Cyclosporin A improves murine pregnancy outcome in abortion-prone matings: involvement of CD80/86 and CD28/CTLA-4

Wen-Hui Zhou1,3, Lin Dong1, Mei-Rong Du1, Xiao-Yong Zhu1 and Da-Jin Li1,2

1Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai 200011, China, 2Department of Obstetrics and Gynecology, The Affiliated Hospital, Hainan Medical College, Haikou 570102, China and 3Department of Obstetrics and Gynecology, Zhongnan Hospital of Wuhan University, Wuhan 430071, China

Correspondence should be addressed to D-J Li; Email: djli@shmu.edu.cn

W-H Zhou and L Dong contributed equally to this work

Abstract

Immune regulation during pregnancy is complex, and thus an optimal therapy for pregnancy complications is always a big challenge to reproductive medicine. Cyclosporin A (CsA), a potent immunosuppressant, prevents rejection of allografts by hosts, but little is known about the modulating effect of CsA on the materno-fetal relationship. Here, pregnant CBA/J females mated with DBA/2 males as an abortion-prone model were administered with CsA on day 4.5 of gestation, and the pregnant CBA/J females mated with BALB/c males were established as successful pregnancy control. It was demonstrated that administration of CsA at the window of implantation significantly up-regulated the expression of CTLA-4, while down-regulating the levels of CD80, CD86, and CD28 at the materno-fetal interface in the CBA/J DBA/2 abortion-prone matings, and the embryo resorption rate of the abortion-prone matings reduced significantly after CsA treatment, implying that modulation of costimulatory molecule expression by CsA might contribute to preventing the fetus from maternal immune attack. In addition, treatment with CsA induced enhanced growth and reduced cell apoptosis of the murine trophoblast cells. Together, these findings indicate that CsA has a beneficial effect on the materno-fetal interface in abortion-prone matings, leading to a pregnancy outcome improvement, which might provide new therapeutics for spontaneous pregnancy wastage.


Introduction

A successful pregnancy is a very complex process involving synchronized molecular and cellular events between the fetus-derived trophoblast cells and the mother-derived immune cells. After the invasion into the decidua, trophoblasts, carrying paternal antigens, are in close contact with the maternal immune cells. Amazingly, the maternal immunocompetent cells do not attack the embryo, and even cooperate with trophoblasts via the production of some cytokines and growth factors to participate in the establishment of a unique materno-fetal immune milieu that helps the fetus to survive and develop in the uterus till parturition (Saito et al. 1993, Jokhi et al. 1994a, 1994b, Saito 2000, Veenstra van Nievenhoven et al. 2003, Higuma-Myojo et al. 2005, Staun-Ram & Shalev 2005). Thus, there is a subtle chorus rather than simple rivalry between the distinguished cells. Dysfunction of trophoblasts or excessive activation of maternal immunocompetent cells is highly linked with some pregnancy failures, such as abortion, pre-eclampsia, fetal growth restriction, and so on (Hustin et al. 1990, Hill et al. 1995, Yamada et al. 2003, Ball et al. 2006, Kadyrov et al. 2006). However, the immune regulation during pregnancy is intricate, and seeking an optimal therapy that gives consideration to both fetus-derived trophoblasts and mother-derived immunocompetent cells is always a big challenge to reproductive medicine.

Cyclosporin A (CsA) is a potent immunosuppressant, which has been widely used to prevent organ rejection (Sketris et al. 1995). It is well accepted that CsA can inhibit activation of T cells through suppressing the $Ca^{2+}$/calcineurin/calmodulin/NFAT signaling pathway that is important to the transcriptional activation for interleukin-2 (Emmel et al. 1989, Liu et al. 1992, Nelson et al. 1993). It has been found that CsA can also influence functions of other immune cells, including natural killer (NK) cells, macrophages, as well as dendritic cells (Wasowska et al. 2001, Sauma et al. 2003, Chen et al. 2004, Poggi & Zacchi 2005). CsA even modulates the expression of two crucial costimulatory...
molecules, CD80 and CD86, which are pivotal in the induction of the alloantigen-specific tolerance (Sayegh & Turka 1995, Jirapongsananuruk & Leung 1999, Lee et al. 1999). The clinical application of CsA has revolutionized organ transplantation and also improved the therapeutic management of some autoimmune diseases.

Pregnancy is a major challenge to the maternal immune system because of the persistence of paternal alloantigens. Although localized mechanisms may contribute to fetal evasion from maternal immune attack, maternal alloreactive lymphocytes do exist at the materno-fetal interface (Abadia-Molina et al. 1996, Trundle & Moffett 2004). Antigens coming from trophoblasts could activate proliferation and interferon-γ production by uterine NK cells (van der Meer et al. 2004, 2007), suggesting that embryo has the potential to stimulate maternal lymphocytes. Over-activation and consequent attack of maternal immune cells to the fetus is postulated to be an important cause of some pregnancy complications, such as recurrent abortion and pre-eclampsia (de Groot et al. 1996, Chao et al. 1999, Laird et al. 2003, Matthiesen et al. 2005). It has been shown that an elaborate ongoing modulation, including Th1/Th2 cytokines, indoleamine 2,3-dioxygenase, human leukocyte antigen (HLA) molecules, regulatory T cells, costimulatory molecules, activation of immunocompetent cells, and so on, occurs in both pregnancy and transplantation, which indicates that there are parallels between models of transplant rejection and pregnancy loss (Hall et al. 1990, Wegmann et al. 1993, Arck et al. 1997, Li et al. 1998, Chao et al. 1999, Gorczynski et al. 2002, Piccinni 2002, Salama et al. 2003, Saito et al. 2005, Zencussen 2005, Wilczynski 2006, Zencussen et al. 2006). Thereby, paradigms from CsA application on transplantation shed some light on investigating the materno-fetal relationship. We speculated that CsA has the potential to suppress the unsuitable maternal immune rejection to the allogeneic fetus just as this drug has done in transplantation.

A successful pregnancy requires not only maternal tolerance to an allogeneic fetus but also the fascinating properties of trophoblast cells. The trophoblast cells are critical for appropriate materno-fetal interactions. Dysfunction of the trophoblast has been confirmed to be associated not only with spontaneous abortion but also with pre-eclampsia and fetal growth restriction (Hustin et al. 1990, de Groot et al. 1996, Ball et al. 2006, Kadyrov et al. 2006). We have demonstrated that CsA can improve the first trimester human trophoblast cell growth and invasiveness in vitro (Yan et al. 2002a, Zhou et al. 2007). Therefore, we wondered whether CsA could also improve the biological functions in vivo of murine trophoblast cells that play key roles in the implantation, placentation, and development of embryo.

In the present study, by recruiting CBA/J×DBA/2 matings as an abortion-prone model and CBA/J×BALB/c as a normal pregnancy model, we observed the effects of CsA, administered at the early stage of gestation, on pregnancy outcome, expression of costimulatory molecules CD80/86 and CD28/CTLA-4, and proliferation and apoptosis of murine trophoblast cells at the materno-fetal interface.

Results

CsA increased fetal survival in abortion-prone matings

In our colony, the spontaneous abortion rate in CBA/J×DBA/2 matings was 28% (25/88), consistent with previous studies (Chaoat et al. 1983, 1985, 1995, Kiger et al. 1985). In order to test whether fetal loss could be reduced by CsA treatment in vivo, different doses of CsA or vehicle were administrated orally to the abortion-prone CBA/J females mated with DBA/2 males on day 4.5 of gestation, at the window of murine implantation. The embryo resorption rate was calculated on day 14.5 of gestation. The results shown in Table 1 clearly demonstrate that different dosage of CsA treatment in abortion-prone matings significantly reduced the resorption rate of (CBA/J×DBA/2) F1 fetuses compared with the non-treated abortion-prone CBA/J matings significantly reduced the resorption rate of (CBA/J×DBA/2) F1 fetuses compared with the non-treated abortion-prone CBA/J matings. Dosage of 1.0 mg/kg CsA was especially significant (P<0.01), and the pregnancy outcome similar to that of the successful pregnancy model (CBA/J×BALB/c matings). These findings indicate that CsA has the potential to improve pregnancy outcome in the abortion-prone matings (Table 1).

In addition, as shown in the above embryo resorption results, when treated with CsA of 1.0 mg/kg, the resorption rate of the abortion-prone matings was the lowest and almost equivalent to that of the normal pregnancy models, thus the following studies were all performed with this optimal dosage.

<table>
<thead>
<tr>
<th>Mating combination</th>
<th>Dosage of CsA (mg/kg)</th>
<th>Number of mice</th>
<th>Implantation sites/ mouse</th>
<th>Number of resorbed fetuses</th>
<th>Percent resorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBA/J×BALB/c</td>
<td>–</td>
<td>10</td>
<td>9.6±1.34</td>
<td>8</td>
<td>8.33 (8/96)</td>
</tr>
<tr>
<td>CBA/J×DBA/2</td>
<td>Vehicle</td>
<td>10</td>
<td>8.8±2.62</td>
<td>25</td>
<td>28.00 (25/88)*</td>
</tr>
<tr>
<td>CBA/J×DBA/2</td>
<td>0.1</td>
<td>10</td>
<td>8.7±1.89</td>
<td>13</td>
<td>14.00 (13/93)†</td>
</tr>
<tr>
<td>CBA/J×DBA/2</td>
<td>1.0</td>
<td>10</td>
<td>8.6±0.97</td>
<td>9</td>
<td>10.47 (9/86)‡</td>
</tr>
<tr>
<td>CBA/J×DBA/2</td>
<td>10</td>
<td>12</td>
<td>8.2±1.47</td>
<td>15</td>
<td>15.30 (15/98)§</td>
</tr>
</tbody>
</table>

The embryo resorption rate was calculated using the formula presented in Materials and Methods. χ² test: *P<0.01, compared with CBA/J×BALB/c matings; † χ² test: †P<0.05; ‡P<0.01, compared with CBA/J×DBA/2 matings treated with vehicle.
Effect of CsA on transcription of CD80/CD86 and CD28/CTLA-4 at the materno-fetal interface

CD80 (B7-1)/CD86 (B7-2) and CD28/CTLA-4, as two main costimulatory molecule pairs, play crucial roles in the activation, proliferation, and differentiation of T lymphocytes, which participate in antigen-specific rejection (Bhatia et al. 2005). In this study, the mRNA level of CD80/CD86 and CD28/CTLA-4 at the materno-fetal interface was determined by real-time RT-PCR. The results in Fig. 1 clearly show that the mRNA level of CD80/CD86 and CD28 at the materno-fetal interface in abortion-prone matings was obviously higher than that of the successful matings, while administration of CsA (1.0 mg/kg) at the window of implantation remarkably reduced the aberrant expression of these costimulatory molecules. On the contrary, compared with the successful matings, the mRNA level of CTLA-4 at the materno-fetal interface in abortion-prone matings was significantly lower, while the mRNA expression of CTLA-4 was up-regulated and almost equivalent to that of the normal pregnancy matings after CsA treatment.

Immunocytochemical identification for the purity of murine ectoplacental-derived trophoblasts

The identification of the ectoplacental-derived trophoblastic cells was confirmed by immunocytochemistry with anti-cytokeratin-7 and anti-vimentin antibody. It is clearly shown in Fig. 2 that the isolated majority of cells were stained positive for cytokeratin-7 (Fig. 2A) and negative for vimentin (Fig. 2B). The purity of the isolated trophoblast cells was above 95%.

CsA-promoted expression of proliferating cell nuclear antigen (PCNA) of murine ectoplacental-derived trophoblasts in abortion-prone matings

The capacity of CsA to promote the murine trophoblast proliferation was investigated through PCNA analysis by flow cytometry. The results showed that the PCNA expression of ectoplacental-derived trophoblastic cells in abortion-prone matings was significantly lower than that of the successful matings, while the number of trophoblasts
expressing PCNA in abortion-prone matings remarkably increased after treatment with 1.0 mg/kg CsA, and the proportion of PCNA-positive cells even approached that of the successful pregnant matings, suggesting that CsA treatment has the potential to promote proliferation in vivo of murine trophoblasts (Fig. 3).

CsA down-regulated apoptosis of ectoplacental-derived trophoblastic cells in abortion-prone matings

Since CsA is able to affect a wide spectrum of cell apoptosis (Borutaite et al. 2003), we observed the effect of CsA on apoptosis of murine trophoblasts. By Annexin V and propidium iodide (PI) staining and subsequent flow cytometric analysis, the number of cells undergoing apoptosis was determined. The apoptosis of ectoplacental-derived trophoblastic cells in abortion-prone matings significantly increased, compared with the successful matings, while CsA (1.0 mg/kg) treatment remarkably reduced the percentage of trophoblast cells undergoing apoptosis, and the proportion of trophoblast cells undergoing apoptosis in CsA-treated abortion models was reduced to that of the successful matings, which suggests that CsA has potential to inhibit apoptosis and improve the function of murine trophoblasts in abortion-prone matings (Fig. 4).

Discussion

At the materno-fetal interface, the expression of CD80/86 and class II antigens is absent on extravillous trophoblast cells, and mainly localized to antigen-presenting cells (APCs) (Athanassakis-Vassiliadis et al. 1990, Vassiliadis et al. 1994, Athanassakis et al. 1995, Petroff et al. 2003). The dendritic cell (DC), the most potent presenter at the materno-fetal interface, has characteristics of itself, including the predominance of myeloid DCs over lymphoid DCs; high proportion of immature DCs to mature DCs, a moderate expression of CD80/86; co-expression of CD86/HLA-DR; more production of interleukin (IL)-10 than that of IL-12, which is beneficial to the development of ‘tolerogenic’ DCs and induction of immune tolerance to the embryo (Kammerer et al. 2000, Gardner & Moffett 2003, Kammerer et al. 2003, Blois et al. 2004, Rieger et al. 2004). The induced class II antigens expression at the spongiotrophoblastic zone of the murine placenta significantly correlates with fetal abortion and developmental abnormalities (Athanassakis-Vassiliadis et al. 1995).
Our present study also found that there was an increased expression of CD80/86 and CD28, while a decreased expression of CTLA-4 at the materno-fetal interface in CBA/J x DBA/2 abortion-prone models. The interaction of CD80/CD86 with CD28 is one of the vital costimulatory regulations that deliver costimulatory signals, activate T cells, and lead to immunorejection (Guinan et al., 1994, June et al., 1994).

It was found, in our previous studies, that at the materno-fetal interface in CBA/J x DBA/2 abortion-prone models, the production of Th1 cytokines up-regulated while that of Th2 cytokines down-regulated (Jin et al., 2004, Zhu et al., 2005, Du et al., 2007). We also found that the embryo resorption rate of CBA/J x DBA/2 abortion-prone models decreased after in vivo CD80/86 mAbs treatment and blocking costimulatory signals CD80/86 could suppress maternal immune attack to the fetus by shifting cytokines from Th1 predominance to Th2 bias at the materno-fetal interface and expanding peripheral CD4^+CD25^+ regulatory T cells (Jin et al., 2004, Zhu et al., 2005). Thus, it is reasonable to propose that a limited and proper expression of costimulatory molecules on some specific cells, for example, DCs at the materno-fetal interface, might contribute to the appropriate and regional activation of T cells via conducting suitable signals, leading to T-cell tolerance to the semi-allogeneic embryo, and an over-expression of costimulatory molecules might play important roles in the pathology of pregnancy loss via excessive activation of maternal immune cells, leading to attack of the fetus.

In the present study, it has been demonstrated that the abnormal expression of both CD80/86 and CD28 at the materno-fetal interface in abortion-prone models (CBA/J x DBA/2 matings) was down-regulated after CsA treatment at the early stage of pregnancy, and the level of CD80/CD86 and CD28 at the materno-fetal interface in CsA-treated abortion-prone models was almost reduced to that of normal models (CBA x BALB/c matings). Our previous study has demonstrated that CsA administrated at the window of implantation could induce a Th2 bias and expansion of CD4^+CD25^+ regulatory T cells in the abortion-prone CBA/J x DBA/2 matings (Du et al., 2007). It has also been shown, in our previous research, that blocking costimulation signals CD80/86 could suppress maternal immune attack to the fetus by shifting cytokines from Th1 predominance to Th2 bias at the materno-fetal interface and expanding...
peripheral CD4+CD25+ regulatory T cells; furthermore, the expression of CD28 and its ligands CD80/CD86 on peripheral lymphocytes was down-regulated (Jin et al. 2004, Zhu et al. 2005). Thus, it could be speculated that the down-regulation of CsA on CD80/CD86 and CD28 might contribute to the induction of a Th2 bias and development of maternal-fetal tolerance, which is beneficial to suppressing maternal rejection to fetus and improving the pregnancy outcome of the abortion-prone matings.

It has become evident that T cells are sensitive to quantitative changes in the molecular interactions that contribute to T-cell antigen recognition, and all these quantitative changes, including binding affinity, kinetics, or surface density, can lead to different T-cell responses (Jameson & Bevan 1995, Sykulev et al. 1995, Alam et al. 1996, Valitutti et al. 1996, Viola & Lanzavecchia 1996, van der Merwe & Davis 2003). As another receptor for ligands delivers a positive signal to T cells that promotes T-cell anergy (Walunas et al. 1994, Greene et al. 1996). The interaction of CD28 with B7 ligands delivers a positive signal to T cells that promotes proliferation and IL-2 secretion (Thompson et al. 1989, Linsley et al. 1991). In contrast, the interaction of CTLA-4 with B7 ligands attenuates T-cell activation and induces T-cell anergy (Walunas et al.1994, Tivol et al. 1995, Waterhouse et al. 1995). Our work showed that CsA down-regulated the expression of CD28, while up-regulating the expression of CTLA-4 at the materno-fetal interface in the abortion-prone matings, and the level of CTLA-4 and CD28 in CsA-treated abortion-prone matings almost approached that of normal models, which suggests that CsA can correct the aberrant expression pattern of CD28 and CTLA-4 in abortion-prone matings. CD80 bound to CTLA-4 and CD28 with Kd values of 0.2 and 4.0 μM respectively; and CD86 bound to CTLA-4 and CD28 with Kd values 20.0 and 2.6 μM respectively, suggesting that CTLA-4 has a higher affinity to CD80/86 than CD28 (van der Merwe et al. 1997, Collins et al. 2002). Thus, the up-regulation of CTLA-4 and down-regulation of CD28 by CsA treatment might further reinforce the competitive ability of CTLA-4 for CD80/86. In this condition, CsA could provide us with twofold guarantees: this drug first suppresses the activation of T cells through down-regulating the abnormal expression of CD80/86 and CD28 in abortion-prone matings, and then delivers activation-induced inhibitory signals through up-regulating the level of CTLA-4, which redounds to the prevention of the fetus from maternal rejection and the induction of maternal-fetal tolerance.

The CD4+CD25+ regulatory T cells (Treg) have recently been described as a unique subpopulation of T cells (Wood & Sakaguchi 2003, Sakaguchi 2004, Waldmann et al. 2004). They have been confirmed to play a major role in preventing autoimmunity and tolerating allogeneic organ grafts (Sakaguchi et al. 1995, Zelenika et al. 2001, Kingsley et al. 2002). The proportion of CD4+CD25+ regulatory T cells at the materno-fetal interface is low, but these cells have been regarded as one of the important mediators inducing embryonic antigen-specific tolerance (Heikkinen et al. 2004, Saito et al. 2005, Zelcnussen 2005, Zenclussen et al. 2006). CTLA-4 is a functional molecule for CD4+CD25+ regulatory T cells, and can deliver inhibitory signals via direct cell–cell interaction (Thornton & Shevach 1998, Takahashi et al. 2000, Bensinger et al. 2001). We determined only the whole level of CTLA-4 at the materno-fetal interface; however, this molecule is constitutively expressed at the surface of CD4+CD25+ regulatory T cells, and the CD4+CD25+ regulatory T cells do exist at the materno-fetal interface (Takahashi et al. 2000, Bensinger et al. 2001). It was found in our present study that the expression of CD80/86 at the materno-fetal interface in abortion-prone models decreased after CsA treatment. Furthermore, our previous research has shown that CsA could expand CD4+CD25+Foxp3+ regulatory T cells in abortion-prone matings, and CD80/86 blockade could also induce expansion and CTLA-4 expression of CD4+CD25+Foxp3+ regulatory T cells in these abortion models (Jin et al. 2004, Zhu et al. 2005, Du et al. 2007). Thus, it is speculated that CsA might enhance both the number and the function of CD4+CD25+ regulatory T cells by down-regulating CD80/86, which might facilitate fetal survival and development in the abortion-prone matings. Of course, this hypothesis deserves further investigation.

Besides maternal tolerance to the embryo, the fascinating property of trophoblast cells is another key player for a successful pregnancy. In mice and other rodents, trophoblast cells are the placental cells in direct contact with endometrial tissues throughout gestation (Billington 1971, Muntener & Hsu 1977). These cells play crucial roles in implantation and placentation (Muntener & Hsu 1977, Bevilacqua & Abrahamsohn 1988, Mehrotra 1988, Kanai-Azuma et al. 1993). After the onset of implantation, the trophoblast cells even have the ability to phagocyte the maternal components (cells interposed in the invasion pathway, as well as substantial numbers of blood cells). It has been reported that this phagocytic activity participates in fetal nutrition prior to complete formation of the placenta, and also plays a role in acquiring space for embryo attachment and development in the endometrium (Welsh & Enders 1987, Bevilacqua & Abrahamsohn 1988). Thus, the accurate function of trophoblasts is crucial to normal pregnancy. It has been demonstrated that an insufficient proliferation or increased apoptosis of trophoblasts is highly linked with both murine and human pregnancy failure (Qumsiyeh et al. 2000, Olivares et al. 2002, Greer 2003, Burdon et al. 2007). The interesting point is that our work showed that the expression of PCNA


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of ectoplacental-derived trophoblastic cells increased and the level of Annexin V in these cells decreased after CsA treatment at the window of murine implantation, compared with the non-treated abortion-prone matings, suggesting that this drug presents a favorable effect on murine trophoblasts via promoting proliferation and suppressing apoptosis of these cells. It was demonstrated, in our previous research, that CsA was able to directly promote in vitro growth of the first trimester human cytotrophoblasts (Yan et al. 2002a). Moreover, our recent study also found that different from its actions on immune cells via the calcineurin/calmodulin/NF-AT signals, CsA enhanced the invasiveness and activity of matrix metalloproteinase (MMP)9 and MMP2 in vitro of first-trimester human trophoblast cells through activation of MAPK/ERK1/2 signaling pathway (Zhou et al. 2007). Therefore, CsA may present different effects on different cells via different signaling pathways, and the pharmacological action of this drug is far from being completely understood and deserves further investigation. It is evident that the decidual local and peripheral lymphocytes are over-activated and produce aberrant Th1-related cytokines in spontaneous abortion, leading to extensive destruction of the trophoblast by inducing apoptosis (Hill et al. 1995, Abadia-Molina et al. 1996, Bates et al. 2002, Olivares et al. 2002). Thus, it is proposed that CsA might modulate the biological function of trophoblast cells in vivo in both a direct (MAPK signaling pathway) and an indirect manner suppressing lymphocyte activation by down-regulating co-stimulatory molecules, which are all beneficial to the improvement of trophoblast functions in the abortion-prone matings.

Our study has demonstrated for the first time that CsA has dual functions: inducing the materno-fetal immunotolerance, and improving the biological functions of trophoblast cells, which is propitious to improve the pregnancy outcome in abortion-prone matings. In addition, our previous study showed that there was no remarkable difference in the weight of viable placentas and fetuses between the CBA/J × DBA/2 abortion-control group and the CBA/J × DBA/2 CsA-treated group, suggesting that CsA treatment on the window of implantation could improve the outcome of the abortion-prone models, while having no obvious inhibition in the weight of placentas and fetuses of the CBA/J × DBA/2 abortion-prone matings. Furthermore, no significant difference in the embryo resorption rate and the weight of placentas and fetuses was observed between the CBA × BALB/c normal group and the CBA × BALB/c normal CsA-treated group, which suggests that a low dosage of CsA treatment at day 4.5 of gestation has no obvious side effect on normal pregnancies (Yan et al. 2002b). Therefore, CsA appears to be useful as an immunological therapy for spontaneous abortion. Of course, the safety for clinical administration deserves further investigation.

Materials and Methods

Mice

Inbred strains female mice of 8-week-old CBA/J (H-2k), male DBA/2 (H-2d), and BALB/c (H-2d) were obtained from the Jackson Laboratories (Bar Harbor, ME, USA), and subsequently maintained in the Laboratory Animal Facility of the Chinese Academy of Sciences (Shanghai, China). The weight of mice ranged from 18 to 22 g. They were usually maintained for 2 weeks in the animal facility before use. The housing and handling of the experimental animals were in accordance with the guidelines of the Chinese Council for Animal Care. The day of appearance of a copulatory plug was arbitrarily designated as day 0.5 of gestation.

CsA administration

The abortion-prone CBA/J females mated with DBA/2 males were administrated orally with 0, 0.1, 1.0, or 10 mg/kg CsA (Novartis, Basel, Switzerland) on day 4.5 of gestation, at the window of murine implantation. The pregnant CBA/J × BALB/c matings were considered as successful pregnancy model. These pregnant CBA/J mice were killed on either day 9.5 or 14.5 of gestation for further investigation.

Embryo resorption

For macroscopic observation of embryo resorption, mice were killed on day 14.5 of gestation, and the uteri were examined for the number of healthy and resorbed embryos. At this stage of gestation, the resorbed embryos were subjected to ischemia, hemorrhage, and necrosis, making them smaller and darker than the larger, pink, viable embryos. Percentage of embryos undergoing resorption was calculated by the formula described previously: \( \%R = \frac{Re}{(Re + F)} \times 100\% \), where Re represents the number of the resorbed embryos and F represents the number of viable embryos (Chaouat et al. 1983, 1985, 1995, Kiger et al. 1985).

Quantitative RT-PCR for mRNA expression of CD80/86 and CD28/CTLA-4 at the materno-fetal interface

Total RNA from the entire implantation site, including placental and decidual tissue, on day 9.5 or 14.5 of gestation was extracted by using Trizol reagent (Invitrogen) and reversely transcribed as previously: \( Re \). The cDNA (5 μl) was amplified by real-time PCR in a final volume of 50 μl containing 25 μl real-time PCR Master Mix (TOYOBO company, Tokyo, Japan), 0.8 μmol/l of each primer, and 5 μmol/l TagMan probe. Each sample was analyzed in duplicates, and the reaction was followed by 40 cycles of 1 min at 95 °C, 15 s at 95 °C, and 1 min at 60 °C using ABI Prism 7000 Sequence Detector. Sequences for primers and probes are shown in Table 2.

Isolation of ectoplacental cone-derived trophoblasts

The detailed description of the procedure used to isolate trophoblasts has been reported in our previous publication (Wu et al. 2004). Briefly, the uterine horns of pregnant CBA/J mice...
on day 9.5 of pregnancy were opened longitudinally, and
the fetoplacental unit was separated from the uterine
implantation sites. After washing in cold Hanks’ balanced salt
solution, the ectloplacental cell cone was further removed from
the fetoplacental unit and cut into small pieces (≈ 1 mm³).
The obtained tissue was digested in four cycles of 10 min by
0.25% trypsin (Bio Basic Inc., Ontario, Canada) and 0.02%
DNase type I (Sigma) at 37 °C with gentle agitation. The cell
suspensions trypsinized each time were pooled and carefully
layered over a discontinuous Percoll Gradient (65–20%, in 5%
steps), and centrifuged at 1000 g (2000 r.p.m.) for 20 min. The
cells sedimenting at densities between 1.048 and 1.062 g/ml
were collected, and washed with DMEM-high glucose medium.
The cells were then diluted to 1×10⁶ cells/ml, maintained in DMEM-high glucose complete medium
containing 10% heat-inactivated FBS (Gibco) and incubated in 24-well plates (precoated with coverlips) at 37 °C with 5% CO₂ for following immunocytochemical characterization.

**Immunochemistry**

After 24 h culture, the trophoblast cells were fixed in 4%
formaldehyde for 20 min at room temperature, washed in
PBS, and permeabilized for 4 min in 0.3% Triton X-100-PBS. The
cells were then incubated with 7% horse serum in PBS for 30 min
to block the nonspecific binding. Three antibodies diluted in PBS containing 1% BSA were added, and anti-mouse
cytokeratin-7 IgG1 (Golden Bridge Biotechnology Co. Ltd,
Beijing, China) and anti-mouse vimentin IgG1 monoclonal
antibody (Golden Bridge Biotechnology Co. Ltd) were used as
markers for cells of trophoblast lineage. Isotype-matched irrelevant IgG (Sino-America Co. Ltd, Shanghai, China) was
used as control. After incubation with the primary antibody
overnight at 4 °C, the cells were washed in PBS-0.1% Tween, and
then incubated with a horseradish peroxidase (HRP)-labeled
secondary antibody (Sino-America Co. Ltd) for 2 h at room
temperature. Streptavidin–HRP was applied for another 30 min at
room temperature. At the end of incubation, a further 400 µl
binding buffer was added, and the cells were analyzed
immediately by flow cytometry (BD Bioscience). The freshly isolated ectoplacental-derived trophoblastic cells were washed and
resuspended in 80 µl binding buffer (10 mM HEPES, 140 mM
sodium chloride, 2.5 mM calcium chloride, pH 7.4). To each
cell suspension was added 10 µl fluorescence-conjugated Annexin V (10 µg/ml) and 10 µl PI reagent (50 µg/ml). The
cells were mixed and then incubated in the dark for 15 min at
room temperature. At the end of incubation, a further 400 µl
binding buffer was added, and the cells were analyzed
immediately by flow cytometry (BD Bioscience). The control
tubes of unstained cells, cells stained with PI alone, and cells
stained with Annexin V only were included for setting up the
flow cytometric compensation.

**Statistical analysis**

All values shown are mean± S.E.M. The significance of
difference in the resorption rates was tested by a χ² test.
Student’s t-test was performed to detect the significance of
difference in the expression of costimulatory molecules at

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**Table 2** The primer sequences and probes for CD80/CD86 and CD28/CTLA-4 by real-time RT-PCR.

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Primers and probes</th>
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<tbody>
<tr>
<td>GAPDH</td>
<td>GAPDH-R: 5'-CACCCCAATTTGATGTTAGT-3'</td>
</tr>
<tr>
<td></td>
<td>GAPDH-F: 5'-CCATTGAGTGCGGAAAG-3'</td>
</tr>
<tr>
<td></td>
<td>GAPDH-probe: 5'-FAM-CAGCCCGCAGAATTGGAAGCTTGTC-TAMRA-3'</td>
</tr>
<tr>
<td>CD28</td>
<td>CD28-R (bp622C): 5'-TGGTAAGGCTTCAGTGAG-3'</td>
</tr>
<tr>
<td></td>
<td>CD28-F (bp499): 5'-ATGGCTTCATGACTTGAGT-3'</td>
</tr>
<tr>
<td></td>
<td>CD28-probe: 5'-FAM-TGACTACATGCAGAAGCTTGTC-TAMRA-3'</td>
</tr>
<tr>
<td>CD80</td>
<td>CD80-R (bp913C): 5'-GAAGACGCTCTGTTCAGCTCA-3'</td>
</tr>
<tr>
<td></td>
<td>CD80-F (bp717): 5'-ACACACTTGTGCTCTTTGGG-3'</td>
</tr>
<tr>
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<td>CD80-probe: 5'-FAM-TCCGCCGCGAGTATAAAGACTGCAGTGC-TAMRA-3'</td>
</tr>
<tr>
<td>CD86</td>
<td>CD86-R (bp958C): 5'-CTCTGTCACCGTTATCTCC-3'</td>
</tr>
<tr>
<td></td>
<td>CD86-F (bp829): 5'-ACAGCTTCAGTTACGCAGT-3'</td>
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<tr>
<td></td>
<td>CD86-probe: 5'-FAM-ATGCTTCAAGAAGAGCGATCGC-TAMRA-3'</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>CTLA-4-R (bp525C): 5'-CAGCTAAGCTGACAGAGCAGA-3'</td>
</tr>
<tr>
<td></td>
<td>CTLA-4-F (bp662C): 5'-TACCTCTGAGGGTGAAC-3'</td>
</tr>
<tr>
<td></td>
<td>CTLA-4-probe: 5'-FAM-CAGCCCGGAATTCTGACCTTCTC-TAMRA-3'</td>
</tr>
</tbody>
</table>

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**Flow cytometry of PCNA expression in murine trophoblasts**

The PCNA is a protein of 36 kDa molecular weight and
synthesized in early G1 and S phases of the cell cycle, thus can
represent the proliferation ability of cells (Das-Bradoo et al.
2006). In the present study, on day 9.5 of gestation, the freshly
isolated ectloplacental-derived trophoblastic cells were washed and
fixed in 70% methanol at 4 °C for 30 min. After blocking
with 10% FBS, the recovered cells were added with anti-mouse
PCNA-PE monoclonal antibody (Biolegend, San Diego, CA,
USA) and anti-mouse cytokeratin-7-FITC monoclonal antibody
(Chemicon, Temecula, CA, USA). After incubation in darkness
for 30 min at room temperature, the cells were analyzed
immediately by flow cytometry (BD Bioscience, Franklin Lakes,
NJ, USA). The experiments were repeated four times.

**Annexin V and PI staining**

To determine apoptosis of murine trophoblast cells, an
Annexin V Detection Kit was used for Annexin V binding and
PI staining (Bender, Burlingame, CA, USA). The freshly isolated
trophoblast-derived trophoblastic cells were washed and
resuspended in 80 µl binding buffer (10 mM HEPES, 140 mM
sodium chloride, 2.5 mM calcium chloride, pH 7.4). To each
cell suspension was added 10 µl fluorescence-conjugated
Annexin V (10 µg/ml) and 10 µl PI reagent (50 µg/ml). The
cells were mixed and then incubated in the dark for 15 min at
room temperature. At the end of incubation, a further 400 µl
binding buffer was added, and the cells were analyzed
immediately by flow cytometry (BD Bioscience). The control
tubes of unstained cells, cells stained with PI alone, and cells
stained with Annexin V only were included for setting up the
flow cytometric compensation.
the materno-fetal interface, and in the level of PCNA and annexin V in murine trophoblasts. Differences were accepted as significant at \( P<0.05 \).

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