

—Original Article—

## Tim-3 and PD-1 regulate CD8<sup>+</sup> T cell function to maintain early pregnancy in mice

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**Abstract.** During pregnancy, CD8<sup>+</sup> T cells are important regulators in the balance of fetal tolerance and antiviral immunity. T-cell immunoglobulin mucin-3 (Tim-3) and programmed cell death-1 (PD-1) are well-recognized negative co-stimulatory molecules involved in viral persistence and tumor metastasis. Here, we demonstrate that CD8<sup>+</sup> T cells co-expressing Tim-3 and PD-1 were down-regulated in the deciduae of female mice in abortion-prone matings compared with normal pregnant mice. In addition to their reduced numbers, the Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells produced lower levels of the anti-inflammatory cytokines interleukin (IL)-4 and IL-10, as well as a higher level of the pro-inflammatory cytokine interferon (IFN)- $\gamma$ , relative to those from normal pregnancy. Furthermore, normal pregnant CBA/J females challenged with Tim-3- and/or PD-1-blocking antibodies were more susceptible to fetal resorption. These findings indicate that Tim-3 and PD-1 pathways play critical roles in regulating CD8<sup>+</sup> T cell function and maintaining normal pregnancy.

**Key words:** CD8<sup>+</sup>T, Miscarriage, Pregnancy, Programmed cell death-1 (PD-1), T-cell immunoglobulin mucin-3 (Tim-3)  
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Physiologically, a successful pregnancy requires the maternal immune system to recognize and tolerate the semi-allogeneic fetus [1]. Pathologic pregnancies such as recurrent spontaneous abortion (RSA) are considered to be associated with dysfunction of maternal immune cells and dysregulation of maternal-fetal tolerance [2]. It has been suggested that the maternal T cell response shifts toward an anti-inflammatory direction during pregnancy; this includes increased production of type 2 T helper cell (Th2-type) cytokines (IL-4, IL-10, etc.) and decreased production of type 1 helper cell (Th1-type) cytokines (IFN- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , etc.), thus contributing to a pregnancy-protecting environment that may be disturbed in diseases like RSA [3, 4].

CD4<sup>+</sup> T cells are found to display a bias toward Th2 and regulatory T cell (Treg) response during normal pregnancy [5]. Relatively less is known about the role of CD8<sup>+</sup> T cells in maternal-fetal tolerance. CD8<sup>+</sup> T cells participate in the adaptive immune response by directly recognizing xenogeneic or allogeneic major histocompatibility complex (MHC) class I molecules, especially in the defense against viral infections. The existence of CD8<sup>+</sup> T cells at the maternal-fetal interface has been confirmed by studies using human tissues and murine models [6, 7]. Human extravillous trophoblast (EVT)

cells, which invade the decidua and interact with maternal immune cells, express embryo-derived allogeneic human leukocyte antigens (HLA)-C and HLA-G [8]. These antigens can induce a cytotoxic response by CD8<sup>+</sup> T cells in circumstances such as autoimmunity and allogeneic organ transplantation [9, 10]. However, the fetus is not attacked by CD8<sup>+</sup> T cells during normal pregnancy. The mechanisms regulating CD8<sup>+</sup> T cells to obtain a tolerant phenotype and to help maintain normal pregnancy remain to be explored.

Negative co-stimulatory (co-inhibitory) molecules are a group of cell-surface molecules that negatively modulate the immune response and have been intensely studied in the fields of oncology and transplantation [11, 12]. Among them, T-cell immunoglobulin mucin-3 (Tim-3) and programmed cell death-1 (PD-1) signals are found to impair CD8<sup>+</sup> T cell proliferation, activation, and function and induce CD8<sup>+</sup> T cell exhaustion, especially in chronic viral infections and tumors [13, 14]. Tim-3 and PD-1 co-expression characterizes the most severely exhausted CD8<sup>+</sup> T cell subset [15], and the combined blocking of Tim-3 and PD-1 can reverse the state of exhaustion and rescue lymphocyte function [16, 17]. *In vitro* research has revealed that Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells accumulate in the human decidua and exhibit high anti-inflammatory cytokine producing capacity, which is dysregulated in RSA [6]. However, information about the function of Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells during pregnancy in murine models is relatively scarce.

In this study, we investigated the expression of Tim-3 and PD-1 on CD8<sup>+</sup> T cells from the decidua and the spleen (representative of the periphery) of pregnant mice from normal and abortion-prone matings, as well as the cytokine production profile of different subsets of decidual and spleen CD8<sup>+</sup> T cells. Importantly, we studied the *in vivo* effects of Tim-3 and/or PD-1 blocking on normal pregnancy

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maintenance. These results underline the significance of the role of the Tim-3 and PD-1 signaling pathways in regulating CD8<sup>+</sup> T cell function, facilitating maternal-fetal tolerance, and maintaining successful pregnancy. Our findings provide novel strategies for the development of immunotherapy in which the enhancement of Tim-3 and PD-1 signals could prevent pregnancy loss by promoting maternal-fetal tolerance.

## Materials and Methods

### Mice

Female CBA/J and male DBA/2 and BALB/c mice were purchased from Huafukang (Beijing, China) and maintained in an animal facility according to institutional and National Institutes of Health guidelines. Eight-week-old CBA/J females were mated to BALB/c males to establish normal pregnancy and were inspected every morning for vaginal plugs. The day of observing a plug was designated as day 0.5 of the pregnancy. Pregnant CBA/J females received intraperitoneal injections of rat anti-mouse PD-1 antibody (clone RMP1-14; BioLegend, San Diego, CA, USA) at doses of 500, 250, and 250 mg at days 4.5, 6.5, and 8.5, respectively; or rat anti-mouse Tim-3 antibody (clone RMT3-23, BioLegend) at doses of 500, 250, and 250 mg at days 4.5, 6.5, and 8.5, respectively; or anti-PD-1 plus anti-Tim-3 antibodies at doses of 500, 250, and 250 mg at days 4.5, 6.5, and 8.5, respectively; or isotype IgG at doses of 500, 250, and 250 mg at days 4.5, 6.5, and 8.5, respectively. Eight-week-old CBA/J females were mated to DBA/2 males to establish an abortion-prone model and were inspected every morning for vaginal plugs. All pregnant mice were monitored at day 10.5 of pregnancy.

### Preparation of mouse cells

The fetal and placental tissues were carefully removed from the uteri of the mice at day 10.5 of gestation and washed in PBS. Minced uteri were digested in RPMI 1640 medium supplemented with collagenase Type IV and DNase I for 30 min at 37°C with gentle agitation. The total suspension was filtrated and enriched by discontinuous Percoll gradient centrifugation (1.130 g/ml, 60%, 40%, 20%; GE Healthcare Life Sciences, Little Chalfont, UK). After centrifugation, the cells between 60% and 40% Percoll (the densities were between 1.062 and 1.077 g/ml, respectively) were roughly separated decidual immune cells (DICs) mixed with a small portion of decidual stromal cells. These cells were then cultured in DMEM/F12 (37°C, 5%CO<sub>2</sub>) for 4 h to remove adherent stromal cells. The DICs were collected from the suspension and characterized by flow cytometry using PE-conjugated anti-mouse CD45 antibody. The spleen was aseptically excised and stored in RPMI 1640 medium. A single-cell suspension was produced using a 10-ml syringe plunger to pass splenic tissue through a 70-mm mesh strainer into fresh medium. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml amphotericin B at 37°C in 5% CO<sub>2</sub>.

### Flow cytometry

The expression levels of cell surface molecules CD8, Tim-3, and PD-1 on decidual and spleen cells from normal pregnant and abortion-prone mice were evaluated using flow cytometry. FITC-conjugated anti-mouse CD8 antibody (BioLegend), PE-conjugated

anti-mouse Tim-3 antibody (BioLegend), and PE-cy7-conjugated anti-mouse PD-1 antibody (BioLegend) were used. The production of intracellular cytokines (TNF- $\alpha$ , IFN- $\gamma$ , IL-4, and IL-10) by Tim-3<sup>+</sup>PD-1<sup>+</sup>, Tim-3<sup>+</sup>PD-1<sup>-</sup>, Tim-3<sup>-</sup>PD-1<sup>+</sup>, and Tim-3<sup>-</sup>PD-1<sup>-</sup> decidual and spleen CD8<sup>+</sup> cells from normal pregnant and abortion-prone mice were also evaluated using flow cytometry. Freshly isolated DICs and splenocytes were treated with brefeldin A (10  $\mu$ g/ml), phorbol 12-myristate 13-acetate (PMA) (50 ng/ml), and ionomycin (1  $\mu$ g/ml) for 4 h, then the cells were harvested and analyzed by flow cytometry. APC-conjugated anti-mouse TNF- $\alpha$  or IL-10 antibodies (BioLegend) and Brilliant Violet 421-conjugated anti-mouse IFN- $\gamma$  or IL-4 antibodies (BioLegend) were used. For intracellular staining, cells were fixed and permeabilized using the Fix/Perm kit (BioLegend). Flow cytometry was performed on a Beckman-Coulter CyAN ADP cytometer and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

### Statistical analysis

The statistical significance of differences between two groups was determined by the *post-hoc* Dunnett *t*-test. Multiple groups were analyzed with GraphPad Prism Version 5 (GraphPad Software, La Jolla, CA, USA) by one-way or two-way ANOVA with Bonferroni post *t*-tests. For all statistical tests, P-values < 0.05 were considered as statistically significant.

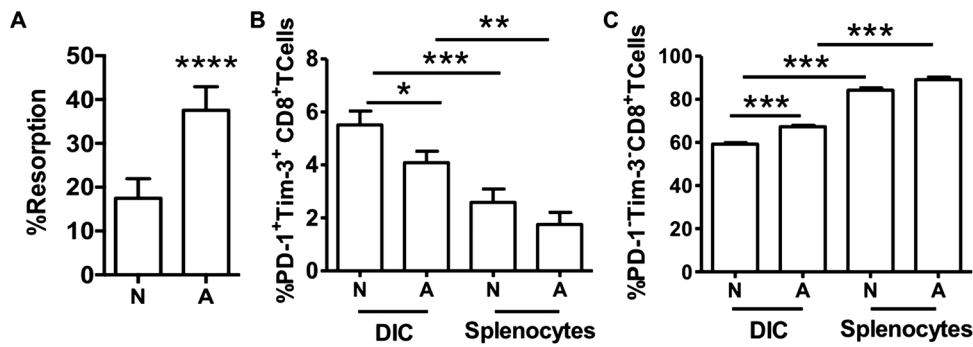
## Results

### Expression of Tim-3 and PD-1 on CD8<sup>+</sup> T cells in normal and abortion-prone matings

To investigate the potential role of Tim-3 and PD-1 in the function of CD8<sup>+</sup> T cells during murine pregnancy, we first established a normal pregnancy model by mating BALB/c males with CBA/J females and an abortion-prone model by mating DBA/2 males with CBA/J females, which is a well-established abortion-prone murine model and has long been used in research. The embryo-resorption rate of the normal pregnancy model and the abortion-prone model were approximately 20% and 40%, respectively (Fig. 1A), which is consistent with previous reports [18, 19]. Then, we examined the expression of Tim-3 and PD-1 on decidual CD8<sup>+</sup> T cells (dCD8<sup>+</sup> T cells) and on spleen CD8<sup>+</sup> T cells (sCD8<sup>+</sup> T cells) in both models. We found that dCD8<sup>+</sup> T cells co-expressing Tim-3 and PD-1 in the normal pregnancy model accounted for approximately 6% of DICs, a higher percentage than in the abortion-prone model (Fig. 1B). In contrast, over 60% of dCD8<sup>+</sup> T cells from the abortion-prone model expressed neither Tim-3 nor PD-1, which is a higher percentage than in the normal pregnancy model (Fig. 1C). In both models, the percentage of Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells in DICs was higher than that in splenocytes (Fig. 1B and C). These results demonstrate that Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells are preferentially distributed in the decidua during normal murine pregnancy and are down-regulated in abortion-prone matings.

### Higher anti-inflammatory cytokine production by Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells in normal pregnancy

To determine whether CD8<sup>+</sup> T cells co-expressing Tim-3 and PD-1 exhibited a tolerant phenotype, we examined their production



**Fig. 1.** Frequency of Tim-3 and PD-1 expression on CD8<sup>+</sup> T cells in normal and abortion-prone matings. Freshly isolated DICs and splenocytes were stained with antibodies against CD8, PD-1, and Tim-3 to assess PD-1 and Tim-3 expression on CD8<sup>+</sup> T cells by flow cytometry. (A) Resorption rate of normal matings (female CBA/J × male BALB/c) and abortion-prone matings (female CBA/J × male DBA/2). n = 9. (B) Frequency of Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells among decidual immune cells (DICs) and splenocytes during early pregnancy of normal and abortion-prone matings. n = 9. (C) Frequency of Tim-3<sup>-</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells among decidual immune cells (DICs) and splenocytes during early pregnancy of normal and abortion-prone matings. n = 9. Data represent mean ± SEM from three independent experiments. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001. N: normal matings; A: abortion-prone matings.

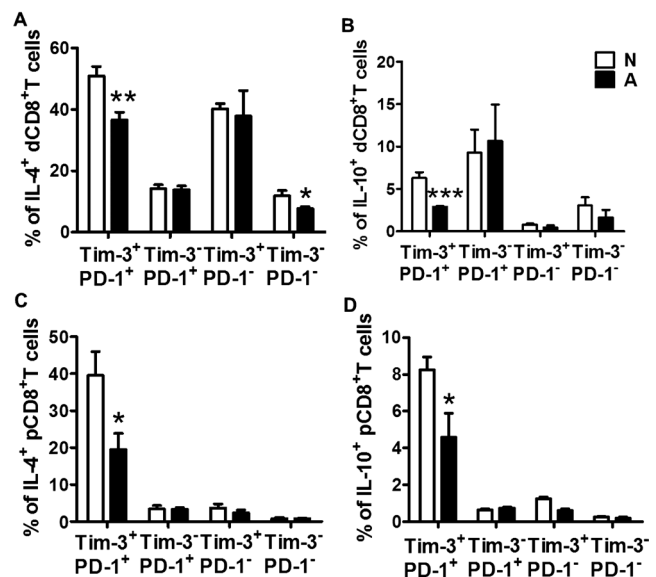
of anti-inflammatory cytokines IL-4 and IL-10 in normal pregnancy and miscarriage. We found that in normal pregnant females, the subset of CD8<sup>+</sup> T cells co-expressing Tim-3 and PD-1 produced the largest amount of IL-4 in both the decidua and the spleen (Fig. 2A and C), as well as the largest amount of IL-10 in the spleen (Fig. 2D), compared with CD8<sup>+</sup> T cells expressing either Tim-3 or PD-1 alone or expressing neither. However, in abortion-prone matings, the production of IL-4 and IL-10 was down-regulated in Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells, both in the decidua and the spleen (Fig. 2). These data indicate that Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells have the capacity of producing more anti-inflammatory and regulatory cytokines during normal pregnancy, which is dysregulated in miscarriage.

#### *Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells produced more IFN- $\gamma$ in abortion-prone matings*

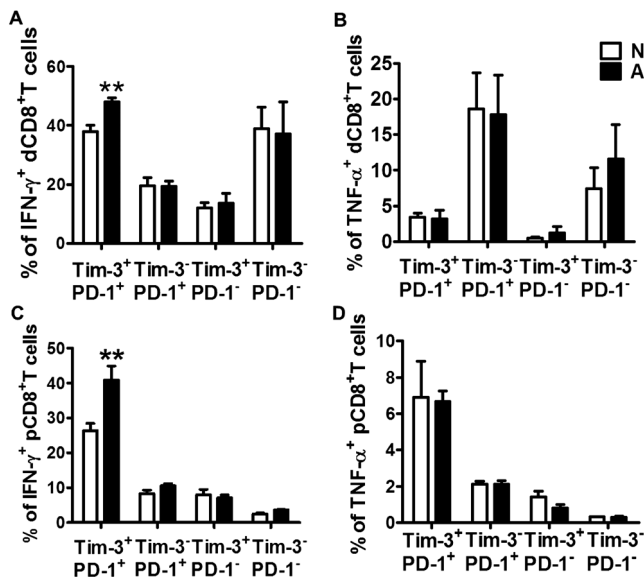
We investigated the production of pro-inflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$  in different subsets of decidual and spleen CD8<sup>+</sup> T cells in normal pregnancy and miscarriage. We found that the production of IFN- $\gamma$  by Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells in both the decidua and the spleen were up-regulated in abortion-prone matings compared with normal matings (Fig. 3A and C). This up-regulation was not observed in other CD8<sup>+</sup> T cell subsets expressing either Tim-3 or PD-1 or in those expressing neither (Fig. 3A and C). There was no significant difference between the production of TNF- $\alpha$  by Tim-3<sup>+</sup>PD-1<sup>+</sup>dCD8<sup>+</sup> T cells and Tim-3<sup>+</sup>PD-1<sup>+</sup>sCD8<sup>+</sup> T cells in normal and abortion-prone matings (Fig. 3B and D). Combined with what we found in Fig. 2, these results suggest that decidual and spleen Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells display a tolerant phenotype in cytokine production during normal pregnancy that is disturbed in miscarriage.

#### *Interception of Tim-3 and PD-1 signals was related with severe embryo resorption*

To further investigate whether Tim-3 and PD-1 signaling pathways participate in normal pregnancy maintenance, we challenged the pregnant female mice from the normal pregnancy model with Tim-3- and/or PD-1-blocking antibodies. Inhibition of either of these



**Fig. 2.** Production of anti-inflammatory cytokines by CD8<sup>+</sup> T cells in normal and abortion-prone matings. (A) Quantitation of flow-cytometric analysis of IL-4 expression by Tim-3<sup>+</sup>PD-1<sup>+</sup>, Tim-3<sup>-</sup>PD-1<sup>+</sup>, Tim-3<sup>+</sup>PD-1<sup>-</sup>, and Tim-3<sup>-</sup>PD-1<sup>-</sup> decidual CD8<sup>+</sup> T cells during early pregnancy of normal and abortion-prone matings. n = 9. (B) Quantitation of flow-cytometric analysis of IL-10 expression by Tim-3<sup>+</sup>PD-1<sup>+</sup>, Tim-3<sup>-</sup>PD-1<sup>+</sup>, Tim-3<sup>+</sup>PD-1<sup>-</sup>, and Tim-3<sup>-</sup>PD-1<sup>-</sup> decidual CD8<sup>+</sup> T cells during early pregnancy of normal and abortion-prone matings. n = 9. (C) Quantitation of flow-cytometric analysis of IL-4 expression by Tim-3<sup>+</sup>PD-1<sup>+</sup>, Tim-3<sup>-</sup>PD-1<sup>+</sup>, Tim-3<sup>+</sup>PD-1<sup>-</sup>, and Tim-3<sup>-</sup>PD-1<sup>-</sup> spleen CD8<sup>+</sup> T cells during early pregnancy of normal and abortion-prone matings. n = 9. (D) Quantitation of flow-cytometric analysis of IL-10 expression by Tim-3<sup>+</sup>PD-1<sup>+</sup>, Tim-3<sup>-</sup>PD-1<sup>+</sup>, Tim-3<sup>+</sup>PD-1<sup>-</sup>, and Tim-3<sup>-</sup>PD-1<sup>-</sup> spleen CD8<sup>+</sup> T cells during early pregnancy of normal and abortion-prone matings. n = 9. Data represent mean ± SEM from three independent experiments. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001. N: normal matings; A: abortion-prone matings.

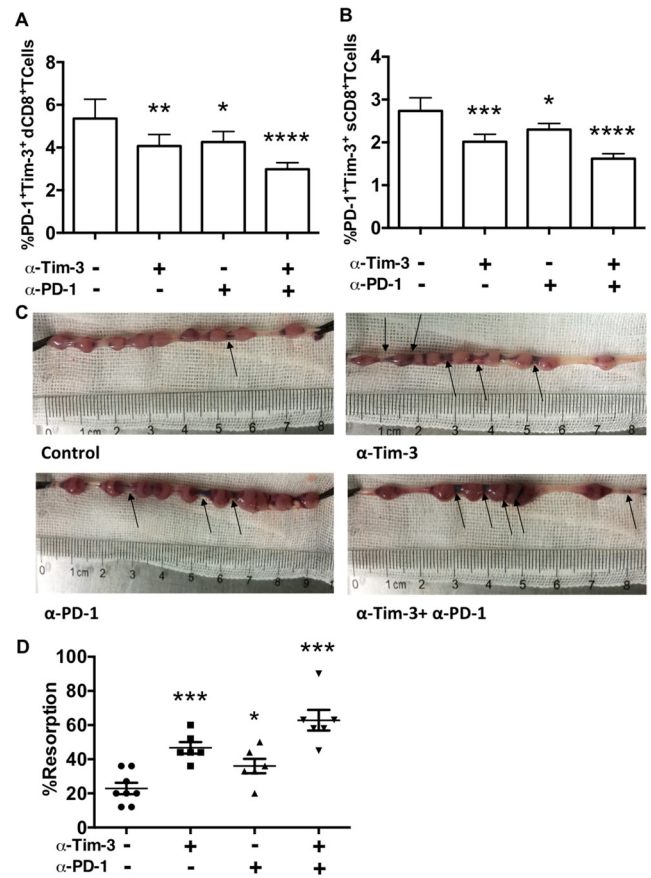


**Fig. 3.** Production of pro-inflammatory cytokines by CD8<sup>+</sup> T cells in normal and abortion-prone matings. (A) Quantitation of flow-cytometric analysis of IFN- $\gamma$  expression by Tim-3<sup>+</sup>PD-1<sup>+</sup>, Tim-3<sup>+</sup>PD-1<sup>-</sup>, Tim-3<sup>-</sup>PD-1<sup>+</sup>, and Tim-3<sup>-</sup>PD-1<sup>-</sup> decidual CD8<sup>+</sup> T cells during early pregnancy of normal and abortion-prone matings. *n* = 9. (B) Quantitation of flow-cytometric analysis of TNF- $\alpha$  expression by Tim-3<sup>+</sup>PD-1<sup>+</sup>, Tim-3<sup>+</sup>PD-1<sup>-</sup>, Tim-3<sup>-</sup>PD-1<sup>+</sup>, and Tim-3<sup>-</sup>PD-1<sup>-</sup> decidual CD8<sup>+</sup> T cells during early pregnancy of normal and abortion-prone matings. *n* = 9. (C) Quantitation of flow-cytometric analysis of IFN- $\gamma$  expression by Tim-3<sup>+</sup>PD-1<sup>+</sup>, Tim-3<sup>+</sup>PD-1<sup>-</sup>, Tim-3<sup>-</sup>PD-1<sup>+</sup>, and Tim-3<sup>-</sup>PD-1<sup>-</sup> spleen CD8<sup>+</sup> T cells during early pregnancy of normal and abortion-prone matings. *n* = 9. (D) Quantitation of flow-cytometric analysis of TNF- $\alpha$  expression by Tim-3<sup>+</sup>PD-1<sup>+</sup>, Tim-3<sup>+</sup>PD-1<sup>-</sup>, Tim-3<sup>-</sup>PD-1<sup>+</sup>, and Tim-3<sup>-</sup>PD-1<sup>-</sup> spleen CD8<sup>+</sup> T cells during early pregnancy of normal and abortion-prone matings. *n* = 9. Data represent mean  $\pm$  SEM from three independent experiments. \*\* *P* < 0.01. N: normal matings; A: abortion-prone matings.

pathways resulted in the down-regulation of Tim3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells in both the decidua and the spleen (Fig. 4A and B), as well as a greater susceptibility to fetal loss manifesting as a higher rate of embryo resorption (Fig. 4C and D). Furthermore, pregnant mice treated with both Tim-3- and PD-1-blocking antibodies represented the most severe cases of embryo resorption (Fig. 4C and D), indicating that the combined blocking of Tim-3 and PD-1 pathways had a synergistic damaging effect on pregnancy maintenance.

## Discussion

CD8<sup>+</sup> T cells are important immune regulators of maternal-fetal tolerance [8]. In the first-trimester human decidua, approximately 10–20% of lymphocytes are CD3<sup>+</sup>TCR<sup>+</sup> T cells. Among them, up to 45–75% are CD8<sup>+</sup> T cells, and 30–45% are CD4<sup>+</sup> T cells [20]. EVT cells invade deep into the decidua during early human gestation, and maternal CD8<sup>+</sup> T cells can directly recognize the embryo-derived antigens expressed by the EVT cells [8]. Nevertheless, cytotoxic responses are not induced in normal pregnancy. Most of the dCD8<sup>+</sup> T cells are not naïve cells, but activated effector memory T cells [21],



**Fig. 4.** The effects of inhibiting Tim-3 and/or PD-1 signal in early pregnancy. Pregnant mice were sacrificed at day 10.5 of pregnancy, the uteri were removed, and the implantation sites and resorbed/live embryos were counted to assess the effect of anti-Tim-3 and anti-PD-1 antibody treatment on pregnancy. (A) Frequency of Tim-3<sup>+</sup>PD-1<sup>+</sup> decidual CD8<sup>+</sup> T cells from CBA/J females mated to BALB/c males following treatment with isotype IgG, anti-Tim-3 antibody, anti-PD-1 antibody, or both antibodies. (B) Frequency of Tim-3<sup>+</sup>PD-1<sup>+</sup> spleen CD8<sup>+</sup> T cells from CBA/J females mated to BALB/c males following treatment with isotype IgG or the indicated blocking antibodies. (C) Representative images of embryos from mice treated with isotype IgG (control) or the indicated blocking antibodies. Arrows point to the resorption sites. (D) The resorption rate of pregnant CBA/J females mated to BALB/c males following treatment with isotype IgG or the indicated blocking antibodies. Data represent mean  $\pm$  SEM of *n* = 6–8 mice per group and are representative of three independent analyses. \* *P* < 0.05, \*\*\* *P* < 0.001, compared with the control group.  $\alpha$ -Tim-3: anti-Tim-3 antibody;  $\alpha$ -PD-1: anti-PD-1 antibody.

suggesting that dCD8<sup>+</sup> T cells interact closely with the invading EVTs and are affected by the allogeneic antigens. Compared with peripheral CD8<sup>+</sup> T cells, dCD8<sup>+</sup> T cells are hypotoxic, expressing significantly lower levels of the cytolytic molecules perforin and granzyme B [21]. Thus, it has been proposed that a population of regulatory or suppressor CD8<sup>+</sup> T cells might exist and contribute to maternal-fetal tolerance during normal human pregnancy [22, 23]. In the junctional zone, which belongs to the fetal compartments of

the mouse placenta, glycogen cells would migrate and invade into the decidua in a similar way as human EVT<sub>s</sub> do [24]. Therefore, in this study, we utilized murine models to investigate the phenotype and function of CD8<sup>+</sup> T cells during pregnancy.

Tim-3 and PD-1 are well known for negative modulation of the immune response and contribution to T cell exhaustion, especially in circumstances such as chronic viral infections and tumors [13, 14]. PD-1<sup>+</sup>Tim-3<sup>+</sup>CD4<sup>+</sup> T cells have been found to have impaired ability of proliferation and cytokine production [25, 26]. Particularly, PD-1<sup>high</sup>Tim-3<sup>+</sup>CD8<sup>+</sup> T cells represent a population with the most severe state of T cell exhaustion [15], exhibiting lowered ability to clear virus or tumor cells. Both in *in vitro* studies and in murine models, the combined blocking of PD-1 and Tim-3 has been reported to reverse tumor-induced T cell exhaustion, rescue lymphocyte activity, and lengthen individual survival [16, 17]. At the maternal-fetal interface, Tim-3-expressing CD8<sup>+</sup> T cells do not display the characteristics of exhaustion. Instead, they are highly proliferative and produce large amounts of anti-inflammatory cytokines [27], and Tim-3 expression on dCD8<sup>+</sup> T cells is significantly higher than on peripheral CD8<sup>+</sup> T cells during early human pregnancy [27]. However, our knowledge of the roles of these co-inhibitory molecules on maternal immune response and pregnancy maintenance is still incomprehensive.

In this study, using normal and abortion-prone murine models, we verified the dual expression of Tim-3 and PD-1 on dCD8<sup>+</sup> and sCD8<sup>+</sup> T cells during early murine pregnancy. In addition, their expression in the decidua was stronger than that in the spleen and was down-regulated in abortion-prone matings compared with normal pregnancy. These results are consistent with earlier findings in a study using human tissues [6]. Thus, we hypothesize that Tim-3 and PD-1 co-inhibitory signaling pathways might be involved in maternal-fetal tolerance and pregnancy maintenance by regulating the maternal CD8<sup>+</sup> T cell response. Further research is warranted to investigate the mechanisms regulating the expression of Tim-3 and PD-1 on CD8<sup>+</sup> T cells in normal pregnancy and miscarriage, where the microenvironment at the maternal-fetal interface, including the hormones, cytokines, growth factors, etc. secreted by decidual stromal cells and trophoblast cells, might be involved, since it has been found that co-culture with human trophoblast cells could increase the expression of Tim-3 and PD-1 on CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the human decidua and peripheral blood [5, 6].

A shift from pro-inflammatory towards anti-inflammatory cytokine production is known to be a key mechanism in the induction of maternal-fetal tolerance [3, 4]. We found that, in normal pregnancy, both in the decidua and the spleen, Tim-3- and PD-1-co-expressing CD8<sup>+</sup> T cells produced larger amounts of anti-inflammatory IL-4 and IL-10 and a smaller amount of pro-inflammatory IFN- $\gamma$  and that this was disturbed in miscarriage. It is unknown how Tim-3 and PD-1 signaling pathways affect the cytokine profile of CD8<sup>+</sup> T cells, i.e. whether it is through direct contact with ligands expressed on invading fetal cells or through indirect influence via interaction with antigen-presenting cells. Whether the pro-inflammatory cytokine profile of CD8<sup>+</sup> T cells in abortion-prone matings results in or results from miscarriage also requires further investigation. *In vivo*, normal pregnant CBA/J females challenged with Tim-3- and/or PD-1-blocking antibodies were found to be more susceptible to fetal loss, with the most severe cases of embryo resorption observed

in the group where both Tim-3 and PD-1 were blocked. In normal pregnancy, Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells might have an anti-inflammatory characteristic, which is disturbed in miscarriage, and the interference of these signals might lead to pregnancy failure.

In summary, we studied the expression of co-inhibitory molecules Tim-3 and PD-1 on decidual and spleen CD8<sup>+</sup> T cells in normal and abortion-prone early murine gestation, as well as the roles of these signals in the maintenance of healthy pregnancy. Our research suggests that the signaling pathways of Tim-3 and PD-1 are crucial for inducing a tolerant phenotype of CD8<sup>+</sup> T cells, and that the subset of Tim-3<sup>+</sup>PD-1<sup>+</sup>CD8<sup>+</sup> T cells in the decidua and the spleen might be involved in the establishment of maternal-fetal tolerance and thus contribute to pregnancy maintenance. These data might be helpful for the development of novel immunotherapies to treat diseases like RSA. Future studies are needed to unveil the underlying mechanisms of how Tim-3 and PD-1 mediate the tolerant state of CD8<sup>+</sup> T cells during early gestation and modulate maternal-fetal immunity.

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

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