Original article

CCR5 is Involved in Interruption of Pregnancy in Mice Infected with *Toxoplasma gondii* During Early Pregnancy

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Abstract

Toxoplasmosis can cause abortion in pregnant humans and other animals; however, the mechanism of abortion remains unknown. C-C chemokine receptor type 5 (CCR5) is essential for host defense in *T. gondii* infection. To investigate the relationship between CCR5 and abortion in toxoplasmosis, we inoculated wild-type and CCR5-deficient ($CCR5^{-/-}$) mice with *T. gondii* tachyzoites intraperitoneally on day 3 of pregnancy (E3). The pregnancy rate decreased as pregnancy progressed in the infected wild-type mice. Histopathologically, no inflammatory lesions were observed in the fetoplacental tissues. Although the wild-type mice showed a higher parasite burden at the implantation sites than the $CCR5^{-/-}$ mice at E6 (3 days post-infection, dpi), *T. gondii* antigen was detected only in the uterine tissue, not in the fetoplacental tissues. At E8 (5 dpi), the embryos in the infected wild-type mice showed poor development compared with the infected $CCR5^{-/-}$ mice and apoptosis was observed in poorly developed embryos. Compared with the uninfected mice, the infected wild-type mice showed increased CCR5 expression at the implantation site at E6 and E8. Furthermore, analyses of mRNA expression in the uterus of non-pregnant and pregnant mice suggested that lack of the *CCR5* gene and downregulation of TNF-α and CCL3 expression at E6 (3 dpi) are important factors for maintenance of pregnancy following *T. gondii* infection. These results suggested that CCR5 signaling is involved in embryo loss in *T. gondii* infection during early pregnancy, and that apoptosis is associated with embryo loss rather than direct damage to the fetoplacental tissues.

INTRODUCTION

Toxoplasmosis is a worldwide zoonosis caused by the protozoan parasite, *Toxoplasma gondii*. *T. gondii* can infect humans and other warm-blooded animals and can cause embryonic death and resorption, fetal death, abortion and stillbirth during pregnancy [1, 2, 3]. The outcome of *T. gondii* infection during pregnancy is thought to depend on the stage of the pregnancy when the infection is contracted; however, the mechanism of abortion and fetal death remains unknown.

Changes in hormones and immune dynamics are closely associated with the maintenance of pregnancy, and hormones such as progesterone and estrogen, which are necessary during pregnancy, can modulate immune cell functions [4, 5]. During *T. gondii* infection, the T helper 1 (TH1) immune response that results in production of interferon-gamma (IFN-γ) or interleukin-12 (IL-12) plays an important role in host defenses, but production of IFN-γ in pregnant mice has been reported to be less than that in non-pregnant mice during infection with *T. gondii* [6]. IL-12 production is reduced by a high progesterone concentration, indicating that downregulation of IL-12 and the TH1 immune response seems to be related to *T. gondii* susceptibility during pregnancy [7]. Additionally, some studies have suggested that *T. gondii* infection, particularly with the type II strains that show moderate virulence in a murine host, induced apoptosis of human trophoblasts that was associated with an increase in IFN-γ [8, 9]. *T. gondii* infection during early pregnancy induced IFN-γ production or inflammation and this was associated with apoptosis in mouse decidual cells, thereby resulting in fetal resorption [10, 11].

C-C chemokine receptor type 5 (CCR5) is a TH1-associated chemokine receptor, and the main CCR5 ligands are chemokine (C-C motif) ligand 3 (CCL3), CCL4 and CCL5 (also call RANTES). In addition to host ligands, secreted *T. gondii* cyclophilin 18 (TgCyp18) can bind to CCR5 [12, 13]. Recombinant TgCyp18 has been reported to enhance RANTES expression in macrophages and control their migration [14, 15]. CCR5 is essential for controlling infection with *T. gondii*; CCR5-deficient ($CCR5^{-/-}$) mice showed high susceptibility to infection, severe tissue damage, low expression of IFN-γ and IL-12, and higher parasite loads compared with wild-type mice [16].

Additionally, RANTES is implicated as a physiological tolerogenic factor in successful implantation [17]. RANTES production is induced by progesterone and can induce apoptosis of maternal $CD3^{+}$ lymphocytes [17, 18]. The frequency of T cell apoptosis was significantly lower in recurrent spontaneous abortion patients than in fertile women [17]. Furthermore, epigenetically inadequate expression of chemokine genes, including the gene encoding RANTES, limits T cell access to the decidua [19].

T. gondii infection in pregnant animals can cause embryonic death, resorption, fetal death, abortion and congenital transmission. Infection with *T. gondii* during early pregnancy has more severe consequences (e.g., decreased offspring survival rates and increased parasite transmission rates) than when it is contracted later in pregnancy [3, 20]. Although the mechanism of abortion caused by *T. gondii* infection is poorly understood, disturbance of many different factors, such as alternations in the immune response and hormone balance, may be associated with embryonic resorption and abortion. CCR5 and RANTES have been reported to be associated with the immune responses required for

host defense against *T. gondii* and a successful pregnancy. In this study, we investigated the role played by CCR5 in abortion caused by *T. gondii* infection using $CCR5^{-/-}$ mice.

RESULTS

Pregnancy rates decreased over time in wild-type mice, but not in CCR5$^{-/-}$ mice, following infection with *T. gondii*

In our preliminary experiments, inoculation of *T. gondii* PLK tachyzoites (5×10^3) could induce clinical signs including weight decrease and rough coat in non-pregnant wild-type mice and CCR5$^{-/-}$ mice, and induced loss of embryos in wild-type mice inoculated at E3, but not in CCR5$^{-/-}$ mice inoculated at E3. Moreover, both groups of mice inoculated with *T. gondii* tachyzoites (5×10^3) at E7 did not induce loss of embryos, when euthanized at E18. Thus, to reveal when embryo loss occurred in wild-type mice, both groups of mice inoculated at E3 were euthanized at E6, E8 and E10 (Fig. 1). The pregnancy rate was 100% in both wild-type (6/6) and CCR5$^{-/-}$ (6/6) groups at E6 (Fig. 1B). However, the rate decreased as pregnancy progressed in the wild-type mice infected with *T. gondii*, and decreased significantly at E10 (1/5, 25%) compared with that at E6 (6/6, 100%) (Fig. 1B). By contrast, the pregnancy rate did not decrease at E8 (5/6, 83.3%) and E10 (6/7, 85.7%) in CCR5$^{-/-}$ mice (Fig. 1B). Although the average numbers of implantation sites were 8.7±1.0 and 7.5±1.3 at E6 and E8, respectively, in wild-type mice, and no normal implantation sites were observed at E10 (Fig. 1A, C). Only one of five wild-type mice exhibited four implantation sites and they were all degenerated. By contrast, there were no significant differences in the number of implantation sites in the CCR5$^{-/-}$ mice at each gestational stage (the average numbers of implantation sites were 8.8±1.0, 9.4±1.7 and 7.8±1.3 at E6, E8 and E10, respectively) (Fig. 1C). These results showed that the loss of an embryo was not due to inhibition of implantation in wild-type mice infected with *T.*

gondii at E3. Furthermore, it was suggested that infection of *T. gondii* at E3 could affect embryos in wild-type mice after E8 and almost all embryos were lost at E10, whereas pregnancy was largely maintained in infected CCR5$^{-/-}$ mice.

Wild-type mice had a lower percentage of developed embryos and a higher percentage of embryo loss compared with CCR5$^{-/-}$ mice

To evaluate the morphological changes and distribution of parasites, implantation sites were analyzed histopathologically and immunohistochemically (Fig. 2). Histopathological changes were most prominent in the *T. gondii*-infected mice at E10. Although only one wild-type mouse had an implantation site at E10 (Fig. 1C), all of the fetoplacental tissues showed necrosis (Fig. 2A). By contrast, many normal-appearance embryos similar to those of the uninfected mice were observed in the CCR5$^{-/-}$ mice (Fig. 2A). In the uterine wall and adipose tissue attached with uterus, there was mild to moderate inflammatory cell infiltration from mononuclear inflammatory cells and neutrophils, and there was evidence of parasitic infection in both groups of mice by immunohistochemistry (Fig. 2B). However, no inflammatory changes and no parasite antigens were detected in the fetoplacental tissues from either of the groups (Fig. 2B). These results suggested that loss of embryos was not induced directly by parasite proliferation or inflammation.

Although inflammatory changes in the uterine wall and adipose tissue were slight to mild in both groups at E6 and E8, there were variations in the growth stage and size of embryonic tissues in the *T. gondii*-infected mice from both groups at E8 (Fig. 3). The

embryonic tissues were classified into the three following categories: (group A) embryos with some structures such as a head fold and amnion, as was observed for many of the embryos from the uninfected control mice at E8; (group B) small embryos with an irregularly folded appearance; (group C) no embryonic tissue in the decidual tissue. Both groups of control mice showed a relatively high percentage of developed embryos (groups A and B) and there was no significant difference in embryo development between wild-type and $CCR5^{-/-}$ mice. The infected wild-type mice had a lower percentage of developed embryos (group A) and a statistically significant higher percentage of embryo loss (group C) compared with the infected $CCR5^{-/-}$ mice. These results suggested that the infected wild-type mice had poorly developed embryos compared with the infected $CCR5^{-/-}$ mice.

To evaluate whether apoptosis at the implantation site could be associated with the poor development of embryos, a TUNEL assay was performed for the implantation sites at E8. In the TUNEL assay, a positive signal was observed around the embryos in the infected and uninfected mice from both groups. When compared with control mice, the prominent signal was observed mainly in small embryos (group B) of infected mice. These results suggested the possibility that apoptosis of fetoplacental tissues may be associated with poor development or loss of embryos in mice infected with *T. gondii*.

Wild-type mice showed higher implantation site parasite burdens compared with $CCR5^{-/-}$ mice

To elucidate whether the number of parasites was associated with poor development or

loss of embryos, the parasite load at implantation sites was determined by quantitative PCR (Fig. 4). The number of parasites found in the implantation sites increased as pregnancy progressed in both wild-type and CCR5$^{-/-}$ mice. Moreover, the parasite load in wild-type mice was significantly higher than that in the CCR5$^{-/-}$ mice at E6 (3 dpi). Although higher parasite load was detected in wild-type mice at E10 (7 dpi), this result was obtained from only one pregnant mouse (Table 1). In non-pregnant mice, there were no significant differences in parasite burden of the uterus between wild-type and CCR5$^{-/-}$ mice at 3 dpi and 7 dpi (Fig. S1). These results suggested that more parasites exist in the implantation sites of pregnant wild-type mice compared with CCR5$^{-/-}$ mice. Although parasite numbers increased at the implantation sites of both types of mice as the pregnancy progressed, we were unable to differentiate whether this was due to migration or proliferation of the parasite. Thus, the parasite number in implantation sites at E6 (3 dpi) is a key factor of poor development or loss of embryos.

CCR5 expression levels in the implantation sites increased significantly in the wild-type mice infected with *T. gondii*

To evaluate the possible involvement of CCR5 and its ligands in embryo loss due to *T. gondii* infection, mRNA expression of CCR5, CCL3, CCL4 and RANTES (CCL5) in the implantation sites was quantitated by real-time RT-PCR (Fig. 5). CCR5 expression levels in the implantation sites increased significantly in the wild-type mice infected with *T. gondii* at E6 (3 dpi) and E8 (5 dpi) compared with those of the uninfected wild-type mice. By contrast, compared with uninfected wild-type mice, expression of CCL3 decreased in

T. gondii-infected CCR5$^{-/-}$ mice at E6, whereas CCL4 expression levels decreased in both types of infected animals. Moreover, the expression of IFN-γ and TNF-α, which are cytokines associated with apoptosis, was evaluated. Although IFN-γ expression increased in both groups of mice infected with *T. gondii* at E8, there were no significant differences among the experimental groups. Compared with uninfected wild-type mice, expression of TNF-α was downregulated in *T. gondii*-infected CCR5$^{-/-}$ mice at E6, whereas decreased expression levels of TNF-α were seen in both types of infected animals at E8. Additionally, we evaluated mRNA expression of IFN-γ, TNF-α, CCL3, CCL4, RANTES (CCL5) and CCR5 in the uterus of non-pregnant mice at 3 dpi (Fig. S2). However, there was no significant difference in mRNA expression of these target genes in the uterus of non-pregnant mice at 3 dpi. Since we observed increased expression of CCR5 in infected wild-type mice, decreased expression of TNF-α and CCL3 in infected CCR5$^{-/-}$ mice and decreased expression of CCL4 in both types of infected mice at E6, compared with uninfected pregnant wild-type mice, we suggest that these reactions may be specific to infected pregnant mice. Together, these results suggest that lack of the *CCR5* gene and downregulation of TNF-α and CCL3 expression at E6 (3 dpi) are important factors for maintenance of pregnancy following *T. gondii* infection.

CCR5$^{-/-}$ mice had higher RANTES levels than the wild-type mice

To evaluate the serum component that could affect pregnancy, the serum levels of RANTES and IFN-γ were quantified (Fig. 6). Serum RANTES levels increased in the *T. gondii*-infected CCR5$^{-/-}$ mice at E6 (3 dpi), E8 (5 dpi) and E10 (7 dpi) (Fig. 6A).

Particularly at E8 and E10, the levels were significantly higher than those of the wild-type group (Fig. 6A). High levels of serum RANTES were observed in non-pregnant CCR5$^{-/-}$ mice infected with *T. gondii* as well as infected pregnant CCR5$^{-/-}$ mice (Fig. S3). Furthermore, the serum IFN-γ level rose in both groups of infected mice to a similar level, and then increased significantly at E10 compared with uninfected pregnant mice (Fig. 6B). Similar patterns of IFN-γ levels were also observed in non-pregnant mice at 3 and 7 dpi (Fig. S3). In the case of progesterone and estradiol, there was no difference between wild-type and CCR5$^{-/-}$ mice or between infected and uninfected mice of the same strain (data not shown). These results suggested that IFN-γ and hormones involved in the maintenance of pregnancy were not associated with embryo loss in wild-type mice inoculated with *T. gondii* at E3.

DISCUSSION

Infection with *T. gondii* during early pregnancy can cause fetal resorption in mice [10, 11]. In this study, implantation sites were observed in all wild-type and CCR5$^{-/-}$ mice at E6; however, the number of mice without embryos increased as pregnancy progressed and no normal embryos were observed at E10 in the wild-type mice (Fig. 1). Bonfá *et al*. showed that CCR5$^{-/-}$ mice were susceptible to *T. gondii* oral infection (ME-49 strain) compared with wild C57BL/6 wild-type mice [16], but then Khan *et al*. reported that C57BL/6 background CCR5$^{-/-}$ mice survived longer than wild-type mice during *T. gondii* (76K strain) infection [21]. In the present study, pregnant mice were used and infection route and strain of parasite were different from those of these reports (oral infection of cyst): it is not easy to compare with those results. However, the present results suggest that CCR5 is associated with the loss of embryos following *T. gondii* infection during early pregnancy. Additionally, RANTES was induced and the levels correlated with IFN-γ levels in pregnant mice infected with the Gram-negative bacterium, *Brucella abortus*; neutralization of RANTES decreased the number of aborted fetuses in *B. abortus*-infected mice [22]. Therefore, while CCR5 is essential for the control of *T. gondii* infection, it is possible that interactions between CCR5 and RANTES during infection are associated with negative outcomes during pregnancy.

Some studies on non-pregnant mice have shown that CCR5$^{-/-}$ mice had high *T. gondii* loads in various tissues including the liver and spleen [16, 21], and that they experienced severe tissue damage compared with wild-type mice [16]. In contrast to these findings, the parasite loads in the implantation sites were significantly higher in

wild-type mice than in CCR5$^{-/-}$ mice in our study of pregnant mice (Fig. 4). Additionally, there were no significant differences in parasite loads in non-pregnant uteri between wild-type and CCR5$^{-/-}$ mice (Fig. S1). As a potential cause of the high parasite load, an increase in parasite proliferation at the implantation site or the migration of infected cells should be considered. If enhanced parasite proliferation occurs locally, prominent tissue damage is likely to be observed in wild-type mice. However, no histopathological differences were found between the wild-type and CCR5$^{-/-}$ mice in the present study (Fig. 2). CCR5 expression in the implantation sites of the wild-type mice increased at E6 and E8 (Fig. 5). Although it is not known whether the high parasite loads in the wild-type mice were uterus-specific, CCR5 may be associated with migration of *T. gondii*-infected cells to the uterus in pregnant mice. Previous studies have shown that regulation of dendritic cells and macrophage migration depends on CCR5, and that inoculation of dendritic cells or peripheral leukocytes infected with *T. gondii* enabled such cells to disseminate more rapidly than occurred following inoculation with free parasites [14, 23, 24]. However, we did not observe prominent infiltration of phagocytes into the uterus. In the presence of some pathogen associated molecular pattern stimuli (PAMPs), such as lipopolysaccharide and peptidoglycan, trophoblast cells restrained early monocyte migration and reduced CCR5 and RANTES expression in monocytes [25]. Although these PAMPs were from bacteria and viruses, PAMPs from parasites may also be associated with the restrained migration of monocytes in trophoblasts. Thus, factors other than parasite proliferation and migration of infected cells may be involved in embryo loss during early pregnancy following infection with *T. gondii*.

Our data showed that there were no significant differences in the number of implantation sites at E6 (Fig. 1C), but the number of parasites in the wild-type mice was higher than that in the $CCR5^{-/-}$ animals at E6 (Fig. 4). The number of implantation sites in the wild-type mice is likely to be lower than that of the $CCR5^{-/-}$ animals if parasite infection affected embryo survival. Therefore, these findings suggest that embryo loss is related to functional changes in the uterus or fetoplacental tissues, such as disorder of the decidual cells and trophoblasts via disturbance of the immune cells (e.g., natural killer cells, regulatory T cells (Treg) and macrophages) that are possibly involved in immune tolerance of the embryos. Furthermore, it is worth considering the role of *T. gondii* secretory molecules other than those that cause tissue damage related to parasite proliferation in the fetoplacental tissues. Senegas *et al*. detected *T. gondii* cysts in decidual tissue at 10 days post-coitum in Swiss Webster mice inoculated orally during early pregnancy; however, no necrosis was observed in the implantation sites [10]. Swiss Webster mice are relatively resistant to *T. gondii* infection and show less Th1 response including IFN-γ production and less tissue damages than C57BL/6 mice during *T. gondii* infection [26, 27]. Additionally, embryonic resorption caused by inoculation of *T. gondii* antigen during early pregnancy suggests that embryonic resorption could be related to a mechanism other than direct proliferation of *T. gondii* in the uterus [28]. Treg cells involved in maternal–fetal tolerance, and CCR5 expression in Treg cells, play an important role in the accumulation of Treg cells in the pregnant uterus [29]. Furthermore, mRNA levels of CCL4, but not RANTES, were elevated in the pregnant uterus and CCL4 attracted $CCR5^{+}$ Treg cells *in vitro*. In mice inoculated with *T. gondii* or *T.*

gondii-excreted-secreted antigens, Treg cell apoptosis was induced at the fetal–maternal interface or the spleen and fetal loss could be prevented partly by adoptive transfer of Treg cells from normal pregnant mice [28, 30, 31]. These findings suggest that a decrease of Treg function could be involved in fetal loss following *T. gondii* infection. CCR5 may be associated with both proinflammatory and anti-inflammatory reactions and a decrease in $CCR5^{+}$ Treg cells with a suppressive function may obstruct maternal–fetal tolerance or enhance proinflammatory reactions in *T. gondii*-infected mice. However, in the present study, no significant difference was observed in mRNA expression of CCL4 in fetoplacental tissues between wild-type and $CCR5^{-/-}$ mice infected with *T. gondii* (Fig. 5). Additionally, no significantly difference was observed in mRNA expression of Foxp3, a marker of Treg cells, in implantation sites between wild-type and $CCR5^{-/-}$ mice at E6 (data not shown). Thus, the role of Treg cells in embryo loss remains unclear.

Although there were no differences in parasite infection and inflammatory changes in the fetoplacental tissues between wild-type and $CCR5^{-/-}$ mice, the wild-type animals displayed poorly developed fetoplacental tissue compared with $CCR5^{-/-}$ animals at E8 (Fig. 3). In the TUNEL assay, the positive signal detected around the embryos probably included trophoblasts, and this signal was observed predominantly in small embryos indicating the involvement of apoptosis in embryo loss (Fig. 3). Apoptosis at the maternofetal interface occurs during normal implantation and gestation, and is associated with appropriate tissue remodeling of the decidua and invasion of the developing embryo. Effective clearance of apoptotic cells and cellular debris by macrophages is thought to be

important for preventing the release of intracellular contents, which may cause tissue damage and inflammatory reactions and promote production of proliferative factors for trophoblasts [32, 33]. Therefore, the imbalance between apoptosis and the clearance of apoptotic cells by macrophages may affect the establishment and maintenance of pregnancy. As described previously, TgCyp18 enhanced RANTES expression in macrophages and controls macrophage migration [14, 15]. Dense granule protein 15 (GRA15), a secretary protein of *T. gondii*, from type II parasites can activate the NF-κB pathway, and this pathway is involved in inflammation, immune responses and anti-apoptosis in various cell types including macrophages and embryonic fibroblasts *in vitro* [34]. Therefore, *T. gondii* infection can affect macrophage function via secretary proteins, and may induce functional aberrations in various types of cells at the implantation site. Furthermore, human extravillous trophoblasts displayed high apoptosis indexes after treatment with the supernatant from macrophages with or without *T. gondii* infection [35]. However, an increase in IL-6 production in monocytes treated with the supernatant from trophoblast cells suppressed *T. gondii* proliferation in monocytes *in vitro* [36]. Further investigations are required to elucidate the mechanism of embryo loss following parasite infection including the role of macrophages and *T. gondii*-derived molecules.

Senegas *et al*. reported that IFN-γ-dependent apoptosis of placental cells was involved in embryo resorption in *T. gondii*-infected mice during early pregnancy, and that serum and uterine IFN-γ levels were increased compared with the uninfected controls [10]. In the present study, the serum IFN-γ level was increased in both wild-type

and CCR5$^{-/-}$ mice; however, there was no significant difference between these groups as the pregnancies progressed (Fig. 6). Additionally, no significant differences were found in serum progesterone and estradiol levels between wild-type and CCR5$^{-/-}$ mice (data not shown). These results suggest that IFN-γ and pregnancy-associated hormones have little association with the apoptosis or embryo resorption associated with CCR5.

Expression of TNF-α and CCL3 in implantation site was significantly lower in infected and uninfected CCR5$^{-/-}$ mice than uninfected wild-type mice at E6 and both groups of mice showed significant downregulation of TNF-α at E8 in the present study. Coutinho *et al*. showed increase of serum TNF-α level and necrotic implantation site in C56BL/6 mice infected with *T. gondii* ME-49 strain: these results suggested that impaired outcome of pregnancy due to *T. gondii* infection may be associated with high TNF-α levels [11]. In contrast, Bonfá *et al*. reported the lower tissue expression of Th1 cytokines including TNF-α and extensive tissue damage in liver of CCR5$^{-/-}$ mice infected with *T. gondii* ME-49 strain compared with wild-type mice [16]: tissue expression of TNF-α does not correlate well with tissue damage in *T. gondii* infection. Although serum TNF-α level was not evaluated, there was no prominent difference in inflammatory changes in uterus between wild-type and CCR5$^{-/-}$ mice in the present study. Therefore, TNF-α is not likely to be associated with pregnancy in our study. Uptake of apoptotic cells suppresses secretion of proinflammatory cytokines including TNF-α from macrophages and promotes the release of anti-inflammatory cytokines during normal pregnancy [37]. TNF-α can inhibit interaction between trophoblast-like cells and maternal endothelial cellular networks, in vitro [38]. Although it is unclear whether

CCL3 has adverse effects except for inflammatory response in implantation site, downregulation of TNF-α and CCL3 in implantation sites may have or reflect positive effect on maintenance of pregnancy in $CCR5^{-/-}$ mice infected with *T. gondii* infection.

Interestingly, $CCR5^{-/-}$ mice showed higher levels of serum RANTES than wild-type mice (Fig. 6). Although mRNA expression of RANTES in the implantation sites increased in both infected wild-type and infected $CCR5^{-/-}$ mice at E8, no significant difference was found between these mouse groups (Fig. 5). However, CCR5 expression in the implantation sites was significantly higher in the infected wild-type mice than in the uninfected controls (Fig. 5). Although not in serum, CCR5 expression in apoptotic neutrophils and T cells was reported to clear CCL3 and RANTES in peritoneal exudates [39]. In a mouse model of proteoglycan-induced arthritis, the serum RANTES level increased significantly in $CCR5^{-/-}$ mice compared with wild-type mice [40]. This indicates that CCR5 expression in cells can control the level of circulating RANTES [40]. In our study, RANTES may have been consumed by the increased level of CCR5 in the wild-type mice infected with *T. gondii,* because RANTES expression increased in the infected wild-type mice but the serum RANTES level did not increase after the infection. Regarding the relationships between CCR5, RANTES and apoptosis, RANTES has been shown to induce apoptosis of CCR5-expressing T cell lines [41]. In trophoblast cells, anti-RANTES antibodies decreased apoptosis of a trophoblast cell line (Swain 71 cells, no expression of CCR5) in co-culture with peripheral blood mononuclear cells from recurrent spontaneous abortions [17], suggesting that CCR5-independent RANTES signaling is needed for apoptosis induction. Thus, RANTES may play a role in apoptosis

induction in embryos following *T. gondii* infection. However, the CCR5–RANTES interaction can inhibit virus-induced apoptosis of macrophages [42]. Therefore, further investigations will be needed to determine whether these findings are consistent across several cell types.

In conclusion, this study has shown that CCR5 is involved in the loss of embryos in mice infected with *T. gondii* during early pregnancy. Additionally, apoptosis around an embryo, rather than direct damage to the fetoplacental tissue from the infection, can be associated with poor embryo development and embryo loss in the presence of CCR5. Although IFN-γ seemed not to be involved in the embryo loss associated with CCR5, wild-type mice had increased levels of CCR5 and RANTES expression at the implantation site and low serum RANTES levels, indicating the possibility of an interaction between CCR5 and RANTES. In addition, our results suggested that lack of the *CCR5* gene and downregulation of TNF-α and CCL3 expression at E6 (3 dpi) may be key factors involved in maintenance of pregnancy following *T. gondii* infection. However, the existence of a direct relationship between CCR5 and apoptosis requires further investigation. Future studies will address the molecular mechanisms of embryo loss including the role of CCR5, RANTES and *T. gondii*-derived molecules in the apoptosis of fetoplacental tissue.

MATERIALS AND METHODS

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 28-57, 25-59, 24-15, 23-61). All surgery for sampling was performed under isoflurane anesthesia, and all efforts were made to minimize animal suffering.

Mice and experimental design

C57BL/6 mice were obtained from Clea Japan (Tokyo, Japan). $CCR5^{-/-}$ mice (B6 129P2-Ccr5 ^{tmlKuz}/J) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained under specific-pathogen-free conditions in the animal facility of the National Research Center for Protozoan Diseases at the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. The mice were used according to the Guiding Principles for Care and Use of Research Animals published by Obihiro University of Agriculture and Veterinary Medicine.

Virgin female mice were housed with males at 10–11 weeks of age, and when a visible vaginal plug was noted we designated this as day 0 of pregnancy (E0). Female mice of both groups were inoculated intraperitoneally with strain PLK *T. gondii* tachyzoites (5×10^3/mouse) at day E3 and then euthanized at days E6 (3 days

post-infection, dpi), E8 (5 dpi) and E10 (7 dpi) (Table 1). Blood and uteri were collected from the mice for quantitative analysis of serum IFN-γ and RANTES, and histopathological analysis. The number of implantation sites was counted. The pregnancy rate was calculated as the ratio of the number of mice with implantation sites to the total number of mice with a vaginal plug in each group. About half (E10) and half the number (E6 and E8) of implantation sites (fetoplacental tissues) were fixed with 4% paraformaldehyde solution for histopathological analysis, and of the others, half were frozen at -80°C prior to DNA and RNA extraction for quantitation of the parasite burden and real-time reverse transcriptase PCR (RT-PCR) analysis, respectively (Table 1).

Preparation of *T. gondii* tachyzoites

T. gondii (strain PLK; type II) tachyzoites were propagated in monkey kidney adherent fibroblasts (Vero cells) cultured in Eagle's minimum essential medium (EMEM, Sigma, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum. To purify the tachyzoites, parasites and host-cell debris were washed in ice cold phosphate-buffered saline (PBS), and the final pellet was re-suspended in cold PBS and passed through a 27-gauge needle and a 5.0 μm pore filter (Millipore, Bedford, MA, USA).

Histopathological and immunohistochemical analyses

After fixation with 4% paraformaldehyde solution, the tissues from the implantation site were routinely embedded in paraffin wax and prepared as 4-μm thick sections. The

sections were stained with hematoxylin and eosin.

Immunohistochemistry of *T. gondii* in the tissues from the implantation site was performed using an anti-*T. gondii* polyclonal antibody (ab15170, Abcam, Cambridge, UK). Briefly, after deparaffinization, the tissues were treated with 0.01 M citrate buffer (pH 6.0) and heated in a microwave (200 W, 5 min, twice) for antigen retrieval. After blocking of endogenous peroxidase with 3% hydrogen peroxide and nonspecific protein with 10% normal goat serum (Histofine SAB-PO Kit, Nichirei Corp, Tokyo, Japan) for 30 min at room temperature, the sections were incubated with the primary antibody (dilution 1:50) overnight at 4°C. After washing, the sections were incubated with the second antibody (EnVision™+ K4003, Dako, Burlingame, CA, USA) for 40 min at 37°C. The sections were treated with 3,3'-diaminobenzidine (DAB; Impact DAB ®, Vector Laboratories Inc., Burlingame, CA, USA) and the chromogenic reaction was stopped with H_2O. Then, the sections were counterstained with Mayer's hematoxylin.

TUNEL analysis of the implantation sites

Cell death in the implantation sites was identified by terminal dUTP nick end-labeling (TUNEL) using an In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) as recommended by the manufacturer.

DNA isolation and quantitative PCR analysis of parasite numbers

DNA from the fetoplacental tissues was extracted using TRI Reagent (Sigma). Amplification of parasite DNA was performed using primers specific for the *T. gondii*

B1 gene (5'-AAC GGG CGA GTA GCA CCT GAG GAG A-3' and 5'-TGG GTC TAC GTC GAT GGC ATG ACA AC-3'), which is present in all known strains of this parasite species [43]. The PCR mixture (25 μl) contained 1 × SYBR Green PCR Buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 400 μM dUTP, 0.625 U of AmpliTaq Gold DNA polymerase, 0.25 U of AmpErase uracil-N-glycosylase (UNG) (AB Applied Biosystems, Carlsbad, CA, USA), 0.5 μM of each primer and 50 ng of genomic DNA. Amplification was performed by a standard protocol recommended by the manufacturer (2 min at 50°C, 10 min at 95°C, 40 cycles at 95°C for 15 s, and 60°C for 1 min). Amplification, data acquisition and data analysis were carried out in an ABI 7900HT Prism Sequence Detector (AB Applied Biosystems), and the cycle threshold values (Ct) were exported to Microsoft Excel for analysis. Parasite loads were estimated by comparison with internal controls, with the level of the internal control calculated per parasite. Briefly, parasite numbers were calculated by interpolation on a standard curve, with the Ct values plotted against a known concentration of parasites. After amplification, the PCR product melting curves were acquired via a stepwise temperature increase from 60°C to 95°C. Data analyses were conducted with Dissociation Curves version 1.0 f (AB Applied Biosystems).

Real-time RT-PCR analysis

Total RNA was extracted from fetoplacental tissues with TRI Reagent (Sigma) according to the manufacturer's instructions. First-strand cDNA synthesis used an oligo (dt) primer and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCR was

performed as described above using the SYBR Green PCR system and an ABI 7700 Prism Sequence Detector instrument. The relative mRNA amounts were calculated using the $\Delta\Delta^{CT}$ method according to the manufacturer's instructions (Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PC, AB Applied Biosystems). The primer sequences (sense and antisense sequences) designed by Primer Express Software (Applied Biosystems, Foster City, CA, USA) were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense primer 5′-TGT GTC CGT CGT GGA TCT GA-3′, GAPDH antisense primer 5′-CCT GCT TCA CCA CCT TGT TGA T-3′; mouse IFN-γ sense primer 5′-GCC ATC AGC AAC AAC ATA AGC GTC-3′, mouse IFN-γ antisense primer 5′-CCA CTC GGA TGA GCT CAT TGA ATG-3′; mouse TNF-α sense primer 5′-GGC AGG TCT ACT TTG GAG TCA TTG C-3′, mouse TNF-α antisense primer 5′-ACA TTC GAG GCT CCA GTG AA-3′; mouse CCL3 sense primer 5′-CCA GCC AGG TGT CAT TTT CCT-3′, mouse CCL3 antisense primer 5′-TCC AAG ACT CTC AGG CAT TCA GT-3′; mouse CCL4 sense primer 5′-CAA CAC CAT GAA GCT CTG CG-3′, mouse CCL4 antisense primer 5′-GCC ACG AGC AAG AGG AGA GA-3′; mouse RANTES (CCL5) sense primer 5′-CCA ATC TTG CAG TCG TGT TTG T-3′, mouse RANTES (CCL5) antisense primer 5′-CAT CTC CAA ATA GTT GAT GTA TTC TTG AAC-3′; mouse CCR5 sense primer 5′-GAC ATC CGT TCC CCC TAC AAG-3′, mouse CCR5 antisense primer 5′-TCA CGC TCT TCA GCT TTT TGC AG-3′. Gene-specific expression was normalized against GAPDH housekeeping gene expression. The optimal reference gene was selected based on the Cotton EST database (http://www.leonxie.com).

Measurements of serum RANTES and IFN-γ

Serum samples were collected for measurement of RANTES and IFN-γ. Serum RANTES levels were measured with a Quantikine mouse RANTES immunoassay kit (R&D systems, Minneapolis, MN, USA). Serum IFN-γ was measured with an OptEIA mouse IFN-γ enzyme-linked immunosorbent assay (ELISA) kit (BD Bioscience, San Jose, CA, USA). Each assay was performed according to the manufacturer's instructions.

Statistical analyses

GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analyses. Data are presented as the mean ± SD. Statistical analyses were performed using the Student's *t* test and one-way analysis of variance (ANOVA), followed by the Tukey–Kramer test for group comparisons. The significance of the differences in the pregnancy rates and embryo structures was analyzed by a χ^2 test. The levels of statistical significance are presented with asterisks and are defined in each figure legend, together with the name of the statistical test that was used. A *P* value of < 0.05 was considered statistically significant.

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Table 1. Mice and experimental design

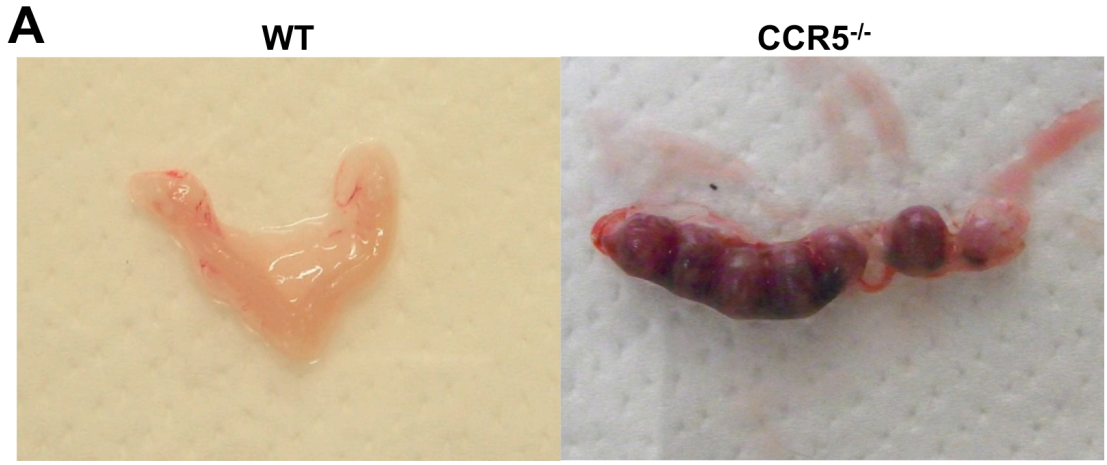
Day of pregnancy (Days post-infection, dpi)	Mouse category		Number of mice	Number of pregnant mice	Pregnancy rate (%)	Number of implantation sites	Number of tissue samples used for histopathology (Fig. 3)	Number of tissue samples used for the detection of parasite numbers (Fig. 4)	Number of tissue samples used for real-time RT-PCR analysis of mRNA (Fig. 5)	Number of serum samples used for the detection of RANTES and IFN-γ (Fig. 6)
E6 (3 dpi)	wild-type	uninfected	4	4	100	$3^{1)},2^{2)},6^{3)},9^{4)}$	$1^{1)},1^{2)},3^{3)},4^{4)}$	$2^{1)},1^{2)},0^{3)},0^{4)\dagger}$	$2^{1)},1^{2)},0^{3)},0^{4)\dagger}$	$2^{\dagger}$
		infected	6	6	100	$9^{1)},9^{2)},7^{3)},10^{4)},9^{5)},8^{6)}$	$4^{1)},4^{2)},3^{3)},5^{4)},4^{5)},4^{6)}$	$5^{1)},5^{2)},4^{3)},5^{4)},5^{5)},4^{6)}$	$5^{1)},5^{2)},4^{3)},5^{4)},5^{5)},4^{6)}$	6
	$CCR5^{-/-}$	uninfected	4	4	100	$10^{1)},6^{2)},9^{3)},10^{4)}$	$5^{1)},3^{2)},4^{3)},5^{4)}$	$5^{1)},3^{2)},0^{3)},0^{4)\dagger}$	$5^{1)},3^{2)},0^{3)},0^{4)\dagger}$	$2^{\dagger}$
		infected	6	6	100	$8^{1)},10^{2)},10^{3)},8^{4)},9^{5)},8^{6)}$	$4^{1)},5^{2)},5^{3)},4^{4)},4^{5)},4^{6)}$	$4^{1)},5^{2)},5^{3)},4^{4)},5^{5)},4^{6)}$	$4^{1)},5^{2)},5^{3)},4^{4)},5^{5)},4^{6)}$	6
E8 (5 dpi)	wild-type	uninfected	2	2	100	$8^{1)},8^{2)}$	$4^{1)},4^{2)}$	$4^{1)},4^{2)}$	$4^{1)},4^{2)}$	2
		infected	6	4	66.7	$0^{1)},9^{2)},8^{3)},0^{4)},7^{5)},6^{6)}$	$0^{1)},4^{2)},4^{3)},0^{4)},4^{5)},3^{6)}$	$0^{1)},4^{2)},4^{3)},0^{4)},3^{5)},3^{6)}$	$0^{1)},4^{2)},4^{3)},0^{4)},3^{5)},3^{6)}$	6
	$CCR5^{-/-}$	uninfected	3	3	100	$8^{1)},9^{2)},8^{3)}$	$4^{1)},4^{2)},4^{3)}$	$4^{1)},5^{2)},4^{3)}$	$4^{1)},5^{2)},4^{3)}$	3
		infected	6	5	83.3	$11^{1)},0^{2)},9^{3)},11^{4)},9^{5)},7^{6)}$	$6^{1)},0^{2)},5^{3)},6^{4)},5^{5)},4^{6)}$	$5^{1)},0^{2)}4^{3)},5^{4)},4^{5)},3^{6)}$	$5^{1)},0^{2)}4^{3)},5^{4)},4^{5)},3^{6)}$	6
E10 (7 dpi)	wild-type	uninfected	3	3	100	$9^{1)},6^{2)},9^{3)}$	$9^{1)},6^{2)},9^{3)}$	$9^{1)},6^{2)},9^{3)}$	ND	3
		infected	5	1	20	$4^{1)},0^{2)},0^{3)},0^{4)},0^{5)}$	$4^{1)},0^{2)},0^{3)},0^{4)},0^{5)}$	$4^{1)},0^{2)},0^{3)},0^{4)},0^{5)}$	ND	5
	$CCR5^{-/-}$	uninfected	3	2	66.7	$7^{1)},7^{2)},0^{3)}$	$7^{1)},7^{2)},0^{3)}$	$7^{1)},7^{2)},0^{3)}$	ND	$2^{\ddagger}$
		infected	7	6	85.7	$10^{1)},8^{2)},7^{3)},0^{4)},6^{5)},8^{6)},8^{7)}$	$10^{1)},8^{2)},7^{3)},0^{4)},6^{5)},8^{6)},8^{7)}$	$10^{1)},8^{2)},7^{3)},0^{4)},6^{5)},8^{6)},8^{7)}$	ND	7

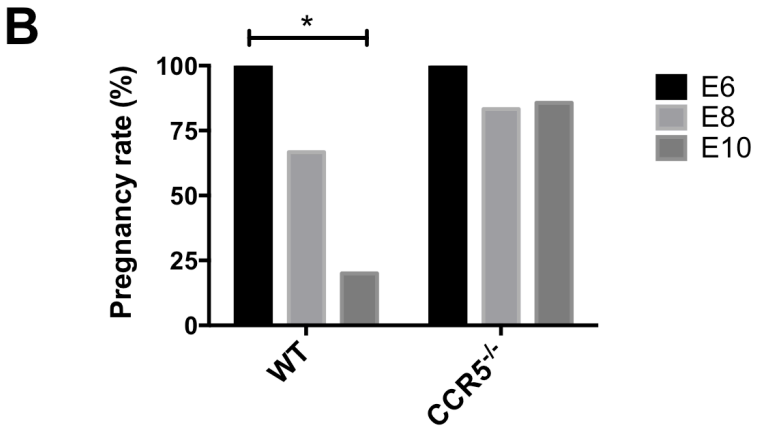
Superscript numbers denote the mouse identification number per experimental group.

† Because two of four uninfected mice in each group were added after the experimental infection, only samples collected in the first experiment were analyzed.

‡ Because no implantation sites were detected in one of the three uninfected mice, this animal was removed from consideration.

Figures


A
WT
CCR5-/-


B
*
Pregnancy rate (%)
100
75
50
25
0
WT
CCR5-/-
E6
E8
E10

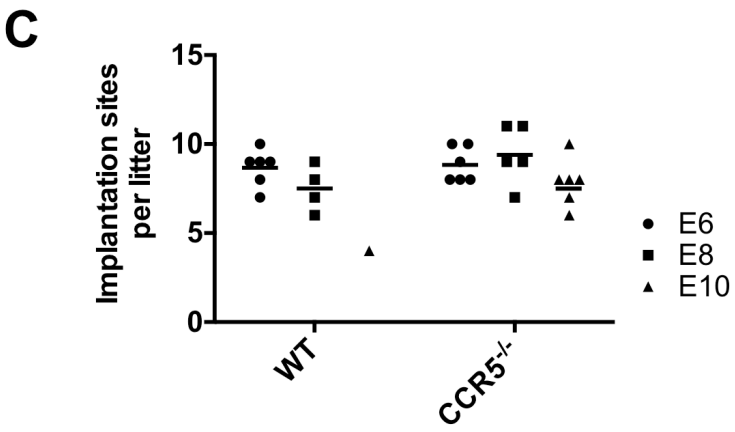

C
Implantation sites per litter
15
10
5
0
WT
CCR5-/-
E6
E8
E10

FIG 1. Uteri (A), pregnancy rate (B) and the number of implantation sites per litter (C) in *T. gondii*-infected mice. (A) Uteri of mice infected with *T. gondii* (5×10^3) at E3 and euthanized at E10. No normal implantation sites were observed in wild-type (WT) mice compared with $CCR5^{-/-}$ mice. (B) Pregnancy rate was measured from 6, 6 and 5 WT pregnant mice and 6, 6 and 7 $CCR5^{-/-}$ pregnant animals at E6, E8 and E10, respectively. (C) Number of implantation sites per litter was measured from 6, 4 and 1 WT dams and 6, 5 and 6 $CCR5^{-/-}$ dams at E6, E8 and E10, respectively. Statistical comparison was performed among different stages of pregnancy within each group of mice. * indicates significant differences as determined by the χ^2 test ($P<0.05$).

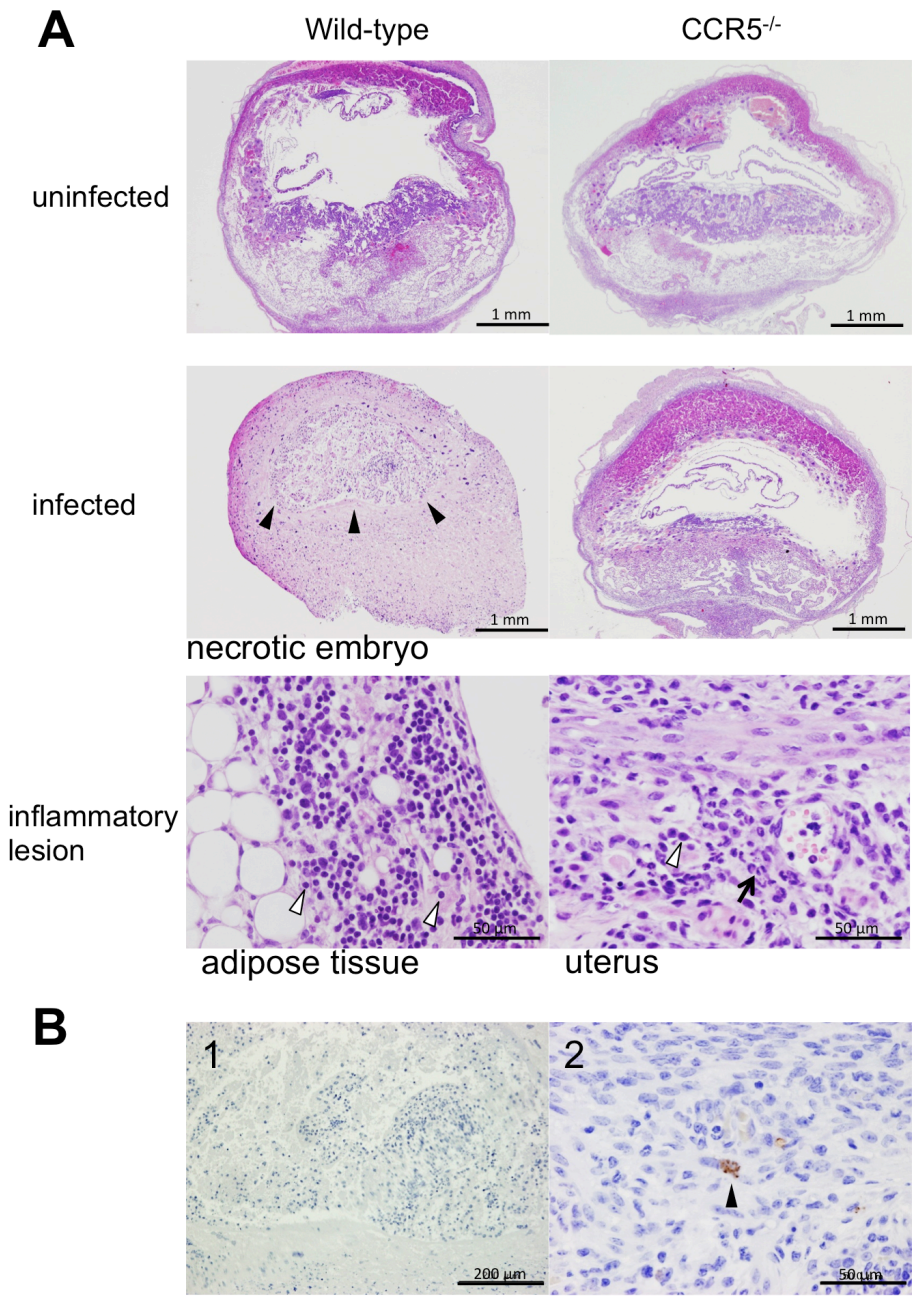
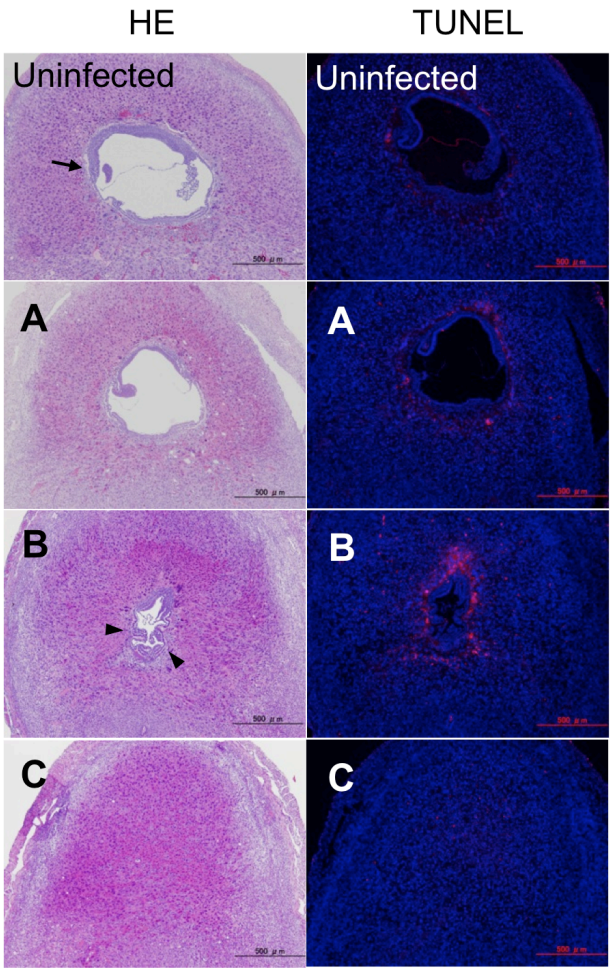

A
Wild-type
CCR5-/-
uninfected
1 mm
1 mm
infected
1 mm
1 mm
necrotic embryo
inflammatory lesion
50 µm
50 µm
adipose tissue
uterus
B
1
200 µm
2
50 µm

FIG 2. Histopathology (A) and immunohistochemistry for *T. gondii* (B) in fetoplacental tissues including uteri at E10. (A) Fetoplacental tissues consisted of embryos with cavity structure and decidua in uninfected wild-type mice. Fetoplacental tissues of uninfected and infected $CCR5^{-/-}$ mice were similar to those of uninfected wild-type mice. All embryos of wild-type mice infected with *T. gondii* showed necrosis (arrow heads). Inflammatory cell infiltration including mononuclear inflammatory cells (open arrow heads) and neutrophils (arrow) was observed in uterus and adipose tissue attached with uterus. (B) *T. gondii* antigen was detected in uterus (2) but not in embryo or decidual tissues from wild-type and $CCR5^{-/-}$ mice (1). The number of evaluated dams (embryos) were 3 (24) and 1 (4) in uninfected and infected WT mice and 2 (14) and 6 (47) in uninfected and infected $CCR5^{-/-}$ mice at E10, respectively.



	A		B		C	
	HE	TUNEL	HE	TUNEL	HE	TUNEL
WT-uninfected (n=8)	7 (87.5%)	-	1 (12.5%)	-	0 (0.0%)	-
CCR5$^{-/-}$-uninfected (n=12)	7 (58.3%)	-	3 (25.0%)	-	2 (16.7%)	-
WT-infected (n=15)	3 (20.0%)	0/3 (0%)	7 (46.7%)	4/7 (57.1%)	5 (33.3%)	1/5 (20.0%)
CCR5$^{-/-}$-infected (n=26)	17 (65.4%) *	3/17 (17.6%)	7 (26.9%)	6/7 (85.7%)	2(7.7%) *	0/2 (0%)

FIG 3. Embryos of *T. gondii*-infected mice at E8. Some structures, including the head fold (arrow), amnion or round cavity, which seemed to be the amniotic cavity, were observed in well-developed embryos of the uninfected control mice at E8. (A) Embryo that developed similarly to the well-developed embryos of the uninfected control mice. Embryos with some structures such as the head fold or amnion were considered as group A. (B) Embryos were small and folded irregularly (arrow heads). (C) No embryo was observed in the decidual tissue. The table shows the number and percentage of embryos classified into each category. A signal in the TUNEL assay was observed around the embryos in both types of mice with or without inoculation. The number of embryos with predominant signals compared with the non-infected control were counted. * indicates significant differences between infected wild-type and $CCR5^{-/-}$ groups as determined by the χ^2 test ($P<0.05$). All images are of wild-type mice. The number of dams (embryos) was 2 (8) and 4 (15) in uninfected and infected WT mice and 3 (12) and 4 (26) in uninfected and infected $CCR5^{-/-}$ mice at E8, respectively.

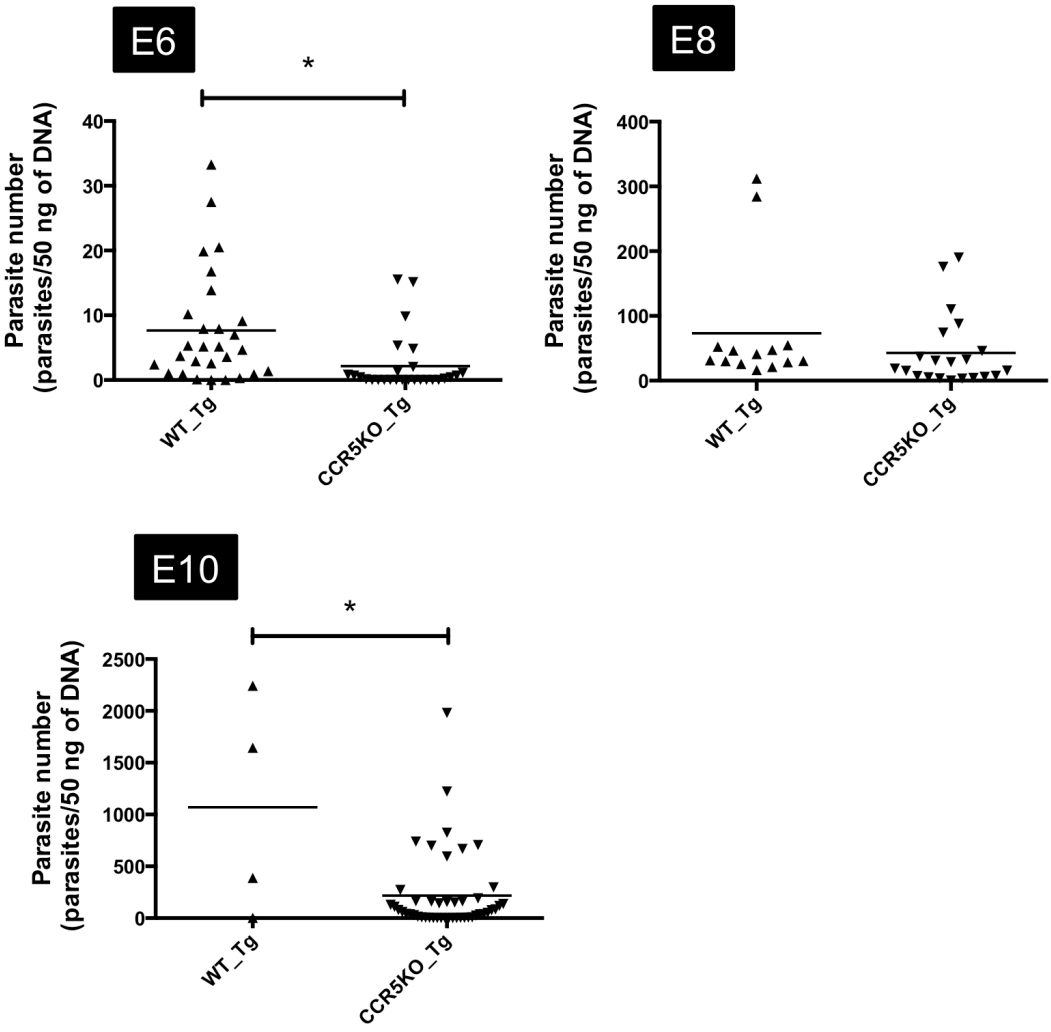

E6
*
Parasite number
(parasites/50 ng of DNA)
40
30
20
10
0
WT_Tg
CCR5KO_Tg
E8
Parasite number
(parasites/50 ng of DNA)
400
300
200
100
0
WT_Tg
CCR5KO_Tg
E10
*
Parasite number
(parasites/50 ng of DNA)
2500
2000
1500
1000
500
0
WT_Tg
CCR5KO_Tg

FIG 4. Parasite load in implantation sites of *T. gondii*-infected mice at E6, E8 and E10. Each point represents data for one mouse, and the bars represent the average value from all data points. The numbers of investigated embryos were 28, 14 and 4 in wild-type (WT) mice and 27, 21 and 47 in $CCR5^{-/-}$ (CCR5KO) mice evaluated at E6, E8 and E10, respectively. * indicates significant differences between *T. gondii*-infected wild-type and $CCR5^{-/-}$ mice as determined by the t-test ($P<0.05$).

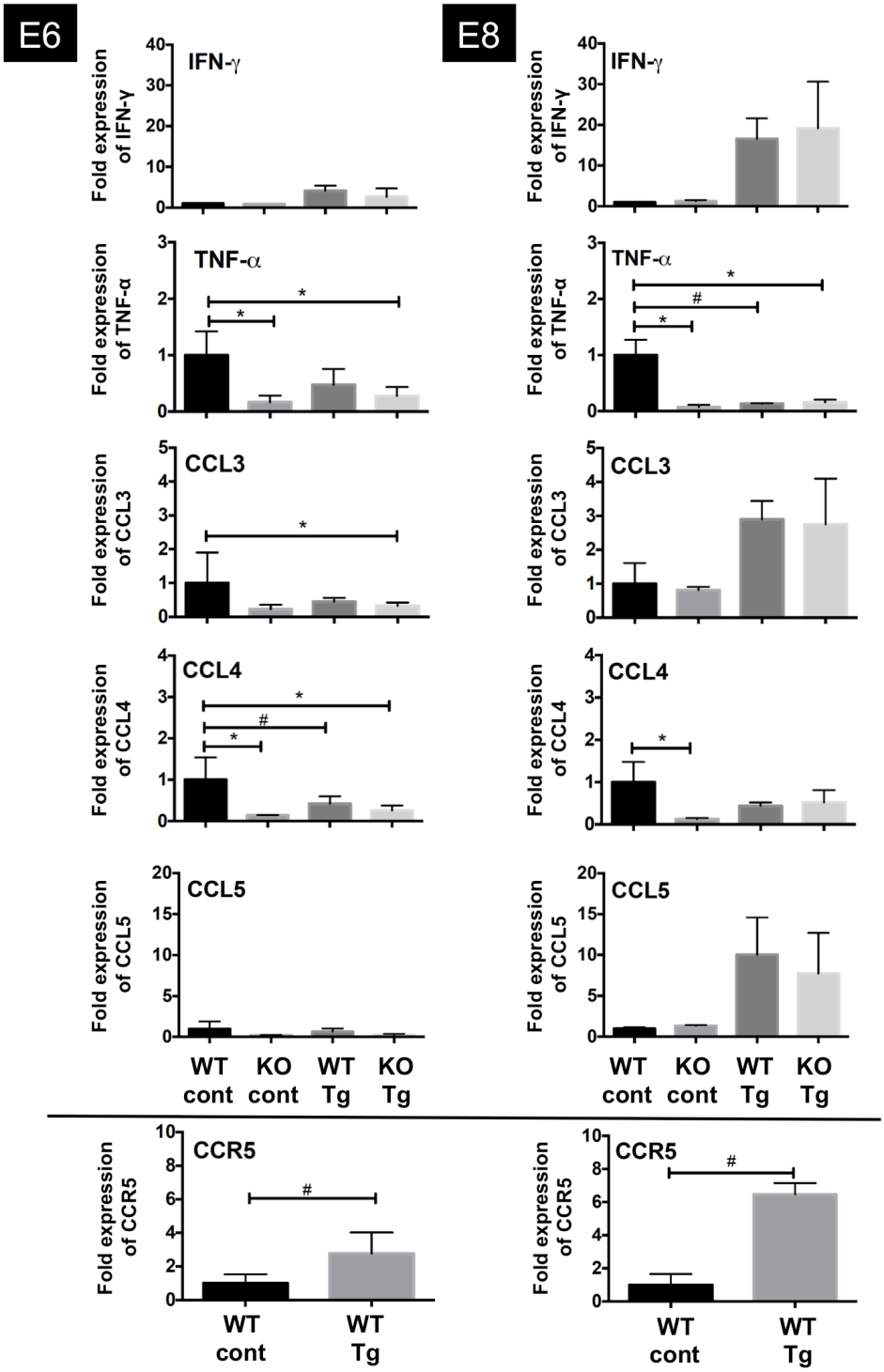

E6
E8
IFN-γ
Fold expression of IFN-γ
TNF-α
Fold expression of TNF-α
CCL3
Fold expression of CCL3
CCL4
Fold expression of CCL4
CCL5
Fold expression of CCL5
WT cont
KO cont
WT Tg
KO Tg
CCR5
Fold expression of CCR5
WT cont
WT Tg

FIG 5. Relative mRNA expression of IFN-γ, TNF-α, CCL3, CCL4, RANTES (CCL5) and CCR5 in implantation sites of *T. gondii*-infected wild-type and $CCR5^{-/-}$ mice at E6 and E8. Wild-type mice (WT), $CCR5^{-/-}$ mice (KO), uninfected (cont), *T. gondii*-infected (Tg). Data are the mean values ± SD. * and # indicates a significant difference as determined by one-way ANOVA plus Tukey–Kramer post-hoc analysis for four experimental groups and by the t-test for two experimental groups ($P<0.05$). * and # indicates between animals of different and same lineages, respectively. The numbers of investigated implantation sites were 3, 8, 28 and 27 at E6 and 8, 13, 14 and 21 at E8 in WT_cont , KO cont, WT_Tg and KO_Tg, respectively.

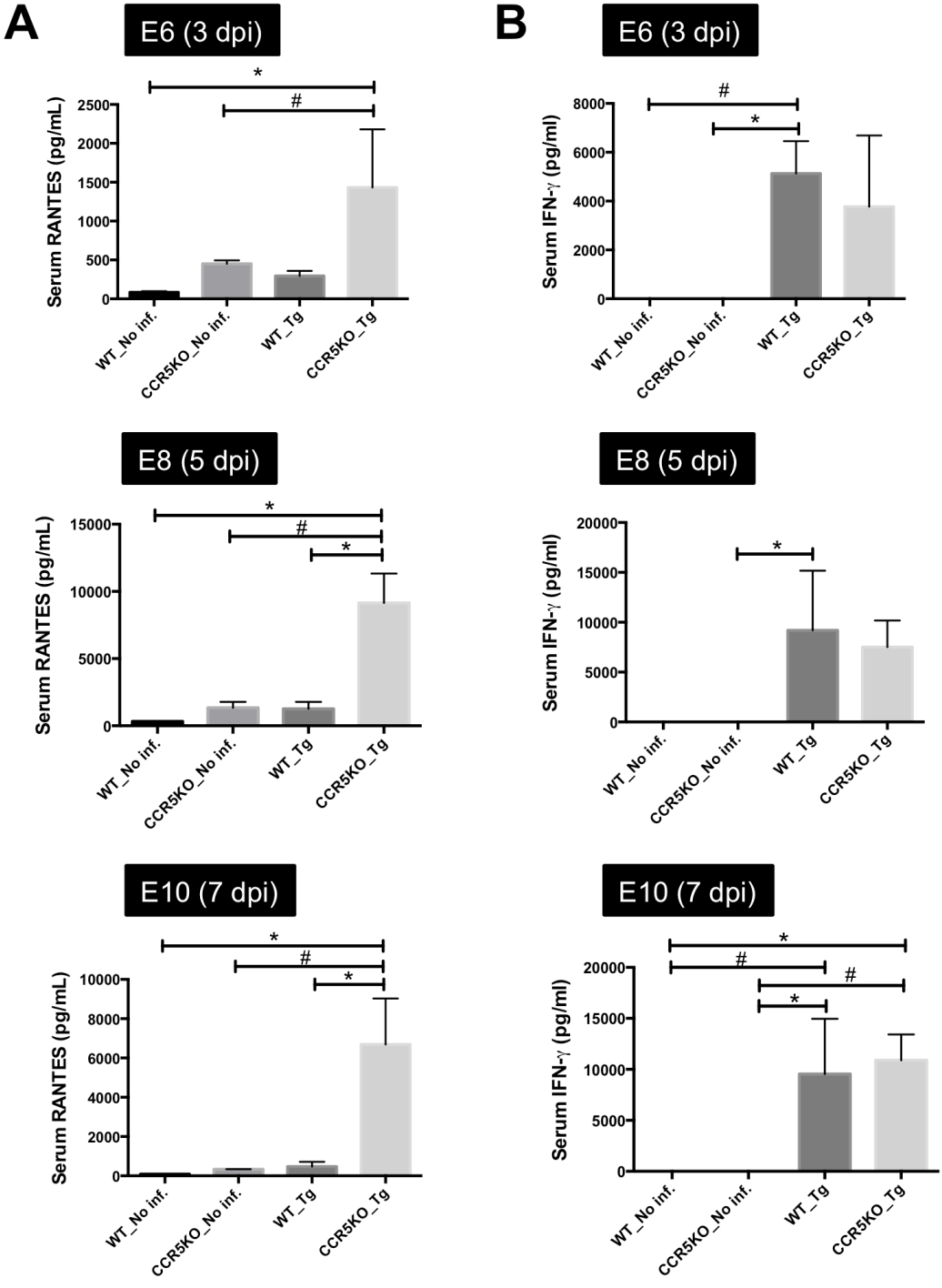

A
E6 (3 dpi)
Serum RANTES (pg/mL)
2500
2000
1500
1000
500
0
*
#
WT_No inf.
CCR5KO_No inf.
WT_Tg
CCR5KO_Tg
B
E6 (3 dpi)
Serum IFN-γ (pg/ml)
8000
6000
4000
2000
0
#
*
WT_No inf.
CCR5KO_No inf.
WT_Tg
CCR5KO_Tg
E8 (5 dpi)
Serum RANTES (pg/mL)
15000
10000
5000
0
*
#
*
WT_No inf.
CCR5KO_No inf.
WT_Tg
CCR5KO_Tg
E8 (5 dpi)
Serum IFN-γ (pg/ml)
20000
15000
10000
5000
0
*
WT_No inf.
CCR5KO_No inf.
WT_Tg
CCR5KO_Tg
E10 (7 dpi)
Serum RANTES (pg/mL)
10000
8000
6000
4000
2000
0
*
#
*
WT_No inf.
CCR5KO_No inf.
WT_Tg
CCR5KO_Tg
E10 (7 dpi)
Serum IFN-γ (pg/ml)
20000
15000
10000
5000
0
*
#
*
#
WT_No inf.
CCR5KO_No inf.
WT_Tg
CCR5KO_Tg

FIG 6. Serum levels of RANTES (A) and IFN-γ (B) at E6, E8 and E10. Wild-type mice (WT), CCR5$^{-/-}$ mice (CCR5KO), uninfected (No inf.), *T. gondii*-infected (Tg). Data are the mean values ± SD. * and # indicates significant differences as determined by one-way ANOVA plus Tukey–Kramer post-hoc analysis (P<0.05). * and # indicates between animals of different and same lineages, respectively. The numbers of used dams were 2, 2, 6 and 6 at E6, 2, 3, 6 and 6 at E8, and 3, 2, 5 and 7 at E10 in WT_No inf, CCR5KO_No inf, WT_Tg and CCR5KO_Tg, respectively.