European Review for Medical and Pharmacological Sciences 2019; 23: 9150-9162

Effect of RMT1-10 on the immunological characteristics of dendritic cells cultured *in vitro* and corneal transplantation *in vivo*

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Abstract. – OBJECTIVE: Corneal allograft rejection is an immunological hypersensitive reaction caused by the antigenicity of the donor cornea. This study aimed to explore the effects of RMT1-10 on the prevention of corneal graft rejection by modifying immunological characteristics of dendritic cells (DCs).

MATERIALS AND METHODS: DCs and CD4+T cells were sorted using flow cytometry and used for in vitro mixed lymphocyte culture. The cultured cells were prepared for the characterization of the DC cell phenotypes using the markers CD11c, CD80, MHC II, CD54, and TIM-4. Cytokine concentrations of IL-4, IL-12, and IL-10 of supernatants were measured by the enzyme-linked immunosorbent assay. CD4+T cells were examined by flow cytometry for apoptosis and proliferation. We also investigated the effect of RMT1-10 in the prevention and treatment of high-risk corneal graft rejection using a mouse model of corneal transplantation.

RESULTS: DCs were identified as the CD-11c+MHC-II–expressing subset. RMT1-10 suppressed the expression of CD11c, CD80, MHC II, CD54, and TIM-4 of DCs using the blockade of TIM-1 signaling. Moreover, TIM-1 blockade inhibited the production of IL-12 and IL-10 in a mixed lymphocyte culture system. However, a TIM-1 blockade had no effect on the apoptosis of CD4+T cells. RMT1-10 suppressed DC maturation, inhibiting the proliferation of CD4+T cells.

CONCLUSIONS: RMT1-10 significantly improved the survival rate of the corneal allografts in mice compared with saline-injected controls. This clinical improvement from RMT1-10 occurred through the inhibition of CD4+T cell proliferation. Moreover, RMT1-10 induced antigen-specific detection of receptor immune tolerance. The cross-linking of TIM-1 on CD4+T cells with the agonist mAb provided a costimulatory inhibition signal for T cell activation or proliferation.

Key Words:
RMT1-10, TIM-1 Signaling, Dendritic cells, CD4+T cells, Corneal transplantation.

Introduction

Corneal blindness is one of the major causes of blindness. Corneal transplantation is the most effective and common treatment for patients with corneal blindness. Each year, more than 65,000 corneal transplantation surgeries take place around the world[1]. Usually, cornea-blinded patients in China have a long wait before transplantation, due to a lack of donors and because they face a series of postoperative complications. Due to immune privilege, the success rate of corneal transplantation is higher than 90% without any preventive measures. However, the rejection rate is as high as 70% within two years after transplantation for high-risk corneal allograft recipients with inflamed and vascularized graft beds[1].

Currently, there is no ideal and effective drug to prevent corneal transplantation rejection[2]. Traditional glucocorticoids and neuro calmodulin inhibitors such as CsA and FK506 delay acute rejection but cannot prevent later loss of function of the transplanted cornea[3]. In recent years, the antibody drugs that specifically bind with targets have had an important protective role in corneal rejection by directly manipulating immune cells without harming any normal tissue or cell types. This method is now widely accepted in the clinical setting[4]. The main reason for corneal rejection is the recognition of the alloantigens and the subsequent activation and proliferation of pathologi-

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cal T cells. Thus, the key for preventing corneal transplant immune rejection is the inhibition of pathological T cells[5].

Antigen-presenting cells (APCs) are the principal mediators of the transplant rejection and tolerance process and the key players in T cell activation[6]. In high-risk corneal transplantation in patients with inflamed and vascularized graft beds, the immature APCs of the donor corneal stroma mature quickly and migrate to the lymphoid tissues to sensitize host T cells[7]. DCs capture antigens, become fully stimulatory and migrate to the T-cell areas of the secondary lymphoid organs. The interactions between the activated DCs and T/B cells are fundamental to the induction of the adaptive immune responses[8].

T cell immunoglobulin and mucin proteins (TIM)[9], a group of proteins expressed by certain T cells and APCs, have recently received widespread attention[10]. TIM-1 was originally described as a hepatitis A virus receptor[11], and it is a new member of the TIM family[12]. While absent on naïve CD4 T cells, TIM-1 expression is quickly upregulated on T cell activation and expressed on the surface of matured Th2 cells. The TIM-1 receptor specifically is expressed on the surface of APCs and binds with TIM-1 to induce T cell proliferation. Thus, we propose that anti–TIM-1 antibody therapy could modulate the corneal transplant rejection[13,14].

Currently, there is no research on targeting TIM-1 via RMT1-10. In this study, we aimed to characterize the therapeutic efficacy and underlying the immunological mechanisms of RMT1-10 in corneal transplant rejection in a mouse model.

Materials and Methods

In Vitro DC Cell Culture

The C57BL/6 donor mice were sacrificed using the cervical dislocation method and immersed in 75% alcohol for one minute. Bone marrow cells were cultured in six-well plates at a concentration of 1×10^6/mL. The cells were cultured for 72 h and supplemented with 10 ng/mL mGM-CS and 1 ng/mL mIL-4. The medium and suspension cells were then discarded, replaced with fresh RPMI-1640 complete medium, and cultured for another 72 h with mGM-CS and mIL-4. The loose adherent cells were collected as stimulating cells. This study was approved by the Animal Ethics Committee of Chengde Medical University Animal Center.

CD4+T Cell Sorting and CFSE Labeling

The same background recipient mice were sacrificed. Their spleens were removed under sterile conditions and mashed and filtered through a 200-μm mesh filter after trituration. They were then split via Tris-NH4CL to prepare a single-cell suspension. CD4+T cells were sorted using flow cytometry, and the concentration of CD4+T cells was adjusted to 1×10^7/mL. Then, 5- or 6- (N-succinimidyloxycarbonyl) -3', 6'-O, O'-diacetylfluorescein, and CFSE were labeled to CD4+T cells using the commercial user guide.

Mixed Lymphocyte Culture System

The cells were cultured *in vitro,* and the cell concentrations were adjusted to 4×10^4, 2×10^4, 1×10^4, and 0.5×10^4/100 μL. The CD4+T cell-only culture was set as the control, and 2×10^5/100 μL of effector CD4+T cells was added to each well. Anti–TIM-1 monoclonal antibody RMT1-10 (Bio-X-Cell, West Lebanon, NH, USA) was added at 100 ng, 200 ng, and 400 ng/mL for 72 h in culture.

Flow Cytometry and Cell Staining

The cultured cells were prepared for cell-surface or intracellular staining using the following reagents: anti-CD11c Alexa Fluor 488, anti-CD80 PE, anti-MHC-II PerCP, anti-CD54 PE, anti-TIM-4 APC, and anti-CD40 PerCP (eBioscience, San Diego, CA, USA). CD4-positive cells were firstly sorted and stained with AnnexinV and 7-AAD. The apoptosis percentage of CD4+T cells was detected using fluorescence-activated cell sorting (FACS). The falcon tubes were prepared and marked as the negative control and test samples. The cells were washed with phosphate-buffered saline twice and used to prepare the 1×10^6/mL suspension with a binding buffer. Then, 100 μL cells were added and put in a dark place at room temperature for 20 to 25 minutes. The binding buffer (400 μL) was added and the sample was processed using flow cytometry. At the same time, CD4+T cell proliferation was measured according to the division of CFSE. The inhibition rate percentage = [(effector cell proliferation without DCs – effector cell proliferation supplemented with DCs)/effector cell proliferation without DCs] × 100%. The flow cytometry data were acquired using the BD FACSCalibur and BD FACSCanto devices (BD Immunocytometry Systems, San Jose, CA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

The cytokine concentrations of IL-4, IL-12, and IL-10 of cell culture supernatants were measured using commercial ELISA kits (RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's protocol. The lower detection limits of IL-4, IL-12, and IL-10 were 0.2 μg/mL, 4.7 μg/mL, and 0.9 μg/mL, respectively. The coefficient variation was < 10%[15].

In Vivo Animal Model of High-Risk Corneal Transplantation

The receptor was randomly classified into four groups: a treatment group (RMT1-10), an isotype control group (Hamster IgG), a positive control group (dexamethasone), and a blank control group, respectively. The treatment group was given a dose of 25 μg/mL before and after surgery, the day before and on the day of surgery, three times a week, and four times a week for four weeks, respectively. The postoperative observation of the eyelid suture was performed on the third day after surgery. The slit lamp and corneal flap turbidity, neovascularization, and edema were scored and recorded. The body temperature, injection site, hair glossiness, abnormal rupture of the beard, decolorization, and other vital signs were recorded twice a week for eight weeks. The mice were sacrificed at zero, one, two, four, and eight weeks after corneal transplantation, and the lymph nodes, spleens, peripheral blood, and corneal grafts were obtained to prepare a single-cell suspension. FACS was used to detect the anti-CD4 PE, anti-CD8 PerCP, anti-TIM-4 FITC, and anti-CD11c APC of cells (Table I).

Statistical Analysis

The data were analyzed using Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). The Student's *t*-test was used to compare the two groups. A comparison between the groups was done using the One-way analysis of variance test followed by the post-hoc test (Least Significant Difference). The difference was considered significant if $p<0.05$. *means $p<0.05$, **means $p<0.01$ and ***means $p<0.001$.

Results

TIM-1 Signaling Suppresses DC Maturation and IL-12 and IL-10 Cytokine Secretion in a Mixed Lymphocyte Culture System

CD11c and MHC II are major maturity markers of DCs[16]. CD11c+MHC-II–expressing cells were identified as DCs. The double-round sorting strategy made it possible to isolate highly pure and viable cells. The percentage of CD11c+MHC II+ cells was 1.27% before sorting, and they increased to 94.4% after sorting enrichment. In addition, a FACSCalibur flow cytometer (Becton Dickinson Immuno-cytometry System, San Jose, CA, USA) was used to sort CD4-positive T cells[17]. The percentage of CD4 T cells increased from 19.2% to 99.1% after sorting.

The TIM-1 signaling pathway triggers CD4+T cells involved in a variety of pathological immune responses in allograft recipients[18]. To analyze the function of TIM-1 signaling, we collected both CD4-positive T cells and CD11c+MHC II+ DC cells and established a co-culture system by mixing 2×10^5/100 μL CD4+T cells with 2×10^4 DC cells. In this system, CD4+T cells served as the effector cells. We used flow cytometry to analyze the expression of DC phenotypes after three days of co-culture. The blockade of TIM-1 signaling by RMT1-10 suppressed the expression of CD11c, CD80, MHC II, CD54, and TIM-4 (Figure 1A). Moreover, this inhibitory effect was dose-depen-

Table I. Criteria for immune rejection.

Score	Opacity index (0-4)	Edema index (0-4)	Neovascularization index (0-4)
0	Corneal grafts are completely clear	No edema with epithelium and basal layer	No new blood vessels
1	Mildly-thick corneal opacity	Mild edema with corneal stroma	Neovascularization around the bed
2	corneal opacity, but iris's texture is visible	diffuse corneal stroma edema	Neovascularization around the graft (up to edge)
3	Aggravated opacity, but pupil is visible and anterior chamber observation is difficult	Diffuse corneal stromal edema with sub-epithelial microcystic vesicles	Neovascularization
4	Severe corneal opacity and anterior chamber observation is impossible	Bullous keratopathy	Corneal grafts were filled with new-born vessels

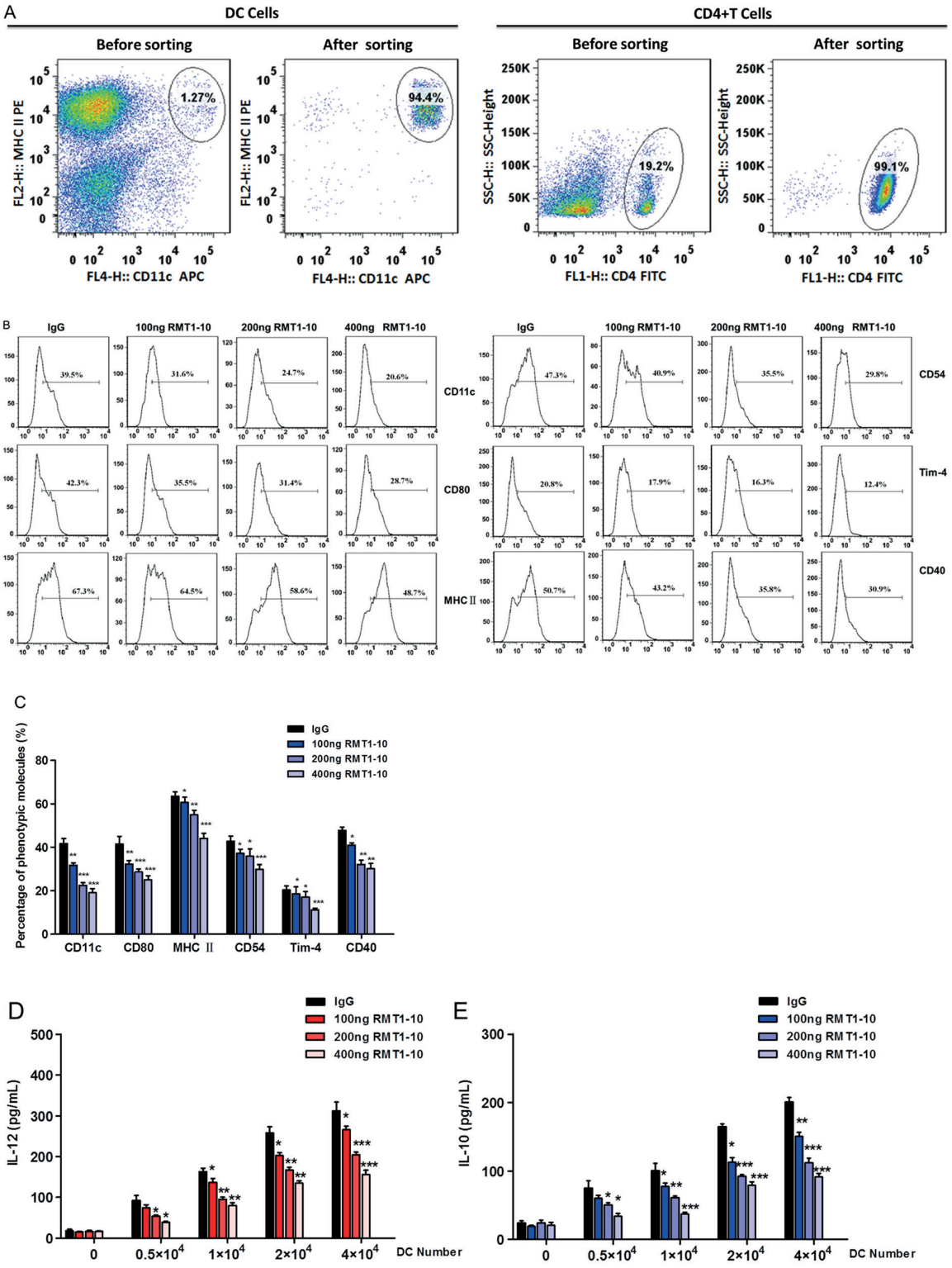

Figure 1. Dendritic cell phenotype and cytokine level analysis for TIM-1 blockade in the mixed lymphocyte culture system. **A**, For phenotype analysis, the expression of CD11c, CD80, MHC II, CD54, TIM-4, and CD40 was detected by fluorescence-activated cell sorting. **B**, Fluorescence-activated cell sorting results show that blocking on TIM-1 signaling by RMT1-10 significantly suppressed the expression of CD11c, CD80, MHC II, CD54, and TIM-4. **C**, Enzyme-linked immunosorbent assay analysis was used to detect the expression levels of IL-12. **D**, Enzyme-linked immunosorbent assay analysis was used to detect the expression levels of IL-10.

dent, and 400 ng RMT1-10 showed a stronger inhibition effect than the 100 ng and 200 ng groups (Figure 1B).

As for IL-12 and IL-10, we used ELISA to analyze the expression levels after three days of co-culture with CD4 T cells. The blockade of TIM-1 signaling suppressed the secretion of both IL-12 and IL-10 compared with the control group. In the 2×10^4 DC cell group, 400 ng RMT1-10 significantly reduced the IL-12 level compared with the control group (139.28±3.27 *vs.* 287.37±4.34 μg/mL, $p<0.001$) (Figure 1C). The group receiving a higher dosage showed an evident lower IL-10 level than the control group (98.72±4.29 *vs.* 211.28±6.23 μg/mL, $p<0.001$) (Figure 1D). These results indicate that TIM-1 signaling is required for the DC maturation and the subsequent cytokine products such as IL-10 and IL-12.

RMT1-10 Inhibits Proliferation of CD4 T Cells

DCs are important bone marrow-derived antigen-presenting cells that induce and regulate T cell responses[19] and are of critical importance in determining the balance between immunity and tolerance[20]. Numerous studies[21] have demonstrated that the tolerogenic DCs cultured *in vitro* have potent modulating effects on autoimmunity. The apoptosis of CD4+T cells is driven by DCs, while from the flow cytometry results (Figure 2A), we

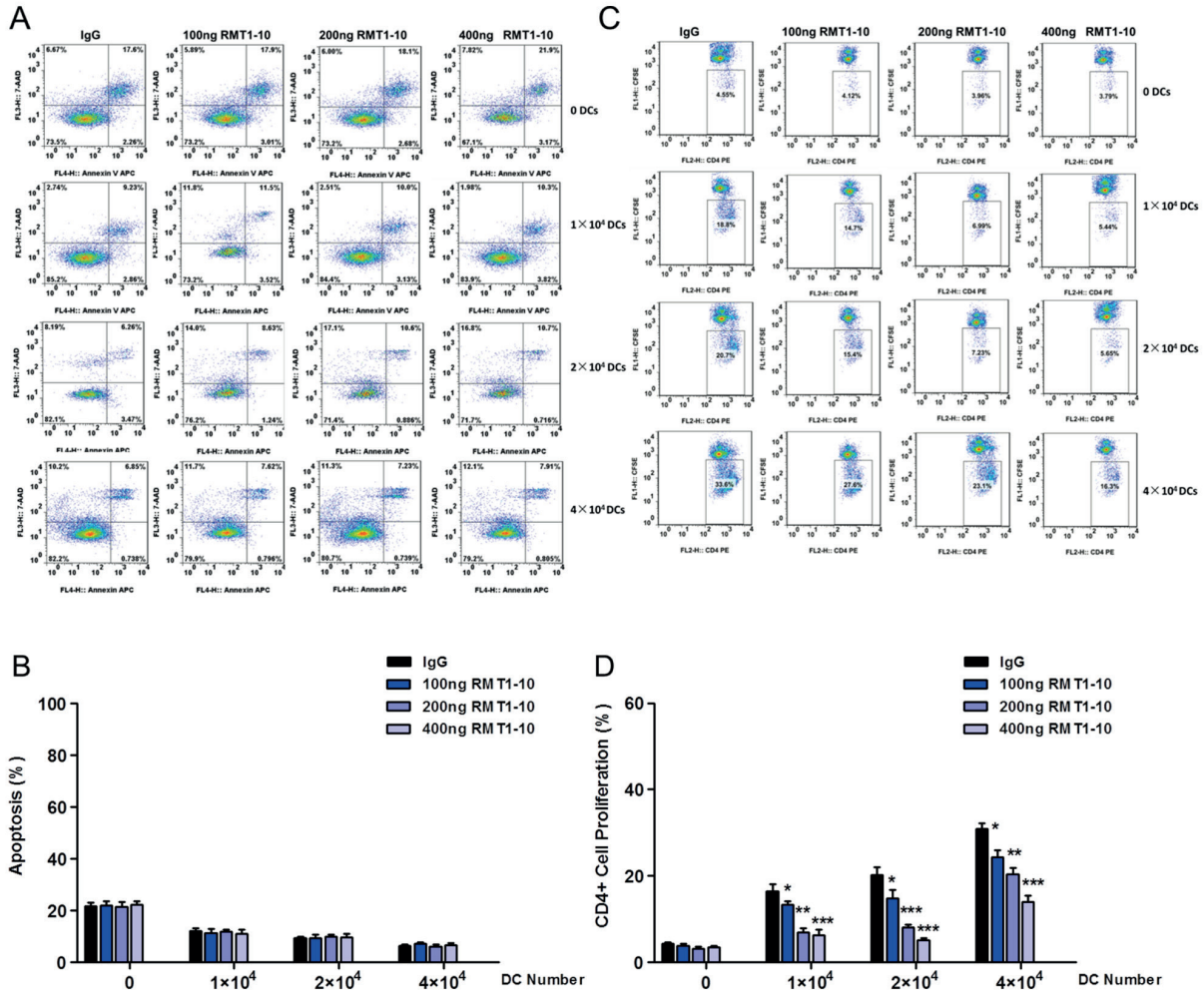

Figure 2. Apoptosis and proliferation analysis of CD4+T cells in the mixed lymphocyte culture system. **A**, Apoptosis of CD4 T cells treated with different concentrations of RMT1-10 cells were detected by fluorescence-activated cell sorting. **B**, RMT1-10 affected apoptosis of CD4+T cells. **C**, Proliferation of CD4+T cells treated with different concentrations of RMT1-10 were detected by fluorescence-activated cell sorting. **D**, RMT1-10 significantly reduced the proliferation percentage of CD4+T cell ($p<0.05$). This inhibitory effect was dose-dependent.

found that RMT1-10 had a little effect on the apoptosis of the CD4-positive T cells (Figure 2B), which further supports the role of RMT1-10 via the TIM-1 pathway.

To understand the mechanism underlying RMT1-10 treatment[22], DCs were co-cultured with isolated T cells in 96-well plates at a 1:10 ratio and detected by flow cytometry (Figure 2C). In the presence of 100 ng RMT1-10, the percentage of proliferating CD4+T cells was significantly lower than that of the control group (21.72±2.37% *vs.* 34.57±3.66%, $p<0.05$) (Figure 2D). This inhib-

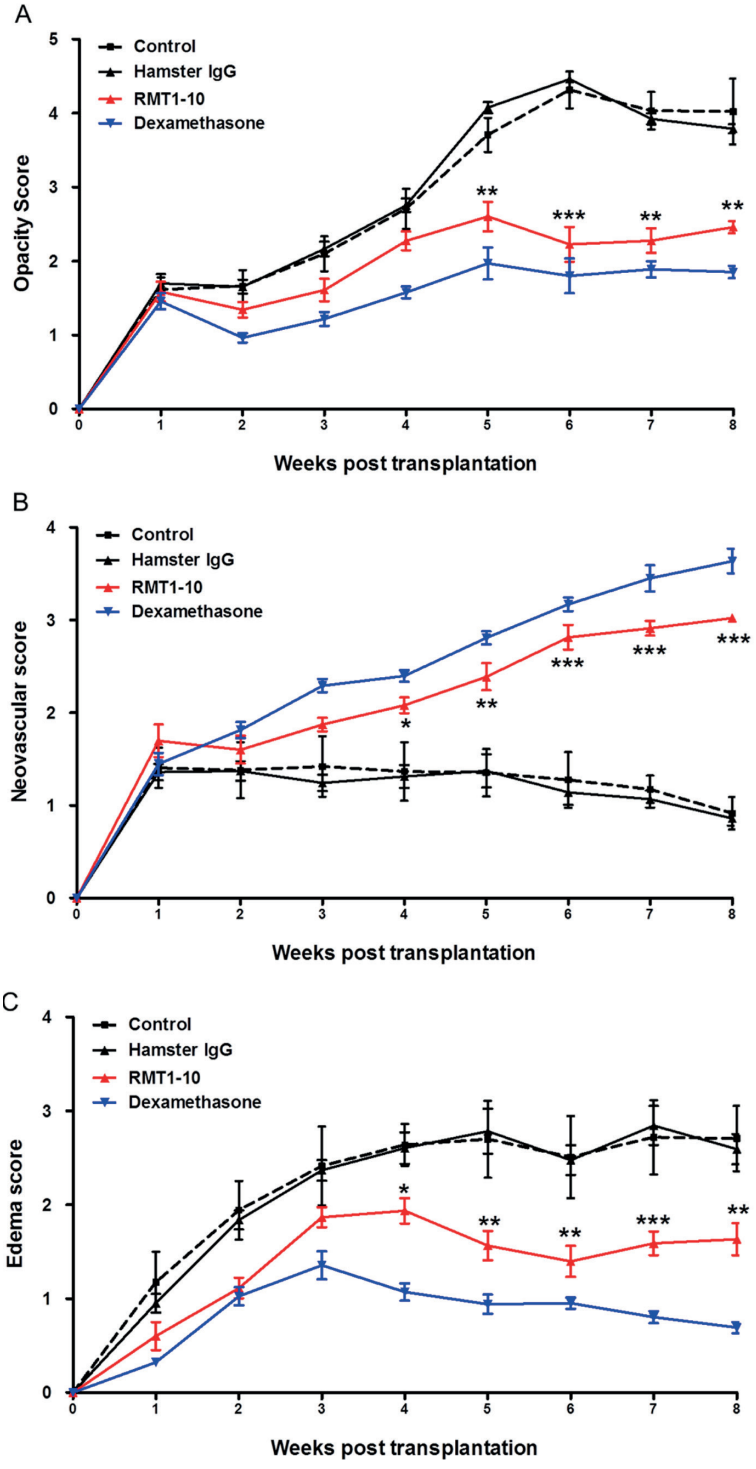

Figure 3. Effect of RMT1-10 in prevention and treatment of high-risk corneal transplantation rejection. Recipient mice were sacrificed in each of eight weeks after corneal transplantation and used to detect the opacity, neovascularization, and edema of corneal grafts. **A**, From five to eight weeks, the opacity scores of different treatment groups. **B**, From four to eight weeks, the neovascularization scores of different treatment groups. **C**, From five to eight weeks, the edema scores of different treatment groups.

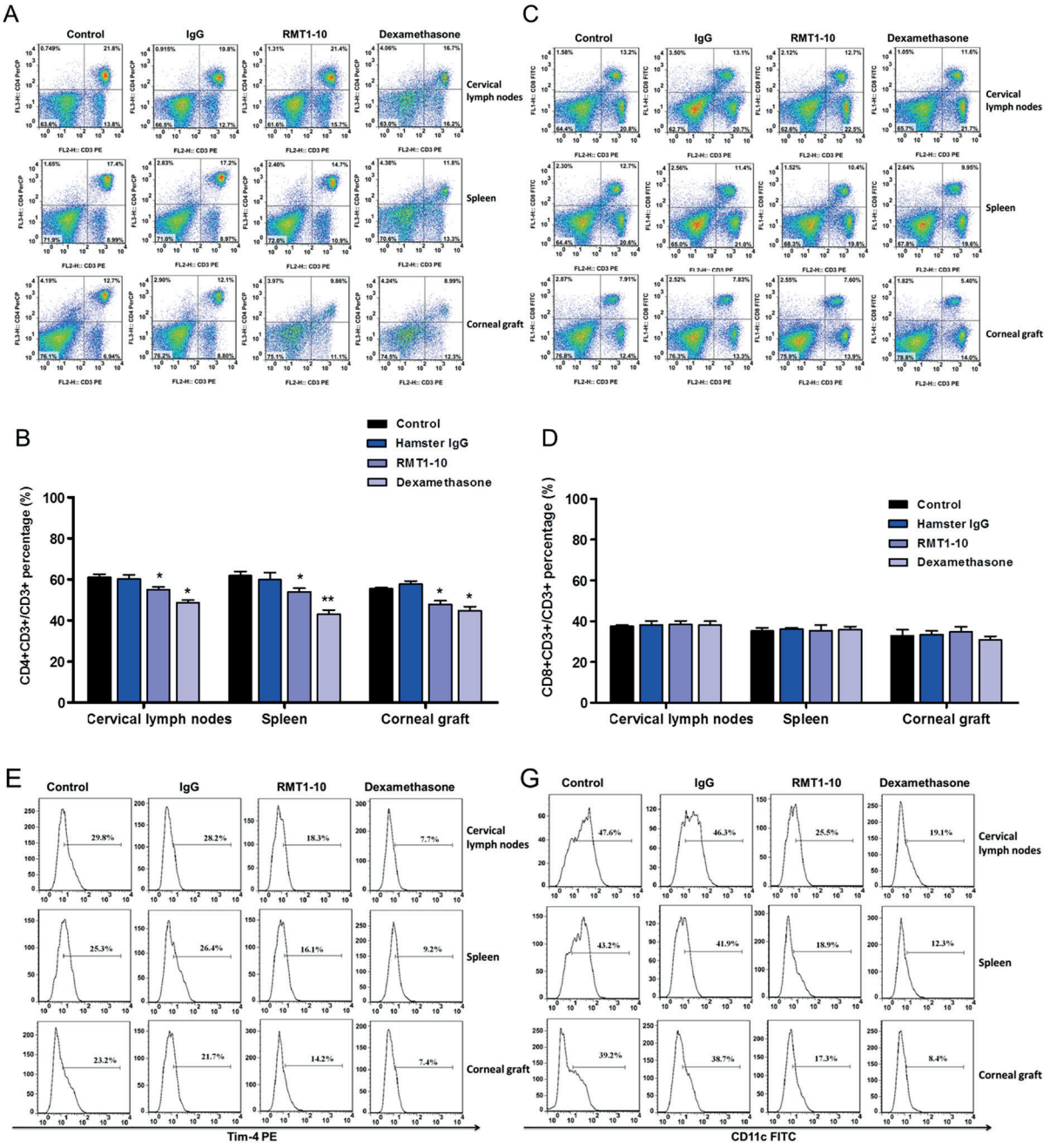

Figure 4. Effect of RMT1-10 on immune cell phenotypes in recipient mice, which were sacrificed after corneal transplantation and used to detect the effect of RMT1-10 on immune cell phenotypes. **A**, Cervical lymph nodes, spleens, and corneal grafts were collected, and a single-cell suspension was prepared to detect the percentage of CD4+T by fluorescence-activated cell sorting. **B**, Fluorescence-activated cell sorting analysis results of the percentage of CD4-positive cells in cervical lymph nodes, spleens, and corneal grafts. **C**, Cervical lymph nodes, spleens, and corneal grafts were collected and prepared for the single-cell suspension to detect the percentage of CD8 by fluorescence-activated cell sorting. **D**, Fluorescence-activated cell sorting analysis results of the percentage of CD8-positive cells in the cervical lymph nodes, spleens, and corneal grafts. **E**, Cervical lymph nodes, spleens, and corneal grafts were collected and prepared for the single-cell suspension to detect the expression of TIM-4 by fluorescence-activated cell sorting. **F**, Fluorescence-activated cell sorting analysis results of the percentage of TIM-4-positive cells in cervical lymph nodes, spleens, and corneal grafts. **G**, Cervical lymph nodes, spleens, and corneal grafts were collected and prepared for the single-cell suspension to detect the expression of CD11c by fluorescence-activated cell sorting. **H**, Fluorescence-activated cell sorting analysis results of the percentage of CD11c-positive cells in the cervical lymph nodes, spleens, and corneal grafts.

Figure continued

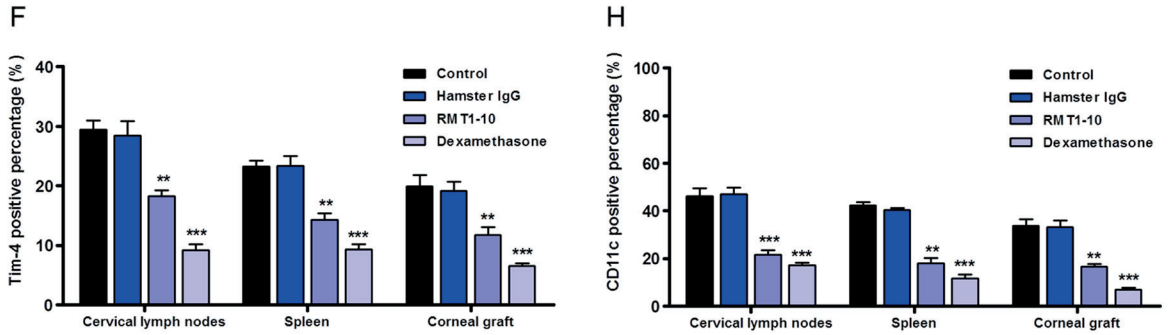

Figure 4. *(Continued).* **F**, Fluorescence-activated cell sorting analysis results of the percentage of TIM-4-positive cells in cervical lymph nodes, spleens, and corneal grafts. **H**, Fluorescence-activated cell sorting analysis results of the percentage of CD11c-positive cells in the cervical lymph nodes, spleens, and corneal grafts.

itory effect was also dose-dependent; 400 ng anti-TIM-1 mAb treatment had a much stronger inhibitory effect than the 100 ng and 200 ng groups. Thus, we learned that RMT1-10 can inhibit DC maturation, enhance the endocytic ability of DCs, and subsequently weaken the stimulatory effect of DCs on T lymphocytes.

RMT1-10 Prevents and Treats High-Risk Corneal Allograft Rejection

APCs play an important role in transplant rejection and tolerance. In this study, we used a mouse model of corneal transplantation to investigate the effect of RMT1-10 on the prevention and treatment of high-risk corneal graft rejection. The recipient mice were sacrificed each week for eight weeks after corneal transplantation and used to detect the opacity, neovascularization, and edema of the corneal graft. From five to eight weeks, the opacity scores of the RMT1-10-treated group were significantly lower than those of the hamster IgG group ($p<0.01$) (Figure 3A). From four to eight weeks, the neovascularization scores of the RMT1-10 treatment group were significantly higher than those of the hamster IgG group ($p<0.05$) (Figure 3B). From five to eight weeks, the edema scores of the RMT1-10 treatment group were significantly lower than those of the hamster IgG group ($p<0.01$) (Figure 3C). These data show that RMT1-10 directly prevents corneal transplant rejection.

The recipient mice were also sacrificed at zero, one, two, four, and eight weeks after corneal transplantation. The cervical lymph nodes, spleens, and corneal graft suspensions were collected and detected by FACS using anti-CD4 PE (Figure 4A), anti-CD8 PerCP (Figure 4C), anti-TIM-4 FITC (Figure 4E), and anti-CD11c APC (Figure 4G). In cervical lymph nodes, the percentage of CD4-positive cells in the RMT1-10 treatment group was significantly lower than that of the control group (53.28±5.23% *vs.* 64.22±6.28%, $p<0.05$) (Figure 4B). Similar results were observed in the spleens and corneal graft cell suspensions. However, there was no difference in CD8 T cells in spleens and corneal graft cell suspensions among groups (Figure 4D). Moreover, both TIM-4-positive cells (9.28±0.88% *vs.* 20.33±1.37%, $p<0.05$) (Figure 4F) and CD11c-positive cells (16.27±2.28% *vs.* 37.29±1.86%, $p<0.05$) (Figure 4H) showed a decreased expression compared with the control groups.

RMT1-10 Treatment Lessens IL-4, IL-10, and IL-12 Levels in Peripheral Blood Serum

At zero, one, two, four, and eight weeks after corneal transplantation, the levels of IL-4, IL-10, and IL-12 in peripheral blood were measured using ELISA. In the fourth week, RMT1-10 treatment significantly reduced the IL-4 level compared with that of the control group (107.27±3.84 *vs.* 169.92±5.18 pg/mL, $p<0.01$) (Figure 5A). In addition, IL-10 levels were also reduced compared with those of the control group (93.84±1.27 *vs.* 148.52±7.18 μg/mL, $p<0.01$) (Figure 5B). Similarly, RMT1-10 treatment also reduced the production of IL-12 to a large extent compared with that of the control group (102.38±3.17 *vs.* 116.73±3.27 μg/mL, $p<0.05$) (Figure 5C).

RMT1-10 Induces Antigen-Specific Detection of Receptor Immune Tolerance

The recipient mice that (had not developed) did not develop rejection at eight weeks after the above-mentioned keratoplasty were sacrificed and a single-cell suspension of lymph nodes was

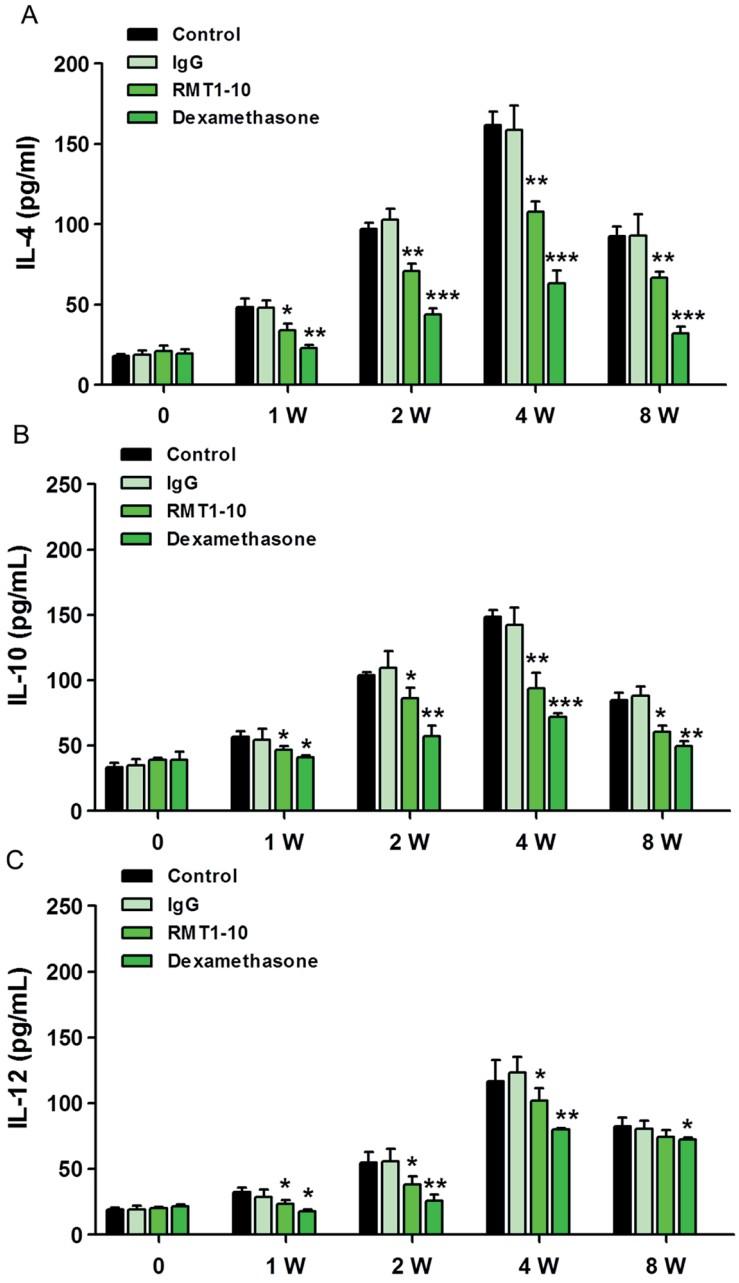

Figure 5. Effect of RMT1-10 on IL-4, IL-10, and IL-12 levels in peripheral blood serum. Enzyme-linked immunosorbent assay analysis was used to detect IL-4, IL-10, and IL-12 levels in peripheral blood serum. **A,** RMT1-10 significantly reduced the IL-4 level compared with that of the control group ($p<0.01$). **B,** RMT1-10 significantly reduced the IL-10 level compared with that of the control group ($p<0.01$). **C**, RMT1-10 also reduced the IL-12 level to a large extent compared with that of the control group ($p<0.05$).

obtained. DCs were sorted and injected into the recipient via tail vein injection 18 h after corneal transplantation. The corneal graft turbidity, neovascularization, and edema of recipient mice were monitored for eight weeks and scored under a slit lamp. In the eighth week, the corneal graft opacity score in the RMT1-10 group was 1.402±0.238, which was higher than that of the dexamethasone group (0.608±0.173, $p<0.001$) (Figure 6A). The neovascularization score of the RMT1-10 group was lower than that of the dexamethasone group (2.962±0.672 *vs.* 3.462±0.782, $p<0.01$) (Figure

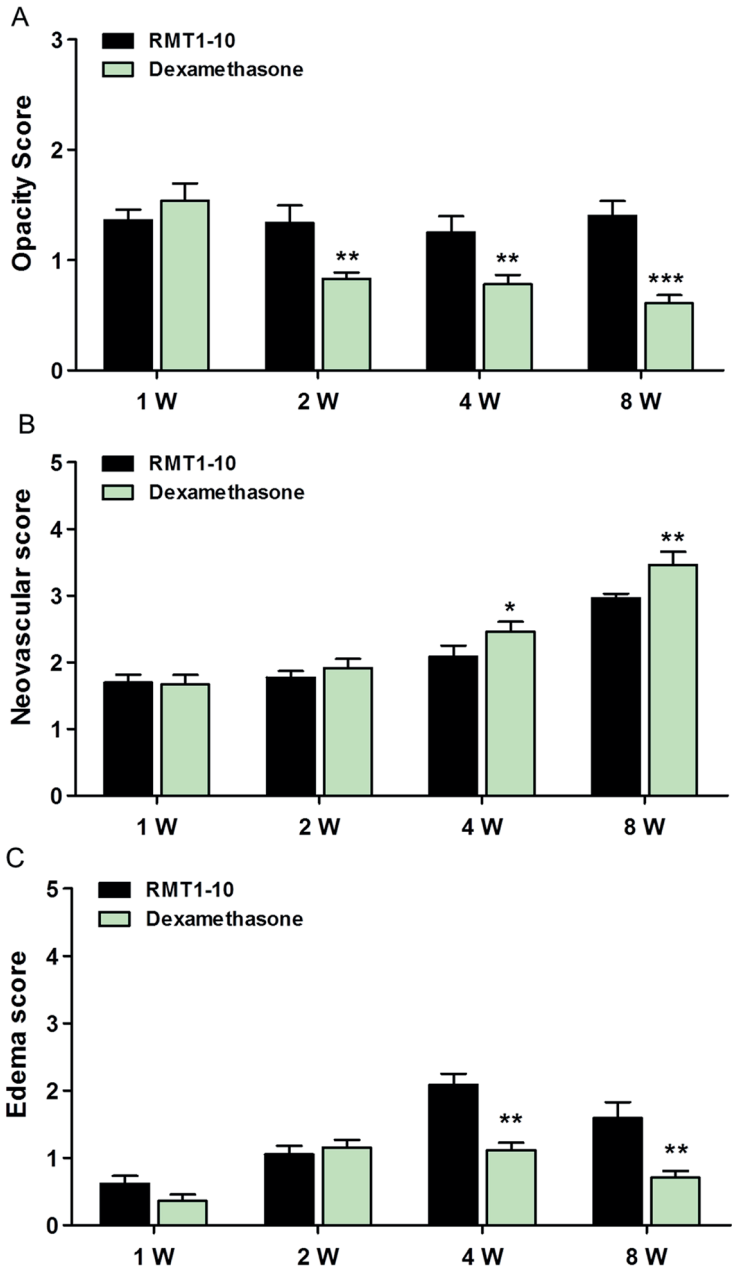

Figure 6. RMT1-10 induced antigen-specific detection of receptor immune tolerance. **A**, The graft opacity score of the RMT1-10 group was significantly higher than that of the dexamethasone group ($p<0.001$) from two to eight weeks. **B**, Neovascularization score of the RMT1-10 group was significantly lower than that of the dexamethasone group ($p<0.01$) from four to eight weeks. **C**, The edema score of the RMT1-10 group was significantly higher than that of the dexamethasone group ($p<0.01$) from four to eight weeks.

6B). The edema score of the RMT1-10 group was higher than that of the dexamethasone group (1.598±0.237 *vs.* 0.706±0.312, $p<0.01$) (Figure 6C). In conclusion, RMT1-10 can induce antigen-specific detection of the receptor immune tolerance.

Discussion

This study aimed to investigate the role of the TIM-1 pathway in the inflammatory reaction and therapeutic efficacy of RMT1-10 in corneal transplantation rejection[23]. We aimed to prove that

treating donor DCs with RMT1-10 can induce tolerogenic maturation-resistant APCs, which leads to a higher survival rate and reduced host allosensitization[24,25].

While a traditional treatment with CsA or FA506 inhibits acute corneal rejection to different extents[26], they cannot control the chronic loss of function of the transplanted corneas and have many limitations, such as high expenses and severe side effects[27]. Nevertheless, here we presented alloantigen-specific tolerance-inducing strategies that can circumvent the above-mentioned limitations[28]. According to our previous studies, the mouse group that received an intraperitoneal injection of RMT1-10 had a corneal survival rate eight times higher than that of the control group. We hypothesized that RMT1-10 inhibits the proliferation of Th1 cells and maintains the function of Tregs cells, which make the corneal microenvironment more immunoregulatory and less pro-inflammatory.

In order to get ideal research results, we used a double-round sorting strategy and flow cytometry to gain a high concentration of DCs and CD4+T cells (supporting information). Then, we aimed to prove the significance of the TIM-1 pathway in immune-mediated corneal transplant rejection. DC phenotypes such as CD11c, CD80, MHC II, CD54, TIM-4, and CD40 and levels of IL-10 and IL-12 were measured after TIM-1 signaling was blocked using RMT1-10. A lower expression of CD11c, CD80, MHC II, CD54, TIM-4, and CD40 was observed in the RMT1-10-treated group compared with the Ig-G treatment group (Figure 1A). The decreased concentrations of IL-10 and IL-12 were also observed (Figure 1B). Notably, both FACS and ELISA experiments showed dose-dependent properties, which means that higher doses of RMT1-10 may have a better treatment efficacy because CD11c, CD80, MHC II, CD54, TIM-4, and CD40 are maturation markers of DCs. Thus, we confirmed that TIM-1 signaling plays an important role in the maturation of DCs, which lead to a proliferation of CD4+T cells[29].

To rule out the possibility that RMT1-10 directly induces less proliferation of CD4+T cells or increased apoptosis, the CD4+T cells were cultured with RMT1-10 at the same time. The groups with different concentrations of RMT1-10 showed a proliferation extent similar to that of the CD4+T cells (Figure 2A), which means that RMT1-10 cannot directly induce the apoptosis of CD4+T cells and that the TIM-1 pathway is the only way to modulate immune responses.

After proving the function of TIM-1 signaling, we aimed to prove the therapeutic efficacy of RMT1-10. Mice were randomly classified into four groups: blank control, hamster-IgG isotype control, RMT1-10 treatment group, or dexamethasone positive control. Anti-CD4 PE, anti-CD8 PerCP, anti-TIM-4 FITC, anti-CD11c APC were used to measure CD4, CD8, TIM-4, and CD11c in local and peripheral DCs of recipient mice[30]. CD4, CD11c, and TIM-4-positive cells in the RMT1-10 treatment group were significantly lower than in the other control group[31]. However, the CD8-positive cells remained at similar levels in the different treatment groups[32] (Figure 3). Furthermore, the concentrations of IL-4, IL-10, and IL-12[33] in peripheral blood serum were measured by ELISA[34]. We also found lower levels[35] of IL-4, IL-10, and IL-12 in the group treated with RMT1-10.

Three days after transplantation surgery, we monitored and scored the graft corneal opacity, neovascularization, and edema. Mice were also randomly classified into four groups: blank control, hamster-IgG isotype control, RMT1-10 treatment group, and dexamethasone positive control group. Using eight weeks of documentation of different indicators after surgery, we found that the group treated with RMT1-10 exhibited excellent tread and score (Figure 5).

Lastly, RMT1-10 antigen-specific induction of immune tolerance was studied. Mice in the RMT1-10 and dexamethasone groups showed no immune rejection eight weeks after surgery, which means that the mice developed an immune tolerance after RMT1-10 treatment[36,37].

Conclusions

We demonstrated that RMT1-10 significantly improves the survival rate of corneal allografts in mice compared with saline-injected controls. This clinical improvement by RMT1-10 occurs through the inhibition of CD4+T cell proliferation. Moreover, RMT1-10 induced antigen-specific detection of the receptor immune tolerance and the cross-linking of TIM-1 on CD4+T cells with the agonist mAb and provided a costimulatory inhibitory signal for T cell activation and proliferation.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Funding Acknowledgements

Natural Science Foundation of Hebei Province (H2015406054). The government of Hebei Province funded the Provincial Excellent Medical Talents Project (361008).

References

1) Armitage WJ, Crewe JM, Tullo AB. Corneal transplantation: how successful are we? Br J Ophthalmol 2001; 85: 122.
2) Baddon A, Jones M, Armitage J, Warwick R, Kaye S. A review of allograft ophthalmic tissue in eye surgery. Cell Tissue Bank 2010; 11: 29-38.
3) Ple-Plakon PA, Shtein RM. Trends in corneal transplantation: indications and techniques. Curr Opin Ophthalmol 2014; 25: 300-305.
4) Armitage WJ, Jones MN, Zambrano I, Carley F, Tole DM, NHSBT Ocular Tissue Advisory Group and Contributing Ophthalmologists OTAG Audit Study 12. The suitability of corneas stored by organ culture for penetrating keratoplasty and influence of donor and recipient factors on 5-year graft survival. Invest Ophthalmol Vis Sci 2014; 55: 784-791.
5) Gillott H, Jackson Spence F, Tahir S, Mytton J, Evison F, Nath J, Sharif A. Smoking history is associated with adverse outcomes for kidney allograft recipients. Exp Clin Transplant 2018; 16: 274-281.
6) Hu ZJ, He JF, Li KJ, Chen J, Xie XR. Decreased microRNA-146a in CD4+T cells promote ocular inflammation in thyroid-associated ophthalmopathy by targeting NUMB. Eur Rev Med Pharmacol Sci 2017; 21: 1803-1809.
7) Tahvildari M, Emami-Naeini P, Omoto M, Mashaghi A, Chauhan SK, Dana R. Treatment of donor corneal tissue with immunomodulatory cytokines: a novel strategy to promote graft survival in high-risk corneal transplantation. Sci Rep 2017; 7: 971.
8) Wang Z, Liang Q, Zhang Y, Yang J, Li M, Wang K, Cui M, Chen H, Fu ZF, Zhao L, Zhou M. An optimized HMGB1 expressed by recombinant rabies virus enhances immunogenicity through activation of dendritic cells in mice. Oncotarget 2017; 8: 83539-83554.
9) Riquelme P, Geissler EK, Hutchinson JA. Alternative approaches to myeloid suppressor cell therapy in transplantation: comparing regulatory macrophages to tolerogenic DCs and MDSCs. Transplant Res 2012; 1: 17.
10) Freeman GJ, Casasnovas JM, Umetsu DT, DeKruyff RH. TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity. Immunol Rev 2010; 235: 172-189.
11) Kaplan G, Totsuka A, Thompson P, Akatsuka T, Moritsugu Y, Feinstone SM. Identification of a surface glycoprotein on African green monkey kidney cells as a receptor for hepatitis A virus. EMBO J 1996; 15: 4282-4296.
12) Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL, Sanicola M. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. J Biol Chem 1998; 273: 4135-4142.
13) Xiao S, Najafian N, Reddy J, Albin M, Zhu C, Jensen E, Imitola J, Korn T, Anderson AC, Zhang Z, Gutierrez C, Moll T, Sobel RA, Umetsu DT, Yagita H, Akiba H, Strom T, Sayegh MH, DeKruyff RH, Khoury SJ, Kuchroo VK. Differential engagement of Tim-1 during activation can positively or negatively costimulate T cell expansion and effector function. J Exp Med 2007; 204: 1691-1702.
14) Nozaki Y, Kitching AR, Akiba H, Yagita H, Kinoshita K, Funauchi M, Matsumura I. Endogenous Tim-1 promotes severe systemic autoimmunity and renal disease MRL-Fas(lpr) mice. Am J Physiol Renal Physiol 2014; 306: F1210-F1221.
15) Dong X, Wang C, Zhang J, Wang S, Li H, Kang Y, Tian S, Fu L. Cholecystokinin expression in the development of postinfarction heart failure. Cell Physiol Biochem 2017; 43: 2479-2488.
16) Kuka M, Ashwell JD. A method for high purity sorting of rare cell subsets applied to TDC. J Immunol Methods 2013; 400-401: 111-116.
17) Mbopi-Kéou FX, Sagnia B, Ngogang J, Angwafo FR, Colizzi V, Montagnier L, Belec L. Validation of a single-platform, volumetric, flow cytometry for CD4 T cell count monitoring in therapeutic mobile unit. J Transl Med 2012; 10: 22.
18) Zhang Y, Ji H, Shen X, Cai J, Gao F, Koenig KM, Batikian CM, Busuttil RW, Kupiec-Weglinski JW. Targeting TIM-1 on CD4 T cells depresses macrophage activation and overcomes ischemia-reperfusion injury in mouse orthotopic liver transplantation. Am J Transplant 2013; 13: 56-66.
19) Stenger EO, Turnquist HR, Mapara MY, Thomson AW. Dendritic cells and regulation of graft-versus-host disease and graft-versus-leukemia activity. Blood 2012; 119: 5088-5103.
20) Watowich SS, Liu YJ. Mechanisms regulating dendritic cell specification and development. Immunol Rev 2010; 238: 76-92.
21) Ezzelarab M, Thomson AW. Tolerogenic dendritic cells and their role in transplantation. Semin Immunol 2011; 23: 252-263.
22) Yang N, Qiao J, Liu S, Zou Z, Zhu L, Liu X, Zhou S, Li H. Change in the immune function of porcine iliac artery endothelial cells infected with porcine circovirus type 2 and its inhibition on monocyte derived dendritic cells maturation. PLoS One 2017; 12: e0186775.
23) Nagata M, Nakamura T, Hata Y, Yamaguchi S, Kaku T, Kinoshita S. JBP485 promotes corneal epithelial wound healing. Sci Rep 2015; 5: 14776.
24) Jin H, Liu L, Ding H, He M, Zhang C, Zhong X. Comparison of femtosecond laser-assisted corneal intrastromal xenotransplantation and the allotransplantation in rhesus monkeys. BMC Ophthalmol 2017; 17: 202.

25) Rowe AM, Yun H, Hendricks RL. Exposure stress induces reversible corneal graft opacity in recipients with herpes simplex virus-1 infections. Invest Ophthalmol Vis Sci 2017; 58: 35-41.

26) Sano Y, Ksander BR, Streilein JW. Minor H, rather than MHC, alloantigens offer the greater barrier to successful orthotopic corneal transplantation in mice. Transpl Immunol 1996; 4: 53-56.

27) Streilein JW. Immunologic privilege of the eye. Springer Semin Immunopathol 1999; 21: 95-111.

28) Azimzade Y, Hong J, Mashaghi A. Immunophysical analysis of corneal neovascularization: mechanistic insights and implications for pharmacotherapy. Sci Rep 2017; 7: 12220.

29) Inomata T, Hua J, Di Zazzo A, Dana R. Impaired function of peripherally induced regulatory T Cells in hosts at high risk of graft rejection. Sci Rep 2016; 6: 39924.

30) Marino J, Paster J, Benichou G. Allorecognition by T lymphocytes and allograft rejection. Front Immunol 2016; 7: 582.

31) Huq S, Liu Y, Benichou G, Dana MR. Relevance of the direct pathway of sensitization in corneal transplantation is dictated by the graft bed microenvironment. J Immunol 2004; 173: 4464-4469.

32) Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392: 245-252.

33) Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest 2008; 118: 3420-3430.

34) Ding Q, Yeung M, Camirand G, Zeng Q, Akiba H, Yagita H, Chalasani G, Sayegh MH, Najafian N, Rothstein DM. Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice. J Clin Invest 2011; 121: 3645-3656.

35) Albacker LA, Karisola P, Chang YJ, Umetsu SE, Zhou M, Akbari O, Kobayashi N, Baumgarth N, Freeman GJ, Umetsu DT, DeKruyff RH. TIM-4, a receptor for phosphatidylserine, controls adaptive immunity by regulating the removal of antigen-specific T cells. J Immunol 2010; 185: 6839-6849.

36) Uchida Y, Ke B, Freitas MC, Ji H, Zhao D, Benjamin ER, Najafian N, Yagita H, Akiba H, Busuttil RW, Kupiec-Weglinski JW. The emerging role of T cell immunoglobulin mucin-1 in the mechanism of liver ischemia and reperfusion injury in the mouse. Hepatology 2010; 51: 1363-1372.

37) Maimaitiming R, Yang X, Wupuer K, Ye N, Kong N, Gu B, Fan Y, Shao L, Pan Z. A retrospective clinical study of Xinjiang Uygur patients with corneal allograft rejection. Int J Clin Exp Med 2015; 8: 4356-4362.