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

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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.

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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.

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 PDL-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 cell lines, 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 PD-L1 immunoglobulin (Ig)-modified bone marrow-derived stem cells (BMSCs) inhibit rat liver transplant rejection and induce liver transplantation immune tolerance, and they display an improved effect compared with BMSCs alone (16-18). However, further investigation into the role of PD-L1 Ig during spontaneous abortion is required.

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 + CD40 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 injected intraperitoneally into CBA/J female mice at days 4, 6, 8, 10 and 12 of gestation. At day 14 of gestation, the CBA/J mice were euthanized with an overdose of sodium pentobarbital (150 mg/kg; intraperitoneally). PD-L1 Ig and 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 absorbed embryos + the number of viable embryos) x100.

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 m Tris-NH_4Cl 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 $2x10^6$ 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, CD80, CD86 and MHC-II were Rat IgG2a κ Isotype control (eBR2a)-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 (eBR2a)-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 ($2x10^5$ cells/well) and 100 μl mitomycin C (50 μg/ml; Sigma-Aldrich; Merck KGaA)-treated stimulator cells ($2x10^5$ 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, ^{3}H-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 is 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.

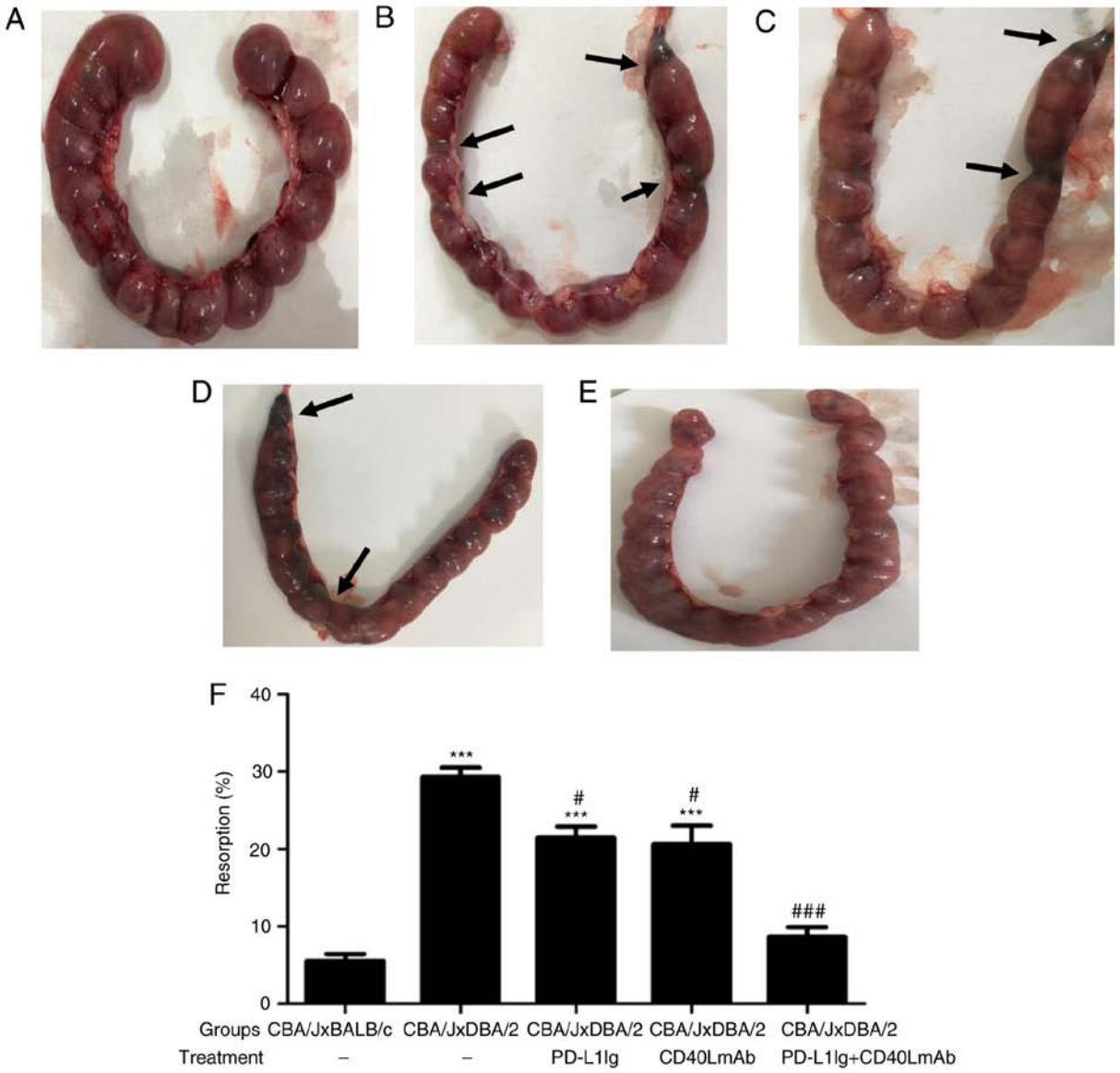

Figure 1. Combined treatment with PD-L1 Ig and anti-CD40L mAbs decreases the embryo resorption rate in the CBA/J x DBA/2 mating model. Representative images of the number of embryos per uterus in (A) the normal group, (B) the spontaneous abortion group, (C) the PD-L1 Ig group, (D) the CD40L group and (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 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.

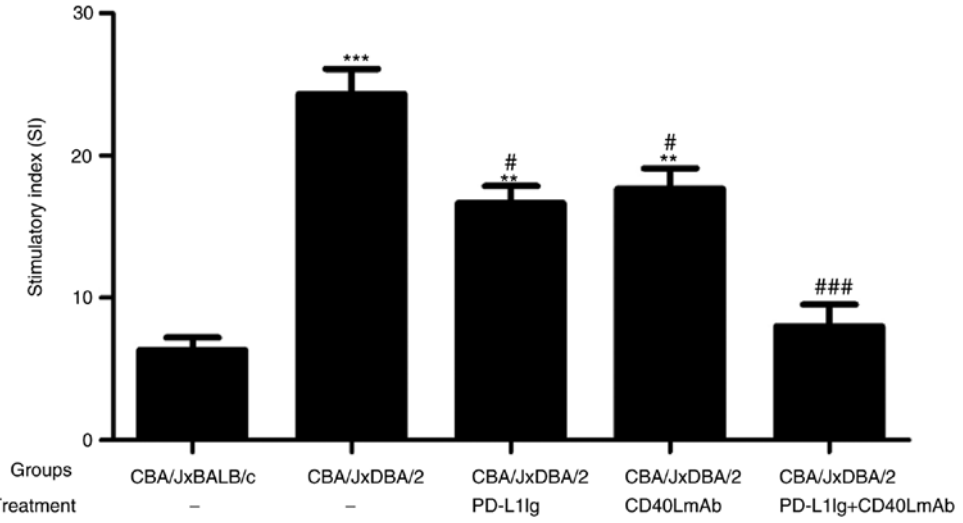

Figure 2. Combined treatment with PD-L1 Ig and anti-CD40L mAbs decreases the proliferation of maternal splenocytes in response to DBA/2 stimulator cells. On day 14 of gestation, maternal splenocytes were co-cultured with mitomycin C-treated paternal DBA/2 splenocytes for 3 days. Proliferation was measured by [^{3}H] thymidine incorporation. **P<0.01 and ***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.

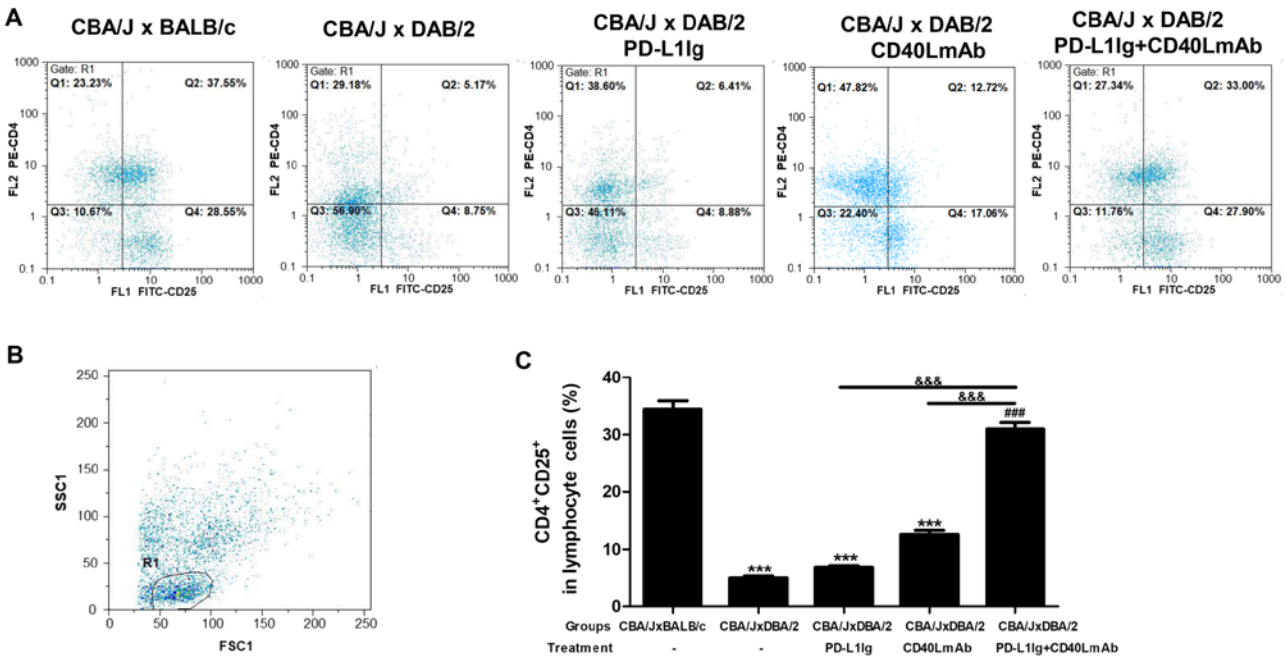

Figure 3. Expansion of the CD4$^+$CD25$^+$ T-cell population is driven by combined treatment with PD-L1 Ig and anti-CD40L mAbs in the CBA/J x DBA/2 model. (A) Number of CD4$^+$CD25$^+$ T cells in splenocytes was determined by flow cytometry. (B) Gating strategy used for flow cytometry, using the FSC/SSC method. R1 represents the T lymphocyte population. (C) Percentage of CD4$^+$CD25$^+$ T cells. ***P<0.001 vs. the normal group (CBA/J x BALB/c). $^{\#\#\#}$P<0.001 vs. the spontaneous abortion group (CBA/J x DBA/2); $^{\&\&\&}$P<0.001 vs. PD-L1 Ig + CD40L mAb group. PD-L1, programmed death-ligand 1; Ig, immunoglobulin; CD40L, CD40 ligand; mAb, monoclonal antibody; FSC, forward scatter; SSC, side scatter; PE, phycoerythrin.

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 MHC-II 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).

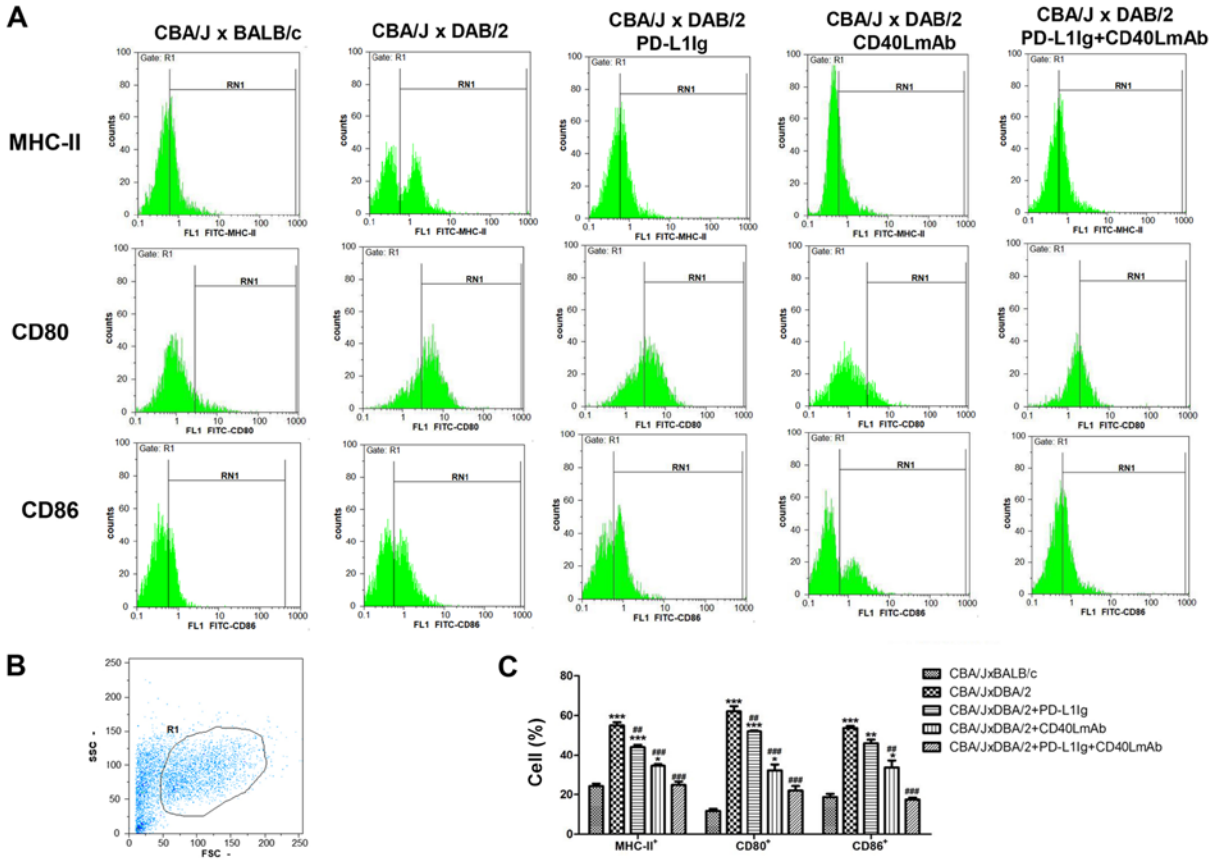

Figure 4. Decreased MHC-II, CD80 and CD86 expression on BMDCs is induced by combined treatment with PD-L1 Ig and anti-CD40L mAbs in the CBA/J x DBA/2 model. (A) Number of MHC-II$^+$, CD80$^+$ and CD86$^+$ cells in the bone marrow, determined by flow cytometry. (B) Gating strategy used for flow cytometry, using the FSC/SSC method. R1 represents the BMDC population. (C) Percentage of MHC-II$^+$, CD80$^+$ and CD86$^+$ cells. *P<0.05, **P<0.01 and ***P<0.001 vs. the normal group (CBA/J x BALB/c). $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the spontaneous abortion group (CBA/J x DBA/2). MHC, major histocompatibility complex; BMDCs, bone marrow-derived dendritic cells; PD-L1, programmed death-ligand 1; Ig, immunoglobulin; CD40L, CD40 ligand; mAb, monoclonal antibody; SSC, side scatter; FSC, forward scatter.

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

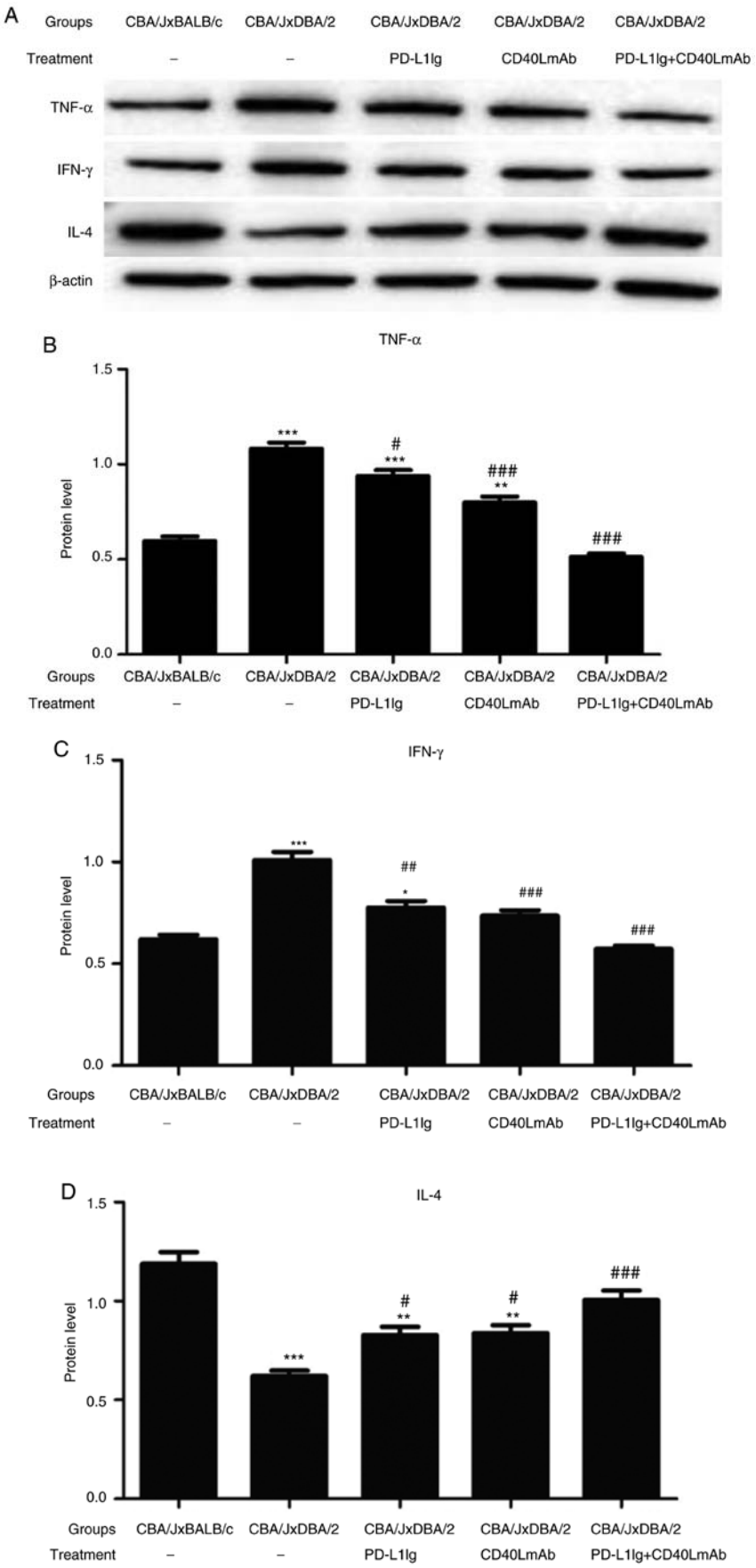

Figure 5. Combined therapy with PD-L1 Ig and anti-CD40L mAbs decreases the expression of TNF-α and IFN-γ expression and increases the expression of IL-4 in the CBA/J x DBA/2 model. Protein expression levels were determined by (A) western blot analysis and quantified for (B) TNF-α, (C) IFN-γ and (D) IL-4. *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 autoantibodies 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 feto-maternal tissues (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 $MHC\text{-}II^+$, $CD80^+$ 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 mAb 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.

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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.

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