supernatant and resuspension of the parasites in phosphate-buffered solution (PBS). The experimental pregnant mice were i.p. inoculated with 400 tachyzoites in 200 μl of sterile PBS on 8 gd, and the control group was inoculated with 200 μl of sterile PBS. At 6 days post-infection (6 dpi), the mice were sacrificed by cervical dislocation and their uteri were removed. Implantation sites with a shrunken placenta and a dissolved or discolored embryo were defined as sites of abortion. The number of implantation and abortion sites was counted. The abortion rate was calculated as the number of abortion sites relative to the total number of implantation sites. The total weight of the litters was also evaluated, and the weight of each fetus was determined.

Flow Cytometric Analysis

Single-cell suspensions were prepared from placentas and uteri by dissecting the tissue into small pieces, grinding and filtering them through sterile nets, and then subjecting them to centrifugation using mouse lymphocyte separation liquid (TBD Tianjin Hao Yang, China). First, the cells were incubated with anti-CD4-PE-Cy7 (GK1.5; eBioscience, USA), anti-PD-1-BV421 (J43; BD, USA), and anti-CTLA-4-APC (UC10-4B9; eBioscience, USA) in the dark for 30 min at 4 °C and then stained with anti-Foxp3-PE (NRRF-30; eBioscience, USA) for 30 min at room temperature after fixation and permeabilization in 1× Fix/Perm buffer (eBioscience, USA) for 30 min at room temperature, in accordance with the manufacturer's instructions. After washing twice with PBS, the cells were resuspended and

analyzed with a FACS flow cytometer equipped with the CellQuest software (BD, USA).

To determine the expression of TGF-β on decidual Treg cells, the cells were first stimulated by leukocyte activation cocktail (BD, USA) and incubated at 37 °C for 4 h. Subsequently, the cells were collected and incubated with anti-CD4-PE-Cy7 (GK1.5; eBioscience, USA) and anti-PD-1-APC (J43; BD, USA) in the dark for 30 min at 4 °C and then stained with anti-Foxp3-PE (NRRF-30; eBioscience, USA), anti-TGF-β-FITC (TW7-16B4; BioLegend, USA), and anti-IL-10-FITC-BV421 (JES5-16E3; BD, USA) for 30 min at room temperature after fixation and permeabilization for 30 min at room temperature. The cells were then washed and resuspended in PBS and subjected to flow cytometry analysis on a FACSCalibur instrument (BD Biosciences).

Scanning Electron Microscopy

For scanning electron microscopy (SEM) analysis, fetuses were removed from the mice on 14 gd, washed in PBS, and fixed by immersion in 2.5% phosphate-buffered glutaraldehyde at 4 °C for 48 h. After immobilization, fetuses were placed on polylysine-coated glass coverslips and dehydrated in a graded ethanol series up to 100% (50%, 70%, 85%, 95%, and 100%); they were immersed for 10 min at each step. Then, the fetuses were dried using the critical point technique (Quorum K850). Thereafter, they were attached to specimen holders, sputter-coated with gold particles, and analyzed under a scanning electron microscope (EVO LS15; Zeiss) operated at 10 kV. Images were obtained at a magnification of × 15 using the Smart SEM user interface software.

Statistical Analysis

Data are presented as mean ± standard error. Statistical analyses were performed using the SPSS 13 statistical software. Unpaired Student's *t* test was used to examine the significance of differences between the two groups. Findings were considered significant or very significant when p values were < 0.05 or < 0.01, respectively.

RESULTS

Adverse Pregnancy Outcomes due to *T. gondii* Infection

At 6 dpi, the normal mice appeared energetic and exhibited normal activities, whereas mental and behavioral disorders, arched back, and erected fur were noted in the infected mice (Fig. 1a). The fetuses and placentas of the normal mice were pink and had normal blood supply, whereas the fetuses of the infected mice were necrotic and hemorrhagic and some were deformed. The placentas of the infected mice had poor blood supply and were smaller in size than those of the normal mice. The abortion rate was significantly higher in the infected group than in the normal group (Fig. 1b), and the weights of the fetuses were significantly lower in the infected group than in the normal group (Fig. 1c). SEM revealed deformed paws, flat eyes, malformed skull, and closed fontanel in the fetuses of the infected mice, whereas the fetuses of the normal mice developed normally (Fig. 1d).

Absolute Percentages of PD-1$^+$ Treg Cells Among Total Decidual CD4$^+$ T Cells Were Significantly Decreased After *T. gondii* Infection

To evaluate the role of PD-1$^+$ Treg cells in *T. gondii* infection, the expression of PD-1 on decidual Treg cells was detected using flow cytometry (Fig. 2a). The results showed that the MFI and percentage of PD-1 expression on decidual Treg cells were clearly increased in the infected mice compared with those in the normal mice (Fig. 2b, c). However, the absolute percentages of PD-1$^+$ Treg cells and total Treg cells among the total decidual CD4$^+$ T cells were both significantly decreased in the infected mice compared with those in the normal mice (Fig. 2d, e).

Change in Foxp3 Expression on Decidual PD-1$^+$ Treg Cells After *T. gondii* Infection

To estimate the function of decidual PD-1$^+$ Treg cells, the expression of Foxp3 was determined (Fig. 3a, b). The results showed that the MFI of Foxp3 in total decidual Treg cells was significantly increased in the infected mice compared with that in the normal mice (Fig. 3c). The MFI of Foxp3 in PD-1$^+$ Treg cells was higher in the infected mice than in the normal mice. In the infected mice, the MFI of Foxp3 was higher in PD-1$^+$ Treg cells than in PD-1$^-$ Treg cells. However, this MFI in PD-1$^-$ Treg cells showed no significant difference between the two groups (Fig. 3d). Although the percentage of Foxp3$^+$PD-1$^+$ Treg cells among decidual Treg cells increased significantly (Fig. 3e), the absolute percentage of Foxp3$^+$PD-1$^+$ Treg cells among CD4$^+$T cells decreased significantly in the infected mice compared with that in the normal mice (Fig. 3f).

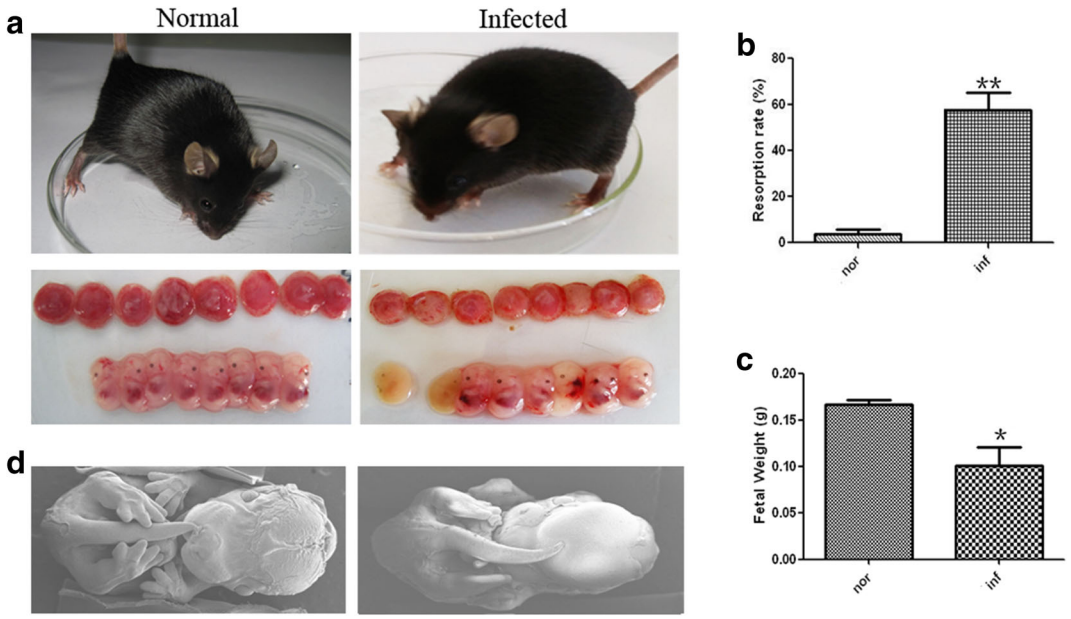

Fig. 1. Adverse pregnancy outcomes due to *T. gondii* infection. **a** Images of normal and *T. gondii*–infected mice with their fetuses and placentas. **b** The abortion rates of normal and *T. gondii*–infected mice were calculated on day 14 of gestation. **c** The weights of fetuses were determined after they were removed from the uteri. **d** The development of fetuses was observed using scanning electron microscopy. Data are expressed as the mean ± standard error from six mice assayed individually for each group. $*p < 0.05$ and $**p < 0.01$ as determined by Student's *t* test.

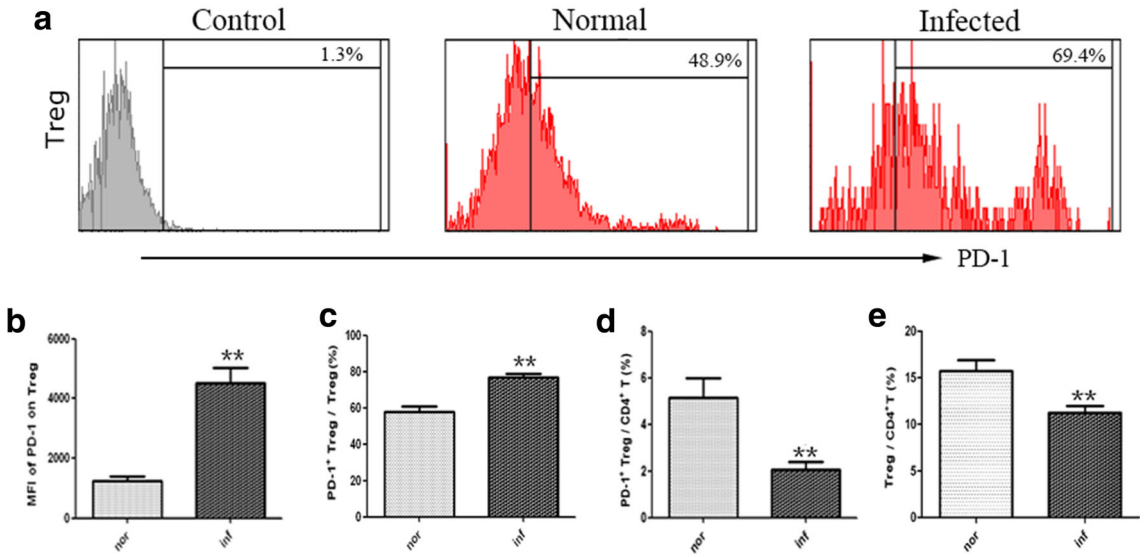

Fig. 2. Expression of PD-1 on decidual Treg cells after *T. gondii* infection. **a** Histogram plots gated on decidual $CD4^+Foxp3^+$ Treg cells showing the percentages of PD-1 expression in infected and normal mice. The **b** MFI and **c** percentages of PD-1 expression on decidual Treg cells in the normal and infected groups. The percentages of **d** $PD\text{-}1^+$ Treg cells and **e** total decidual Treg cells among decidual $CD4^+$ T cells in the normal and infected groups. Data are expressed as the mean ± standard error from six mice assayed individually for each group. $*p < 0.05$ and $**p < 0.01$ as determined by Student's *t* test.

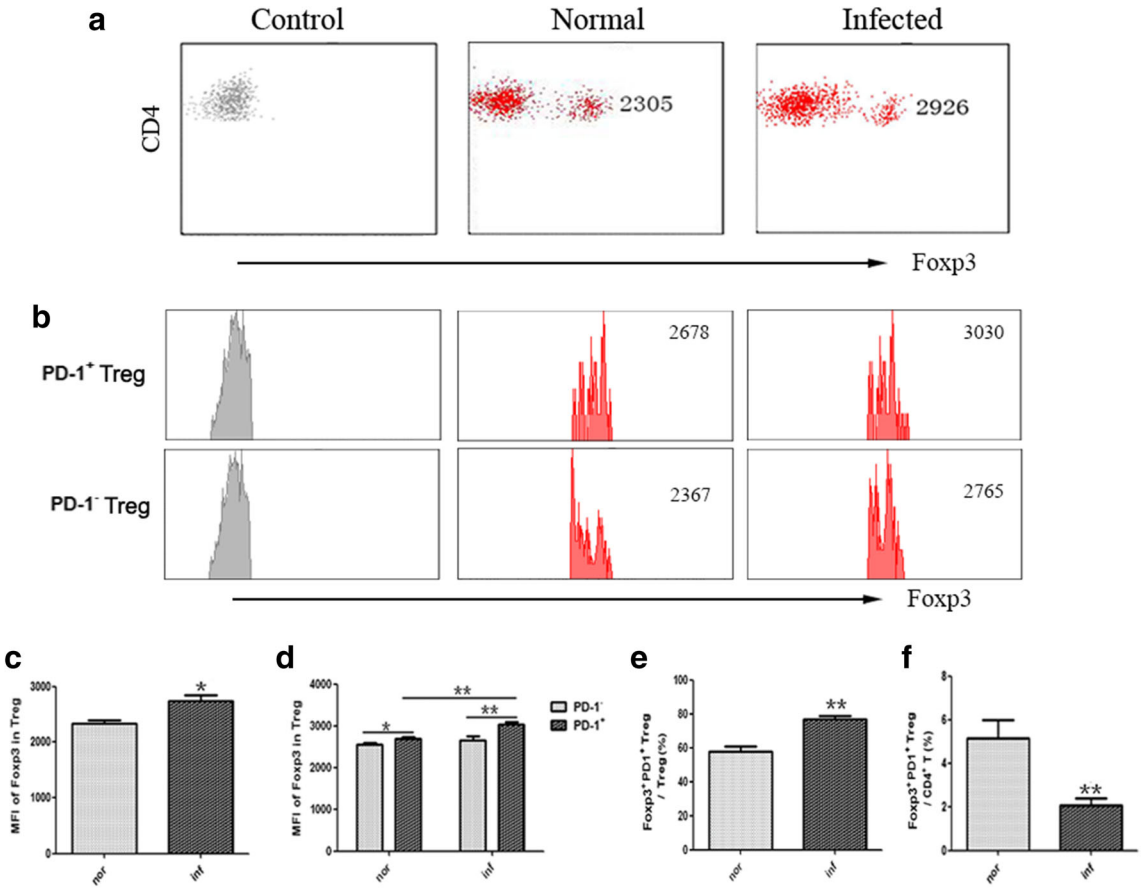

Fig. 3. Expression of Foxp3 on decidual PD-1$^+$ Treg and PD-1$^-$ Treg cells in *T. gondii*–infected mice. **a** FACS plots gated on decidual CD4$^+$ T cells showing the MFI of Foxp3 in decidual Treg cells of infected and normal mice. **b** FACS plots gated on decidual PD-1$^+$ Treg and PD-1$^-$ Treg cells showing the MFI of Foxp3 in infected and normal mice. The MFI of Foxp3 in **c** decidual Treg cells and in **d** PD-1$^+$ Treg and PD-1$^-$ Treg cells was analyzed in infected and normal mice. **e** The percentage of Foxp3$^+$PD-1$^+$ Treg cells among decidual Treg cells and **f** the absolute percentage of Foxp3$^+$PD-1$^+$ Treg cells among the total decidual Treg cells in decidual CD4$^+$ T cells were analyzed in infected and normal mice. Data are expressed as the mean ± standard error from six mice assayed individually for each group. *$p < 0.05$ and **$p < 0.01$ as determined by Student's *t* test.

Changes in CTLA-4 Expression on Decidual PD-1$^+$ Treg Cells After *T. gondii* Infection

The expression of the surface CTLA-4 on decidual PD-1$^+$ Treg and PD-1$^-$ Treg cells was determined using flow cytometry (Fig. 4a). The percentage of CTLA-4 expression on PD-1$^+$ Treg cells increased in the infected mice compared with that in the normal mice. In the infected mice, the percentage of CTLA-4 expression increased in PD-1$^+$ Treg cells compared with that in PD-1$^-$ Treg cells; however, the percentage of CTLA-4 expression on PD-1$^-$ Treg cells showed no significant difference between the two groups (Fig. 4b). The absolute percentages of decidual CTLA-4$^+$PD-1$^+$ Treg and CTLA-4$^+$PD-1$^-$ Treg cells among CD4$^+$T cells were significantly lower in the infected mice than in the normal mice; notably, the extent of the decrease was much greater in decidual CTLA-4$^+$PD-1$^+$ Treg cells than in decidual CTLA-4$^+$PD-1$^-$ Treg cells (Fig. 4c).

Variations in TGF-β and IL-10 Expressions on Decidual PD-1$^+$ Treg Cells After *T. gondii* Infection

The main inhibitory cytokines TGF-β and IL-10 were detected in decidual PD-1$^+$ Treg and decidual PD-1$^-$ Treg cells, respectively, using flow cytometry (Figs. 5a and 6a). The results showed that the MFI (Figs. 5b and 6b) and percentages (Figs. 5c and 6c) of both the cytokines in PD-1$^+$ Treg cells were significantly higher in the infected mice than in the normal mice. In the infected mice, the MFI and

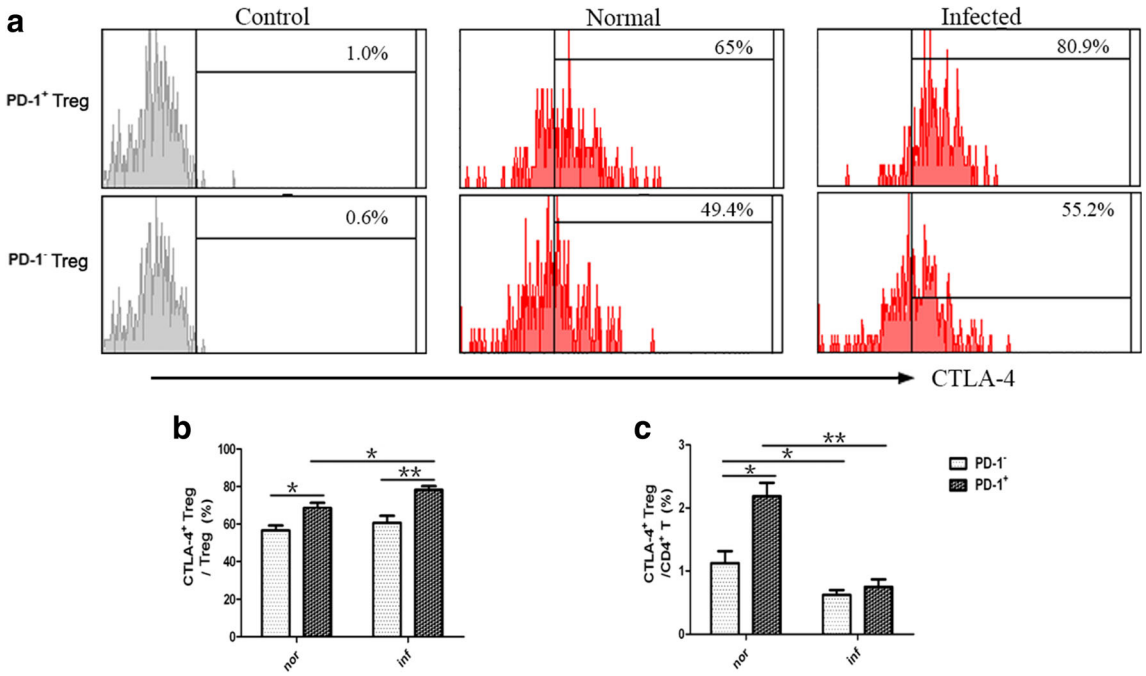

Fig. 4. Expression of CTLA-4 on decidual PD-1$^+$ Treg and PD-1$^-$ Treg cells in *T. gondii*–infected mice. **a** Histogram plots of CTLA-4 expression gated on decidual PD-1$^+$ Treg and PD-1$^-$ Treg cells of infected and normal mice. **b** The percentages of CTLA-4 expression on PD-1$^+$ Treg cells and PD-1$^-$ Treg cells in the normal and infected groups. **c** The percentages of CTLA-4$^+$PD-1$^+$ Treg and CTLA-4$^+$PD-1$^-$ Treg cells among total decidual CD4$^+$ T cells in the normal and infected groups. Data are expressed as the mean ± standard error from six mice assayed individually for each group. $*p < 0.05$ and $**p < 0.01$ as determined by Student's *t* test.

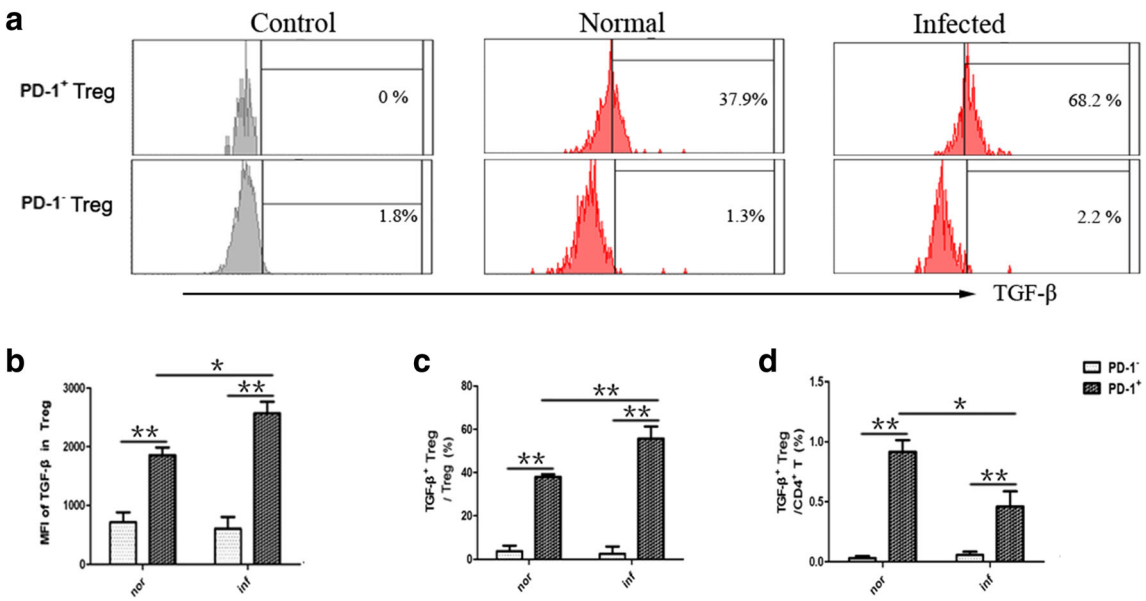

Fig. 5. Expression of TGF-β on decidual PD-1$^+$ Treg and PD-1$^-$ Treg cells in *T. gondii*–infected mice. **a** Respective histogram plots of TGF-β expression gated on decidual PD-1$^+$ Treg and PD-1$^-$ Treg cells in infected and normal mice. The **b** MFI and **c** percentage of TGF-β expression on decidual PD-1$^+$ Treg and PD-1$^-$ Treg cells of infected and normal mice. **d** The percentages of TGF-β$^+$PD-1$^+$ Treg and TGF-β$^+$PD-1$^-$ Treg cells among total decidual CD4$^+$ T cells in the infected and normal groups. Data are expressed as the mean ± standard error from six mice assayed individually for each group. $*p < 0.05$ and $**p < 0.01$ as determined by Student's *t* test.

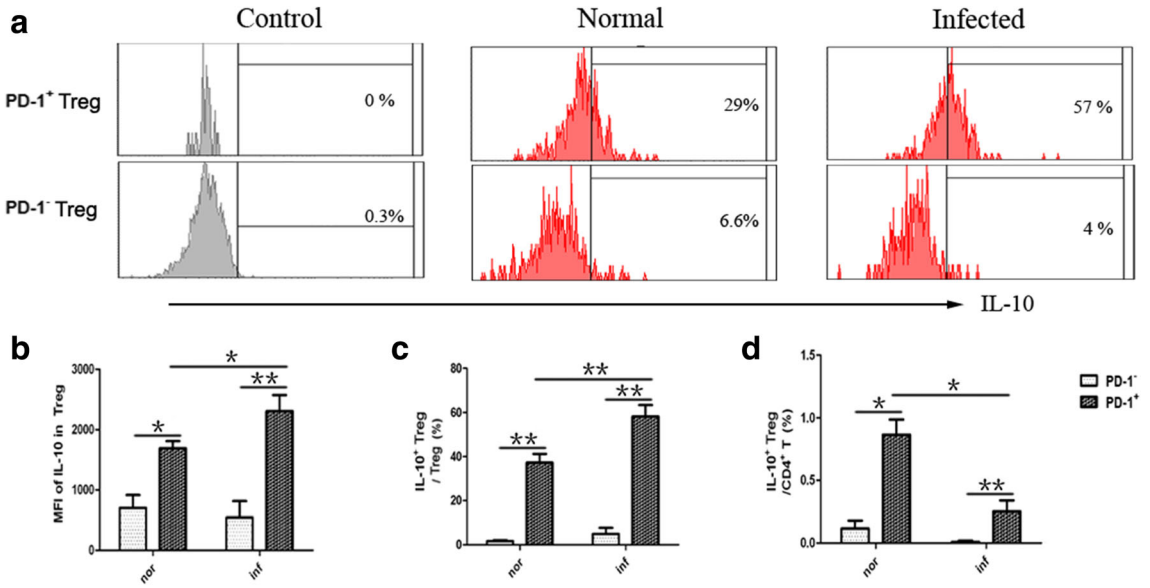

Fig. 6. Expression of IL-10 on decidual PD-1$^+$ Treg cells and PD-1$^-$ Treg cells in *T. gondii*–infected mice. **a** Histogram plots of IL-10 expression gated on decidual PD-1$^+$ Treg and PD-1$^-$ Treg cells in infected and normal mice. The **b** MFI and **c** percentage of IL-10 expression on decidual PD-1$^+$ Treg and PD-1$^-$ Treg cells of infected and normal mice. **d** The percentages of IL-10$^+$PD-1$^+$ Treg and IL-10$^+$PD-1$^-$ Treg cells among total decidual CD4$^+$ T cells in the infected and normal groups. Data are expressed as the mean ± standard error from six mice assayed individually for each group. *$p < 0.05$ and **$p < 0.01$ as determined by Student's *t* test.

percentages of both the cytokines were significantly higher in PD-1$^+$ Treg cells than in PD-1$^-$ Treg cells; however, the MFI and percentages did not significantly differ in PD-1$^-$ Treg cells between the two groups. The absolute percentages of TGF-β$^+$PD-1$^+$ Treg and IL-10$^+$PD-1$^+$ Treg cells among decidual CD4$^+$T cells were significantly decreased in the infected group compared with those in the normal group, and the absolute percentages of TGF-β$^+$PD-1$^-$ Treg and IL-10$^+$PD-1$^-$ Treg cells did not significantly differ between the two groups (Figs. 5d and 6d).

DISCUSSION

Accumulating evidence has indicated that PD-1 plays an immunoinhibitory function in normal pregnancy as a negative regulator of immune responses [24, 25]. It has also been reported that the suppressive capacity of Treg cells depends on their PD-1 expression [11, 12]. The blockade of PD-1 significantly abrogates Treg-mediated fetal protection, suggesting its importance in Treg-mediated suppression of the maternal–fetal tolerance [26]. The abnormal expression of PD-1 on Treg cells has shown to be associated with pre-eclampsia [15]. PD-1$^+$ Treg cells were reported to exert a higher suppressive function than PD-1$^-$ Treg cells [14]. They also play an important role in maintaining normal pregnancy given that the majority of Treg cells in the peripheral blood of healthy pregnant women were found to be positive for PD-1 [24]. Our previous studies have found that *T. gondii* infection can result in the abnormal expression of PD-1 on total decidual Treg cells and impairment of decidual Treg cell function, which contributes to adverse pregnancy outcomes [8]. However, the role of PD-1$^+$ Treg cells in adverse pregnancy outcomes due to *T. gondii* infection is still unclear. In the present study, the infected pregnant mice were successfully established and used to explore the role of PD-1$^+$ Treg cells in abnormal pregnancy outcomes during *T. gondii* infection. The results of flow cytometry showed that the percentage of PD-1 expression on decidual Treg cells was clearly increased in the infected mice compared with that in the normal mice, suggesting that the PD-1$^+$ subset of Treg cells plays an important role in abnormal pregnancy during *T. gondii* infection. Meanwhile, the MFI of PD-1 on decidual Treg cells was also significantly higher in the infected mice than in the normal mice, suggesting that the immunosuppressive function of decidual PD-1$^+$ Treg cells is enhanced during *T. gondii* infection. However, the absolute percentages of PD-1$^+$ Treg cells among the total decidual CD4$^+$ T cells were significantly

decreased in the infected mice, which resulted in a sharp decline in the inhibitory ability of Treg cells at the maternal–fetal interface and then contributed to adverse pregnancy outcomes.

As a specific transcriptional regulator, Foxp3 is associated with the development and regulatory ability of Treg cells [22]. It has been reported that only Foxp3$^+$ Treg cells possess immunoinhibitory capacity [27, 28]. During normal pregnancy, the expression of Foxp3 was reported to be significantly higher in deciduae than in the endometrium of nonpregnant women [29], whereas a decrease in the expression of Foxp3 was reportedly associated with primary infertility in humans [30]. PD-1 can enhance the suppressive activity of Treg cells by promoting the expression of Foxp3 [31]. To evaluate the immunosuppressive function of decidual PD-1$^+$ Treg cells in *T. gondii*–infected mice, the expression of Foxp3 was determined using flow cytometry. The results showed that the MFI of Foxp3 in decidual Treg cells was significantly higher in the infected mice than in the normal mice. The MFI of Foxp3 in decidual PD-1$^+$ Treg cells increased significantly, which contributed to the significantly increased expression of Foxp3 on decidual Treg cells after *T. gondii* infection, but the MFI of Foxp3 in PD-1$^-$ Treg cells showed no significant difference between the two groups. This suggested that *T. gondii* infection mainly influences the suppressive activity of PD-1$^+$ Treg cells but not of PD-1$^-$ Treg cells. The MFI of Foxp3 was higher in PD-1$^+$ Treg cells than in PD-1$^-$ Treg cells in the infected mice, thus suggesting that PD-1$^+$ Treg cells, and not PD-1$^-$ Treg cells, play important roles in adverse pregnancy outcomes due to *T. gondii* infection. Taken together, these results also indicate that *T. gondii* infection mainly influences the suppressive activity of PD-1$^+$ Treg cells, which in turn contributed to adverse pregnancy outcomes.

CTLA-4 is one of the inhibitory molecules on the Treg cell surface [16]. The expression of CTLA-4 has been shown to be associated with the suppressive capacity of Treg cells [32]. Moreover, the expression of CTLA-4 was found to be increased in normal pregnancy, and the blockade of CTLA-4 has shown to increase the abortion rate in mice [33]. In this study, the percentages of CTLA-4 expression on PD-1$^+$ Treg cells, but not on PD-1$^-$ Treg cells, were increased in the infected mice compared with those in the normal mice, suggesting that the function of PD-1$^+$ Treg cells is influenced by *T. gondii* infection. The percentage of CTLA-4 expression on PD-1$^+$ Treg cells was higher than on PD-1$^-$ Treg cells in the infected mice, which suggested that PD-1$^+$ Treg cells exert more powerful immunosuppressive functions than that exerted by PD-1$^-$ Treg cells during *T. gondii* infection. However, the absolute percentage of decidual CTLA-4$^+$PD-1$^+$ Treg cells decreased significantly after infection. This indicated that *T. gondii* infection downregulates the immunosuppressive function of decidual CTLA-4$^+$PD-1$^+$ Treg cells on the maternal–fetal surface and impairs the immunotolerant microenvironment, thus contributing to adverse pregnancy outcomes.

Treg cells can also exert an inhibitory activity by secreting cytokines such as TGF-β and IL-10 [18, 19], which are pivotal in the development of the maternal–fetal tolerance [17, 34]. It was also reported that PD-1$^+$ Treg cells can produce more TGF-β and IL-10 than can PD-1$^-$ Treg cells to inhibit allograft rejection [20, 21]. The results of the present study showed that the expressions of TGF-β and IL-10 on PD-1$^+$ Treg cells, but not on PD-1$^-$ Treg cells, were upregulated significantly after *T. gondii* infection, which indicated that such infection affects the ability of decidual PD-1$^+$ Treg cells, but not of PD-1$^-$ Treg cells, to secrete cytokines in response to the abnormal secretion of TGF-β and IL-10. The expressions of TGF-β and IL-10 were much higher on PD-1$^+$ Treg cells than on PD-1$^-$ Treg cells in the infected mice, suggesting that PD-1$^+$ Treg cells, but not PD-1$^-$ Treg cells, play an important role during *T. gondii* infection by upregulating the secretion of TGF-β and IL-10. However, the absolute percentages of both TGF-β$^+$PD-1$^+$ Treg and IL-10$^+$PD-1$^+$ Treg cells were decreased significantly after infection. This indicated that *T. gondii* infection downregulates the immunosuppressive function of decidual TGF-β$^+$PD-1$^+$ Treg and IL-10$^+$PD-1$^+$ Treg cells, weakens the maternal–fetal immunotolerance, and consequently contributes to adverse pregnancy outcomes.

In this paper, the results indicate that *T. gondii* infection significantly upregulates the suppressive function of PD-1$^+$ Treg cell population and the percentage of PD-1$^+$ Treg cell in total decidual Treg cells. Meanwhile, *T. gondii* infection significantly downregulates the number of whole decidual Treg cell population. So, the absolute number of PD-1$^+$ Treg cells is decreased actually. After *T. gondii* infection, the higher function of PD-1$^+$ Treg cells could not counteract their reduced number, which is responsible for the weakened immune suppressive function at maternal–fetal interface, and consequently contributes to adverse pregnancy outcomes.

In conclusion, this study demonstrated that decidual PD-1$^+$ Treg cells, rather than PD-1$^-$ Treg cells, play an important role at the maternal–fetal interface during *T. gondii* infection. This infection mainly influences the expressions of the functionally associated molecules Foxp3, CTLA-4, TGF-β, and IL-10 on decidual PD-1$^+$ Treg cells,

which decreases the immunosuppressive function of decidual PD-1$^+$ Treg cells, thus leading to adverse pregnancy outcomes. We believe that our study provides new insights into the immune mechanism associated with adverse pregnancy outcomes due to *T. gondii* infection.

FUNDING INFORMATION

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COMPLIANCE WITH ETHICAL STANDARDS

All experiments were performed in accordance with the ethical standards formulated by the Institutional Animal Experimental Ethics Committee of Binzhou Medical University. The animal protocols were approved by the Institutional Animal Care Committee of Binzhou Medical University.

Conflict of Interest. The authors declare that they have no conflicts of interest.

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