Effects of combined treatment with PD-L1 Ig and CD40L mAb on immune tolerance in the CBA/J x DBA/2 mouse model

GUANFEI LI*, LIHUA YANG*, DAN LI, JINHONG ZHANG, LING DU, LIBIN XIA, YUNHUA LIU and WANQIN HU

Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650101, P.R. China

Received August 29, 2019; Accepted January 1, 2020

DOI: 10.3892/mmr.2020.10977

Abstract. The embryo is a natural allograft and is the only exception to immune rejection, which reflects maternal immune tolerance towards the embryo. However, pregnancy loss is primarily caused by maternal immune rejection of the embryo. The aim of the present study was to explore the effects of combined treatment of programmed death-ligand 1 (PD-L1) immunoglobulin (Ig) and CD40-ligand (CD40L) monoclonal antibody (mAb) on immune tolerance in an abortion-prone mating model. Mice were divided into the normal, spontaneous abortion, PD-L1 Ig, CD40L mAb and the PD-L1 Ig + CD40L mAb groups. On day 14 of gestation, the embryo resorption abortion rates of all the groups was observed. The maternal hypo-responsiveness to paternal antigens was determined using a mixed lymphocyte response and the splenic CD4+CD25+ T-cell population, major histocompatibility complex (MHC)-II+, CD80+ and CD86+ cell populations in pregnant female CBA/J mice were analyzed using flow cytometry. The expression levels of intracellular cytokines in the splenic tissues of pregnant CBA/J female mice were analyzed using western blotting. The PD-L1 Ig + CD40L group displayed the lowest resorption rate compared with the other groups. A significant decrease in the proliferative response of maternal splenic immunocompetent cells against paternal antigens, and a significant increase in the proliferative response of maternal splenic CD4+CD25+ T cells was observed in the PD-L1 Ig + CD40L group compared with the spontaneous abortion group. The number of MHC-II+, CD80+ and CD86+ bone marrow-derived dendritic cells (DCs) generated by female mice, and the levels of tumor necrosis factor-α and interferon-γ in the spleens of female mice were significantly decreased in the PD-L1 Ig + CD40L mAb group compared with the spontaneous abortion group. By contrast, interleukin-4 levels were significantly increased in the PD-L1 Ig + CD40L mAb group compared with the spontaneous abortion group. The results suggested that the administration of PD-L1 Ig + CD40L mAb on day 4 of gestation, the period of peri-implantation, may induce paternal antigen-specific immunotolerance, leading to the embryo resorption rate of the abortion-prone model being similar to that of the normal pregnancy model. The results indicate that the combined treatment of PD-L1 Ig and anti-CD40L mAbs may serve as a potential therapeutic for pregnancy loss.

Introduction

Normal pregnancy involves a special type of alloimmune tolerance. The fetus, a semi-allograft, escapes from maternal immune attack, survives and develops until delivery; a process that is dependent on maternal immune tolerance (1). Abortion results from the immune rejection of the natural implant by the mother. Normal physiological pregnancy is similar to allotransplantation; as a natural allograft, the embryo is not immune to maternal rejection, but may be the only exception to immune rejection, which reflects maternal immune tolerance to the embryo. However, pregnancy failure is primarily caused by maternal immune rejection of the embryo (1). During successful pregnancy, maternal decidual cells and fetal trophoblasts can produce various chemokines, such as CC chemokine ligand 4 and cytokines, including interleukin (IL)-4/5, which contribute to a unique maternal-fetal immune environment that prevents fetal alloantigens from inducing maternal immune attack (2).

Spontaneous abortion occurs before 28 weeks of gestation, when the embryo or fetus is <1,000 g in weight. Spontaneous abortions account for 0.4-0.8% of all abortions in women of childbearing age and account for 10-15% of the total number of abortions. Early abortions account for the remaining 80% of total abortions (2). Fetal loss caused by maternal immune attack has been intensively studied for years (3). However, the mechanism of spontaneous abortion is complicated and is not completely understood.

*Contributed equally

Correspondence to: Dr Wanqin Hu, Department of Obstetrics and Gynecology, The Second Affiliated Hospital of Kunming Medical University, 374 Diamian Road, Kunming, Yunnan 650101, P.R. China

E-mail: huwanqin0723@163.com

Key words: programmed death-ligand 1 immunoglobulin, anti-CD40 ligand monoclonal antibody, T cell, dendritic cell, abortion-prone model, immunotolerance
Programmed cell death 1 (PD-1), also named PDCD1 and CD279, is a type I transmembrane protein consisting of 288 amino acid residues that belongs to the B7-CD28 receptor superfamily (4). PD-1 is expressed on the surface of bone marrow cells, dendritic cells (DCs), natural killer cells (NKs), monocytes, CD4-CD8-thymus cells, regulatory T cells, B cells and antigen-presenting cells (5). The PD-1 gene was identified in a study conducted by Ishida et al (4) in 1992 aiming to identify the gene that induces programmed cell death. In 1998, Nishimura et al (6) reported that mice lacking the PD-1 gene developed lupoid autoimmune disease, and the negative immune regulatory function of PD-1 was not present. Subsequently, the two PD-1 ligands, programmed death-ligand (PD-L)1 and PD-L-2, were discovered (7-9). PD-1 is an inhibitory immunoreceptor that is expressed on the surface of T cells under certain conditions (10). PD-L1 has a wide tissue expression profile and is expressed in certain malignant tumor cells, such as ovarian cancer and head and neck squamous cell carcinoma, which may be related to the tumor immune escape mechanism (11-13). A number of studies have reported that the PD-1/PD-L signaling pathways play a role in the negative regulation of the immune response (14,15). Previous studies have reported that the activation and maturation of B cells and the production of self-reactive antibodies in a mouse heart transplantation model. Furthermore, acute rejection and the production of self-reactive antibodies in a mouse heart transplantation model. Additionally, the negative regulation of alloantigens, reducing the risk of rejection (21,22). The CD40 ligand (CD40L), also known as CD154, is a member of the tumor necrosis factor superfamily (19). CD40L is mainly expressed on the surfaces of activated CD4+ T cells, providing synergistic stimulation signals necessary for the activation of T and B cells. CD40L is also expressed on the surface of CD8+ T cells, B cells, macrophages and dendritic cells, as well as on the surface of non-immune cells, including endothelial cells and activated platelets (20). Larsen et al (21) reported that blocking the CD40-CD40L signaling pathway with an anti-CD40L monoclonal antibody (mAb) could prevent acute rejection and the production of self-reactive antibodies in a mouse heart transplantation model. Furthermore, Coenen et al (22) suggested that anti-CD40L mAbs could induce the proliferation of CD4+CD25+ T cells in vitro. Blocking the CD40-CD40L signaling pathway can block the activation of CD4+ T cells directly, or indirectly by blocking the activation and maturation of B cells and the production of alloantigens, reducing the risk of rejection (21,22). However, a limited number of studies have examined the role of anti-CD40L mAbs during spontaneous abortion (21-25). Therefore, the present study aimed to investigate the effects of PD-L1 Ig and anti-CD40L mAbs on spontaneous abortion in a CBA/J x DBA/2 abortion-prone mouse model.

Materials and methods

Animals and groups. A total of 50 female CBA/J, 20 male DBA/2 and 5 male BALB/c mice (age, 8-10 weeks; weight, 12-15 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were maintained under controlled conditions at 19-23°C with 12-h light/dark cycles and 40-60% humidity, with free access to drinking water and food. Animal experiments were approved by the Institutional Animal Care and Use Committee of Kunming Medical University.

The mice were divided into the following five groups: the normal group (10 CBA/J mice), the spontaneous abortion group (10 CBA/J mice), the PD-L1 Ig group (0.1 mg/kg PD-L1 Ig; 10 CBA/J mice), the CD40L mAb group (0.2 mg/kg anti-CD40L mAbs; 10 CBA/J mice) and the PD-L1 Ig + CD40L mAb group (0.1 mg/kg PD-L1 Ig and 0.2 mg/kg anti-CD40L mAbs; 10 CBA/J mice). The normal group was mated with male BALB/c mice (n=5). The spontaneous abortion, PD-L1 Ig, CD40L mAb and PD-L1 Ig + CD40L mAb groups were mated with male DBA/J mice (n=5/group). The day the vaginal plug was observed was recorded as day 0 of gestation. PD-L1 Ig and/or anti-CD40L mAbs were purchased from R&D Systems.

Analysis of the embryo absorption rate. Pregnant CBA/J female mice in each group were euthanized on day 14 of gestation, and the number of absorbed embryos and surviving embryos were counted. The embryo resorption rate was calculated according to the following formula: resorption rate (%) = the number of absorbed embryos/the number of viable embryos x 100.

Isolation of splenocytes. At day 14 of gestation, the spleens of five pregnant CBA/J mice and five paternal mice from each group were aseptically removed and mechanically teased out of the stroma in PBS. The cell suspensions were filtered through a 100-mm pore size nylon mesh and centrifuged at 1,000 x g for 10 min at 4°C. Subsequently, the supernatant was removed. Following the addition of Lymphocyte Isolation Fluid (Beijing Solarbio Science & Technology Co., Ltd.), the spleen cells were centrifuged at 800 x g for 30 min at 4°C. After centrifugation, the splenic mononuclear cells were carefully isolated and washed twice with a 3-fold volume of PBS. The cells were counted and the cell concentration was adjusted to 1x10^7 cells/ml with PBS.

Isolation of bone marrow-derived dendritic cells (BMDCs). At day 14 of gestation, BMDCs were generated from five pregnant CBA/J mice from each group. Briefly, femora and tibiae were removed from CBA/J mice and were mechanically isolated from the surrounding tissues. The samples were centrifuged at 1,000 x g for 10 min at room temperature and the supernatant was discarded. Subsequently, 5 ml Tris-NH4Cl solution was added to the cells and the samples were incubated at room temperature for 2 min to fully lyse the red blood cells. The samples were centrifuged at 1,000 x g for 5 min at room temperature, the supernatant was discarded and 5 ml PBS was added to resuspend the cells. The samples were centrifuged at 1,000 x g for 5 min at room temperature, the supernatant was discarded and the samples were washed three times with PBS. RPMI-1640 complete medium (Logan; GE Healthcare Life Sciences), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), was used to adjust the cell concentration to 1x10^7 cells/ml with PBS.
concentration to 2x10^6 cells/ml. The cells were incubated in a culture dish at 37°C overnight. Subsequently, the unadhered cells and culture medium were discarded and the adherent cells were washed twice with PBS. Fresh culture medium was added to the adherent cells and changed every other day by removing and replacing half of the volume; the adherent cells were used for subsequent experimentation.

Flow cytometry. The expression of cell surface molecules was evaluated using a Sysmex Partec CyFlow® space flow cytometer (Sysmex Partec GmbH) and FloMax version 2.8 software (Sysmex Partec GmbH). Cells were fixed with 70% ethanol at 4°C for 2 h and blocked with 2% BSA (Beijing Solarbio Science & Technology Co., Ltd.) at 4°C for 30 min. The splenic cells were labeled with both anti-mouse CD25 FITC (cat. no. BG-07312-50-100; BioGems) and anti-mouse CD4 PE (cat. no. 85-12-0041-81; eBioscience; Thermo Fisher Scientific, Inc.) in the dark at 4°C for 30 min. The BMDCs were labeled with anti-mouse MHc-II FITC (cat. no. 85-11-5321-81; eBioscience; Thermo Fisher Scientific, Inc.), anti-mouse CD80 FITC (cat. no. 85-11-0801-81; eBioscience; Thermo Fisher Scientific, Inc.) and anti-mouse CD86 FITC (cat. no. 85-11-0862-81; eBioscience; Thermo Fisher Scientific, Inc.) in the dark at 4°C for 30 min. Subsequently, the cells were centrifuged at 800 x g at 4°C for 5 min and the supernatant was removed. The cells were washed twice with PBS and resuspended in PBS for flow cytometry analysis. The control cells were stained with the corresponding isotype-matched antibody for the same duration and temperature as the other cells. The isotype-matched antibodies for CD25, CD4, CD86, and MHc-II were Rat IgG2a κ Isotype control (eB2a)-FITC (cat. no. 11-4321-80; eBioscience; Thermo Fisher Scientific, Inc.), Rat IgG2b κ Isotype control (eB149/10H5)-PE (cat. no. 12-4031-82; eBioscience; Thermo Fisher Scientific, Inc.), Armenian Hamster IgG Isotype control (eBio299Arm)-FITC (cat. no. 11-4888-81; eBioscience; Thermo Fisher Scientific, Inc.), Rat IgG2a κ Isotype control (eB2a)-FITC and Rat IgG2b κ Isotype control (eB149/10H5)-FITC, respectively. Cells were analyzed using a flow cytometer. The flow cytometry results are presented as the percentage of cells positive for the surface marker evaluated. The experiment was repeated three times.

**Mixed lymphocyte response.** Splenocytes from five pregnant CBA/J mice from each group on day 14 of gestation were used as responder cells, and paternal splenocytes were used as stimulator cells. Firstly, 100 µl responder cells (2x10⁶ cells/well) and 100 µl mitomycin C (50 µg/ml; Sigma-Aldrich; Merck KGaA)-treated stimulator cells (2x10⁶ cells/well; stimulator cells were incubated with mitomycin C at 37°C for 30 min) were aliquoted into 96-well plates. Responder cells cultured with complete medium alone in 96-well plates were used as the control. After a 3-day incubation at 37°C, ³H-thymidine (20 µCurie/well) was added to the cells and incubated for 6 h at 37°C. The cells were harvested onto glass-fiber paper using a cell harvester, and the count per minute (cpm) was measured using a liquid scintillation counter. The proliferative capacity was presented as the stimulatory index (SI), calculated according to the following equation: SI=(cpm of stimulated cultures-cpm of control cultures)/cpm of control cultures. The experiment was repeated three times.

Western blotting. Total protein was extracted from spleen tissues isolated from five pregnant CBA/J mice from each group on day 14 of gestation using RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein (30 µg/lane) was quantified using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) and separated by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 10% skim milk at room temperature for 4 h. Subsequently, the membranes were incubated at 4°C overnight with the following primary antibodies: Anti-FoxP3 (cat. no. bs-0269R; 1:1,000; BIOSS), anti-TNF-α (cat. no. A0277; 1:1,000; ABclonal Biotech Co., Ltd.), anti-IFN-γ (cat. no. bs-0480R; 1:1,000; BIOSS), anti-IL-4 (bs-0581R; 1:1,000; BIOSS) and anti-β-actin (cat. no. AC026; 1:2,000; ABclonal Biotech Co., Ltd.). Subsequently, the membranes were incubated with a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. bs-0295G-HRP; BIOSS). Protein bands were visualized using ECL Plus Western Blotting Detection reagents (EMD Millipore). Blots were performed in triplicate and protein expression was quantified using ImageJ 2x software (National Institutes of Health) with β-actin as the loading control.

**Statistical analysis.** Data are expressed as the mean ± standard deviation. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test. All statistical analyses were performed using GraphPad Prism software (version 5.0a; GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Combined therapy with PD-L1 Ig and anti-CD40L mAbs reduces the embryo resorption rate of abortion-prone CBA/J x DBA/2-mated mice.** To investigate whether combined treatment with PD-L1 Ig and anti-CD40L mAbs decreases the rate of fetal abortion in vivo, PD-L1 Ig and/or anti-CD40L mAbs were intraperitoneally injected into pregnant CBA/J females on days 4, 6, 8, 10, and 12 of gestation. The embryo resorption rate was determined on day 14 of gestation. Absorbed embryos (Fig. 1B-D) displayed hemorrhage, ischemia and necrosis, and were smaller and darker compared with healthy embryos (Fig. 1A and E). Treatment of pregnant CBA/J females with PD-L1 Ig or anti-CD40L mAbs significantly reduced the resorption rate compared with the spontaneous abortion group (Fig. 1F). Combined treatment with PD-L1 Ig and anti-CD40L mAbs also significantly reduced the resorption rate compared with the spontaneous abortion group (Fig. 1F). There was no significant difference between the normal group and the PD-L1 Ig + CD40L mAbs group (Fig. 1F). The results suggested that combined treatment with PD-L1 Ig and anti-CD40L mAbs was effective in preventing maternal rejection of the embryo.

**Combined treatment with PD-L1 Ig and anti-CD40L mAbs induces maternal hyporesponsiveness to paternal antigens in the CBA/J x DBA/2 mating model.** To further investigate the inhibitory effects of PD-L1 Ig and anti-CD40L mAb treatment on the maternal responses to paternal antigens, a mixed lymphocyte reaction proliferation assay was performed.
Combined treatment with PD-L1 Ig and anti-CD40L mAbs significantly decreased the proliferation of CBA/J splenocytes in response to DBA/2 stimulator cells compared with the spontaneous abortion group. Furthermore, the inhibitory effect of the combined treatment resulted in lower proliferation of CBA/J splenocytes compared with the PD-L1 Ig or CD40L mAb group (Fig. 2). The results indicated that combined treatment with PD-L1 Ig and anti-CD40L mAbs successfully induced maternal hyporesponsiveness to paternal antigens. Therefore, the results suggested that combined therapy inhibited maternal T-cell activation to prevent overactivation of the immune system in vivo.

Combined treatment with PD-L1 Ig and anti-CD40L mAbs expands the peripheral CD4+CD25+ T-cell population in the CBA/J x DBA/2 mating model. To further investigate the mechanisms involved in the inhibitory effects of PD-L1 Ig and anti-CD40L mAbs on abortion, the splenic CD4+CD25+ T-cell population in pregnant female CBA/J mice was analyzed by flow cytometry (Fig. 3A-C). The spontaneous abortion group displayed a significant decrease in the percentage of CD4+CD25+ T cells within the CD4+ T-cell population compared with the normal group (Fig. 3C). The percentage of CD4+CD25+ T cells within the CD4+ T-cell population in the spleens of the PD-L1 Ig + CD40L mAb group was significantly increased compared with the spontaneous abortion, PD-L1 Ig and CD40L mAb groups (Fig. 3C). There was no significant difference between the PD-L1 Ig or CD40L mAb group, and the normal group (Fig. 3C). The results indicated that combined treatment with PD-L1 Ig and anti-CD40L mAbs activated the splenic CD4+CD25+ T cells in the CBA/J x DBA/2 mating model.

Figure 1. Combined treatment with PD-L1 Ig and anti-CD40L mAbs decreases the embryo resorption rate in the CBA/J x DBA/2 mating model. Representative images of the number of embryos per uterus in (A) the normal group, (B) the spontaneous abortion group, (C) the PD-L1 Ig group, (D) the CD40L group and (E) the PD-L1 Ig + CD40L group. The black arrows indicate embryos that displayed hemorrhage, ischemia and necrosis. PD-L1 Ig and/or anti-CD40L mAbs were injected intraperitoneally into pregnant CBA/J female mice on days 4, 6, 8, 10, and 12 of gestation. (F) Embryo resorption rates were calculated on day 14 of gestation (n=10). ***P<0.001 vs. the normal group (CBA/J x BALB/c), #P<0.05 and ###P<0.001 vs. the spontaneous abortion group (CBA/J x DBA/2). PD-L1, programmed death-ligand 1; Ig, immunoglobulin; CD40L, CD40 ligand; mAb, monoclonal antibody.
Combined treatment with PD-L1 Ig and anti-CD40L mAbs decreases the MHCII+, CD80+ and CD86+ cell populations in BMDCs. To further investigate the mechanisms involved in the inhibitory effects of PD-L1 Ig and anti-CD40L mAbs on spontaneous abortion, the MHCII+, CD80+ and CD86+ cell populations in BMDCs were determined by flow cytometry. In the spontaneous abortion group, 57.55% of the BMDC population expressed MHCII molecules, 62.07% expressed CD80 and 53.73% expressed CD86 (Fig. 4a-c). The percentage of MHCII+, CD80+ and CD86+ cells in the spontaneous abortion group was significantly increased compared with the normal group (Fig. 4C). The PD-L1 Ig, CD40L mAb and PD-L1 Ig + CD40L mAb groups displayed a significant decrease in the percentages of MHC-II+, CD80+ and CD86+ cells compared with the spontaneous abortion group (Fig. 4C). However, the PD-L1 Ig + CD40L mAb group displayed the lowest percentages of MHC-II+, CD80+ and CD86+ cells out of the three treatment groups. Immature DCs expressed low levels of MHC-II, CD80 and CD86, and mature DCs expressed high levels of MHC-II, CD80 and CD86 (26,27).
The results indicated that combined treatment with PD-L1 Ig and anti-CD40L mAbs decreased DC maturation in the CBA/J x DBA/2 mating model.

Combined treatment with PD-L1 Ig and anti-CD40L mAbs decreases TNF-α and INF-γ expression and increases IL-4 expression in the CBA/J x DBA/2 mating model. The expression of intracellular cytokines, including TNF-α, INF-γ and IL-4, in the spleen tissues of pregnant CBA/J female mice were determined by western blotting (Fig. 5A-D). The levels of TNF-α and INF-γ in the spontaneous abortion group were significantly increased, and the level of IL-4 was significantly decreased compared with the normal group (Fig. 5B-D). The PD-L1 Ig + CD40L mAb group displayed lower expression levels of TNF-α and INF-γ, and higher expression levels of IL-4 compared with the spontaneous abortion, PD-L1 Ig and CD40L mAb groups (Fig. 5B-D). The results suggested that combined treatment with PD-L1 Ig and anti-CD40L mAbs decreased TNF-α and INF-γ expression and increased IL-4 expression in the CBA/J x DBA/2 mating model.

Discussion

Normal pregnancy is a complex physiological process that is similar to successful allotransplantation. The maternal immune system is stimulated by paternal human leukocyte antigens (HLA) carried by the fetus, resulting in a corresponding immune response, however, the mother often develops immune tolerance to the fetus. If the immune tolerance is disrupted, it can lead to the occurrence of abortion (1). The mechanism of maternal and fetal immune tolerance is a focus for research in the field of reproductive immunology.

PD-L1, in combination with PD-1, can significantly regulate the expression of cytokines to inhibit the function of T cells and promote T cell apoptosis (11). PD-L1 combined with PD-1 inhibits cell proliferation and cytokine production (28,29). CD40 and CD40L are costimulatory molecules that are involved in the specific immune response system in vivo, which is required for the humoral and cellular immune responses of the body. CD40 and CD40L play a role in B cell activation, proliferation, differentiation, antibody production and homotypic transformation. The two molecules also have a regulatory role in T cell activation and the secretion of effector cytokines (30-32). Abnormalities in the CD40-CD40L signaling pathway can lead to pathological reactions, such as inflammation and atherosclerosis, in the body (33,34). Furthermore, blocking this costimulatory pathway, by using anti-CD40L mAbs for example, has been identified as an immunotherapy strategy. Larsen et al (21) reported that blocking the CD40-CD40L signaling pathway
Figure 5. Combined therapy with PD-L1 Ig and anti-CD40L mAbs decreases the expression of TNF-α and IFN-γ expression and increases the expression of IL-4 in the CBA/J x DBA/2 model. Protein expression levels were determined by (A) western blot analysis and quantified for (B) TNF-α, (C) IFN-γ and (D) IL-4. *P<0.05, **P<0.01 and ***P<0.001 vs. the normal group (CBA/J x BalB/c). #P<0.05, ##P<0.01 and ###P<0.001 vs. the spontaneous abortion group (CBA/J x DBA/2). PD-L1, programmed death-ligand 1; Ig, immunoglobulin; CD40L, CD40 ligand; mAb, monoclonal antibody; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL-4, interleukin-4.
with anti-CD40L mAbs could prevent acute rejection and self-reactive antibody generation in a mouse heart transplantation model.

Therefore, the CD40-CD40L signaling pathway plays a role in the formation of antibodies in the body and blocking this pathway can reduce the production of pathogenic auto-antibodies or unrelated antibodies, which might be a novel approach for the clinical treatment of related autoimmune diseases. Furthermore, it has been reported that combined treatment of anti-CD40L mAbs and CTLA-4 Ig in a mouse skin and heart transplantation model, as well as in a non-human primate kidney transplantation model, could significantly prolong the survival time of the graft (20,35,36). However, the effects of combined treatment with PD-L1 Ig and anti-CD40L mAbs on spontaneous abortion are not completely understood, and the underlying mechanisms remain unclear.

Spontaneous abortion in abortion-prone CBA/J x DBA/2-mated female mice is related to systemic maternal immune inflammation, increased lymphocyte trafficking, complement deposition and costimulatory molecules, and the activation of NK cells, macrophages and T cells in the spontaneous abortion group (37-40). In the present study, an abortion-prone model with CBA/J female mice and DBA/2 male mice was constructed to investigate the effects of PD-L1 Ig and anti-CD40L mAb treatment on spontaneous abortion. CBA/J x BALB/c mating pairs were used to model normal pregnancy. On days 4, 6, 8, 10 and 12 of gestation, 0.1 mg/kg PD-L1 Ig and/or 0.2 mg/kg anti-CD40L mAbs were injected into pregnant CBA/J female mice. On day 14 of gestation, the spleens, femora and tibiae were isolated from pregnant CBA/J female mice for subsequent experimentation. The resorption rate in the spontaneous abortion group was higher compared with all other groups. However, combined treatment with PD-L1 Ig and anti-CD40L mAbs reduced the resorption rate compared with the PD-L1 Ig or CD40L mAb groups. The proliferation assay suggested that the peripheral immune cells in the spleens of pregnant mice in the spontaneous abortion group displayed a significantly enhanced proliferation response to paternal antigens compared with the normal group. Furthermore, combined treatment with PD-L1 Ig and anti-CD40L mAbs during implantation significantly decreased the proliferation response of the peripheral immune cells in the spleen to paternal antigens in the spontaneous abortion group. The combined treatment group displayed the most significant decrease in proliferation out of the three treatment groups.

To investigate the potential mechanism involved in maternal immune tolerance, the splenic CD4+CD25+ T-cell population was assessed by flow cytometry. Increasing evidence suggests that regulatory T cells, in particular CD4+CD25+ regulatory T cells, play a role in the formation of maternal and fetal tolerance (41,42). The expansion of CD4+CD25+ T cells or the augmentation of their activity can suppress allograft rejection (43,44). The present study indicated that compared with the normal group, the proportion of CD4+CD25+ T cells in the spleens of the spontaneous abortion group was significantly reduced. This suggested that the number of regulatory T cells in the spontaneous abortion group was abnormal, which may provide an explanation for the increased embryo uptake rate in the spontaneous abortion group compared with the normal group. Combined PD-L1 Ig and anti-CD40L mAb treatment significantly increased the proportion of the CD4+CD25+ T cell population compared with either treatment administered as a monotherapy. Therefore, it could be hypothesized that combined treatment with PD-L1 Ig and anti-CD40L mAbs inhibited embryo resorption by increasing the proportion of CD4+CD25+ T cells in the spleen.

Dendritic cells (DCs) are the sentinel cells of the immune system that regulate both innate and acquired immune responses (45). Mature DCs can promote the immune response and immature DCs can inhibit the immune response; therefore, DCs are involved in immune tolerance and rejection of grafts (46). DCs were collected from the bone marrow of pregnant mice and the levels of MHC-II, CD80 and CD86 cells were determined by flow cytometry. The DCs in the spontaneous abortion group had higher MHC-II, CD80 and CD86 expression compared with all other groups, and the DCs in the combined treatment group had lower MHC-II, CD80 and CD86 expression compared with the spontaneous abortion, PD-L1 Ig and CD40L mAb groups. The results suggested that combined treatment with PD-L1 Ig and anti-CD40L mAbs inhibited the maturation of DCs and increased the number of immature DCs. Therefore, combined treatment with PD-L1 Ig and anti-CD40L mAbs might have inhibited embryo resorption by inhibiting DC maturation.

T helper (Th) cells are involved in the immune tolerance mechanism during pregnancy, and abnormal Th1- and Th2-type cytokine levels are associated with the occurrence of abortion. Th2 cytokines are the dominant type during normal pregnancy, however Th1/Th2-type cytokine balance disorders in patients experiencing abortions are typically characterized by a skew toward Th1 bias (47,48). Evidence suggests that fetal rejection can be prevented by increasing the ratio of Th2 to Th1 cytokines produced by maternal leukocytes (49). In the present study, the expression of the Th1 cytokines, TNF-α and INF-γ, and the Th2 cytokine, IL-4, were determined by western blotting. Combined treatment with PD-L1 Ig and anti-CD40L mAbs decreased the production of TNF-α and INF-γ and increased the expression of IL-4 in the spontaneous abortion group. The results suggested that combined treatment with PD-L1 Ig and anti-CD40L mAbs altered the local immune microenvironment to aid with immune tolerance and further decrease the embryo resorption rate. Therefore, combined treatment with PD-L1 Ig and anti-CD40L mAbs decreased the bias towards Th1 cell responses and increased the bias towards Th2-cell responses to maintain pregnancy.

In conclusion, in the present study, a normal pregnancy model was constructed with female CBA/J and male BALB/c mice and a spontaneous abortion model was constructed with female CBA/J and male DBA/2 mice. Subsequently, PD-L1 Ig and/or anti-CD40L mAbs were injected into pregnant CBA/J female mice. The combined treatment with PD-L1 Ig and anti-CD40L mAbs significantly reduced the embryo resorption rate by inhibiting MHC-II, CD80 and CD86 expression in DCs, decreasing TNF-α and IFN-γ levels, and increasing the CD4+CD25+ T cell population and IL-4 levels; these effects are beneficial to the maintenance of pregnancy. Thus, these findings indicated that combined treatment with PD-L1 Ig and anti-CD40L mAbs may result in maternal immune tolerance and inhibit maternal immune rejection of allogeneic embryos.
improving the outcome of pregnancy in an abortion-prone mouse model. The combined treatment with PD-L1 Ig and anti-CD40L mAb also inhibited the maturation of DCs, expanded the peripheral CD4⁺CD25⁺ T cell population and promoted a shift in cytokine polarization from Th1 to Th2. The results of the present study may aid in designing therapeutic approaches for immunological pregnancy complications and also extended the existing knowledge of how an allograft is tolerated in a foreign environment. Further investigation into the role of PDL-1 Ig and anti-CD40L mAbs in uterine immune tolerance is required.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Special Project of Yunnan Science and Technology Department-Kunming Medical University Applied Basic Research [grant no. 2017FE467(-062)] and the Medical Science Leaders Training Project of Yunnan Health commission (grant no. D-201633).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GL, LY and WH conceived and designed the study; GL, LY, DL and JZ performed the experiments; JZ, LD, LX and YL analyzed the data; LX and YL wrote the manuscript; GL, LY and WH reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the animals ethics Committee of Kunming Medical University and the Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Trowsdale J and Betz AG: Mother's little helpers: Mechanisms of maternal-fetal tolerance. Nat Immunol 7: 241-246, 2006.
2. Munoz-Suano A, Hamilton AB and Betz AG: Gimme shelter: The immune system during pregnancy. Immunol Rev 241: 20-38, 2011.
3. Bonney EA and Brown SA: To drive or be driven: The path of a mouse model of recurrent pregnancy loss. Reproduction 147: R153-R167, 2014.
4. Ishida Y, Agata Y, Shibahara K and Honjo T: Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J 11: 3887-3895, 1992.
5. Okazaki T and Honjo T: PD-1 and PD-1 ligands: From discovery to clinical application. Int Immunol 19: 813-824, 2007.
6. Nishimura H, Minato N, Nakano T and Honjo T: Immunological studies on PD-1 deficient mice: Implication of PD-1 as a negative regulator for B cell responses. Int Immunol 10: 1563-1572, 1998.
7. Carrreno BM and Collins M: The B7 family of ligands and its receptors: New pathways for costimulation and inhibition of immune responses. Annu Rev Immunol 20: 29-53, 2002.
8. Lázár-Molnár E, Yan Q, Magiera K, Dömling A, Dubin G and Holak TA: Structural biology of the immune checkpoint receptor PD-1 and its ligands PD-L1/PD-L2. Proc Natl Acad Sci USA 105: 10483-10488, 2008.
9. Zak KM, Grudnik P, Magiera K, Dömling A, Dubin G and Holak TA: Structural biology of the immune checkpoint receptor PD-1 and its ligands PD-L1/PD-L2. Structure 25: 1163-1174, 2017.
10. Nishimura H and Honjo T: PD-1: An inhibitory immunoreceptor involved in peripheral tolerance. Trends Immunol 22: 265-268, 2001.
11. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, et al: Tumor-associated B7-H1 promotes T-cell apoptosis: A potential mechanism of immune evasion. Nat Med 8: 793-800, 2002.
12. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T and Minato N: Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc Natl Acad Sci USA 99: 12293-12297, 2002.
13. Dong H and Tan L: B7-H1 pathway and its role in the evasion of tumor immunity. J Mol Med (Berl) 81: 281-287, 2003.
14. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, et al: Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192: 1027-1034, 2000.
15. Carter L, Fouser LA, Jussif J, Fitz L, Deng B, Wood CR, Collins M, Honjo T, Freeman GJ and Carreno BM: PD-1:PD-L inhibitory pathway affects both CD4⁺ and CD8⁺ T cells and is overcome by IL-2. Eur J Immunol 32: 634-643, 2002.
16. Gao L, Liu J, Tan L, Liu T, Chen Z and Shi C: The immunosuppressive properties of non-cultured dermal-derived mesenchymal stromal cells and the control of graft-versus-host disease. Biomaterials 35: 3582-3588, 2014.
17. Vanikar AV, Trivedi HL, Gopal SC, Kumar A and Dave SD: Pre-transplant co-infusion of donor-adipose tissue derived mesenchymal stem cells and hematopoietic stem cells may help in achieving tolerance in living donor renal transplantation. Ren Fail 36: 457-460, 2014.
18. Hong J, Yeom HJ, Lee E, Han KH, Koo TY, Cho B, Ro H, Oh KH, Ahn C and Yang J: Islet allograft rejection in sensitized mice is refractory to control by combination therapy of immune-modulating agents. Transpl Immunol 28: 86-92, 2013.
19. Foy TM, Aruffo A, Bajorath J, Buhlmann J et and n oelle r J: Therapeutic interventions targeting the co-stimulatory interactions play a critical role during allograft rejection. Transplantation 61: 4-9, 1996.
20. Vermeiren J, Cuuppers JL, Haegel-Kronenberger H, De Boer M, Boon L and Van Gool SW: Blocking B7 and CD40 co-stimulatory molecules decreases antiviral T cell activity. Clin Exp Immunol 153: 253-258, 2008.
21. Im SH, Barchan D, Maiti PK, Fuchs S and Souroujon MC: Blockade of CD40 ligand suppresses chronic experimental myasthenia gravis by down-regulation of Th1 differentiation and up-regulation of CTLA-4. J Immunol 166: 6893-6898, 2001.
22. Law CL and Grewal IS: Therapeutic interventions targeting CD40L (CD154) and CD40: The opportunities and challenges. Adv Exp Med Biol 647: 8-36, 2009.
26. Lewis KL and Reizis B: Dendritic cells: Arbiters of immunity and immunological tolerance. Cold Spring Harb Perspect Biol 4: a007401, 2012.

27. Pardee AD, Yano H, Weinstein AM, Ponce AA, Ethridge AD, Normolle DP, Vujanovic L, Mizejewski GJ, Watkins SC and Butterfield LH: Route of antigen delivery impacts the immunostimulatory activity of dendritic cell-based vaccines for hepatocellular carcinoma. J Immunother Cancer 3: 32, 2015.

28. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, Sasmal DK, Huang J, Kim JM, Mellman I and Vale RD: T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. Science 355: 1428-1433, 2017.

29. Patsoukis N, Brown J, Petkova V, Liu F, Li L and Boussiotis VA: Selective effects of PD-1 on Akt and Ras pathways regulate molecular components of the cell cycle and inhibit T cell proliferation. Sci Signal 5: ra46, 2012.

30. Graf D, Müller S, Korthäuer U, van Kooten C, Liu YJ, Rousset F and Saeland S: The CD40 antigen and its ligand. Annu Rev Immunol 12: 881-922, 1994.

31. Hamissian SH and Geha RS: Jak3 is associated with CD40 and is critical for CD40 induction of gene expression in B cells. Immunity 6: 379-387, 1997.

32. Schönböck U, Sukhova GK, Shimizu K, Mach F and Libby P: Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice. Proc Natl Acad Sci USA 97: 7464-7469, 2000.

33. Yin D, Ma L, Shen J, Byrne GW, Logan JS and Chong AS: CTLA-41g in combination with anti-CD40L prolongs xenograft survival and inhibits anti-gal ab production in GT-Ko mice. Am J Transplant 2: 41-47, 2002.

34. Platt JL: New directions for organ transplantation. Nature 392 (6679 Suppl): S11-S17, 1998.

35. Lin H, Mosmann TR, Guilbert L, Tuntipiprat S and Wegmann TG: Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J Immunol 151: 4562-4573, 1993.