·Basic Research·

Mechanism of immune tolerance induced by donor derived immature dendritic cells in rat high-risk corneal transplantation

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Abstract

• **AIM: To study the role of immature dendritic cells (imDCs) on immune tolerance in rat penetrating keratoplasty (PKP) in high-risk eyes and to investigate the mechanism of immune hyporesponsiveness induced by donor-derived imDCs.**

• **METHODS: Seventy-five SD rats (recipient) and 39 Wistar rats (donor) were randomly divided into 3 groups: control, imDC and mature dendritic cell (mDC) group respectively. Using a model of orthotopic corneal transplantation in which allografts were placed in neovascularized high-risk eyes of recipient rat. Corneal neovascularization was induced by alkaline burn in the central cornea of recipient rat. Recipients in imDC group or mDC group were injected donor bone marrow-derived imDCs or mDCs of 1×10^6 respectively 1 week before corneal transplantation *via* tail vein. Control rat received the same volume of PBS. In each group, 16 recipients were kept for determination of survival time and other 9 recipients were executed on day 3, 7 and 14 after transplantation. Cornea was harvested for hematoxylin-eosin staining and acute rejection evaluation, Western blot was used to detect the expression level of Foxp3.**

• **RESULTS: The mean survival time of imDC group was significantly longer than that of control and mDC groups (all $P<0.05$). The expression level of Foxp3 on $CD4^+CD25^+$ T cells of imDC group (2.24±0.18) was significantly higher than that in the control (1.68±0.09) and mDC groups (1.46±0.13) (all $P<0.05$).**

• **CONCLUSION: Donor-derived imDC is an effective treatment in inducing immune hyporesponsiveness in rat PKP. The mechanism of immune tolerance induced by imDC might be inhibit T lymphocytes responsiveness by regulatory T cells.**

• **KEYWORDS:** immature dendritic cell;high-risk keratoplasty; regulatory T cells
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INTRODUCTION

In orthotopic corneal transplantation in humans, a surprisingly high proportion of grafts, especially in so-called low-risk situations, are successful, compared with other types of organ transplants [1]. Nevertheless, immunological allograft rejection is reported to be the leading cause of corneal graft failure [2]. The incidence of corneal allograft performed in high-risk hosts, characterized by inflamed and vascularized recipient beds, is significantly increased [3]. Considering the high failure rate from rejection in these high risk corneas, development alternative methods of immunosuppressant are still needed for corneal transplantation, especially for use in these HR hosts.

Dendritic cells (DCs) are bone marrow-derived antigen-presenting cells (APCs). DCs have a major role in initiating immune responses by activation of naive T cells [4,5] and in maintaining T cell tolerance, and thus modulate homeostasis and prevention of autoimmunity [5-7]. Depending on their maturational state and their location, DCs perform different functions within the immune system [8]. DCs normally reside and traffic through nonlymphoid tissues in an immature form, poised for antigen capture. Non-activated immature DCs (imDCs) reside in various tissues of the body and are capable of recognizing and capturing antigens bearing pathogen-associated molecular patterns [9]. Upon activation, the DCs up-regulate co-stimulatory molecules, process and present antigens in context of MHC molecules, and produce cytokines to provide efficient priming and stimulation of

antigen-specific naive T cells [10]. Because the differential polarization of T cells in response to antigen presentation is dependent on the maturational state of DCs, we hypothesized that the adoptive transfer of imDCs would prolong corneal graft survival.

Regulatory T cells (Tregs) are a subset of $CD4^+$T cells crucial for protecting the host from autoimmunity by suppressing self-reactive T cells and preventing immunopathology by restraining immune responses directed against foreign antigen [11,12]. The peripheral Treg pool is comprised of naturally arising Tregs (nTregs) that develop in the thymus and inducible Tregs (iTregs) that are converted from conventional $CD4^+$T cells in the periphery [13]. Naturally occurring nTregs play an active role in establishing and maintaining immunological unresponsiveness to self-constituents and negative control of various immune responses to non-self-antigens [14]. The concept of Tregs for immunologists is certainly a plausible therapeutic target for immunologic tolerance[15,16].

Little is known, however, about the DCs and the interactions that can influence the development and/or peripheral expansion of Tregs, although evidence certainly suggests that their role may be important[17-19]. Here we discuss the capacity of DCs to expand antigen-specific Tregs.

MATERIALS AND METHODS

Materials Wistar and SD male rats 8-10 weeks, weighing 160-180g were purchased from the animal institute of the Xinjiang Medical Academy, Xinjiang, China. All animals were treated in accordance with the ARVO statement on the use of animals in ophthalmic and vision research.

Reagents, cytokines, and antibodies RPMI-1640 nonessential amino acids, penicillin/streptomycin, fetal bovine serum (FBS) were purchased from Gibco-Life Technologies, Inc.(Grand Island, NY,USA). Recombinant rat granulocyte macrophage-colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) and tumor necrosis factor alpha (TNF-α) were purchased from Peprotech (Rocky Hill, NJ, USA). Fluorescein isothiocynanate (FITC)-conjugated monoclonal antibody (mAb) OX-62 (anti-integrin alpha E), HIS19 (anti-MHC class Ⅱ), and phycoerythrin(PE)-conjugated mAb 3H5 (anti-CD80 [B7-1]), 24F (anti-CD86 [B7-2]) were purchased from eBioscience (San Diego, CA, USA). Anti-FoxP3 was purchased from Invitrogen (Carlsbad, CA, USA).

Methods

Generation of immature and mature DCs from rat bone marrow progenitors The generation of rat normal DCs was performed according to the previous report with some modifications[20]. The femurs and tibias from the hind limbs of Wistar rats were removed, cleaned of muscle tissue, and placed in sterile petri dishes containing complete medium (RPMI 1640 with 5% FBS, pen/strep (100U/mL and 100μg/mL), and 2mmol/L L-glutamine). The ends of the bones were cut, and the marrow was flushed out with culture medium (CM) in a 10-mL syringe capped with a 23-gauge needle. The marrow was dispersed and filtered through a sterile, nylon screen to remove debris and clumps. The cells were pelleted at 400g for 5 minutes, and red blood cells in the pellet were lysed by hypotonic treatment with 17mmol/L Tris, pH 7.2, for 2 minutes. Isotonicity was restored by the addition of 10 volume of RPMI 1640/15% FBS. After pelleting, the BM cells were resuspended in DC CM. The DC CM was supplemented further with GM-CSF, (20ng/mL) and IL-4 (10ng/mL). Cells (3mL) were then plated into six-well trays at 2×10^6/mL and were maintained in a humidified incubator at 37℃ with 5% CO_2/95% air. The cultures were fed every other day by removing 50% of the supernatant and replacing it with fresh DC CM plus cytokines. On day 6, cells were harvested as imDCs and mDCs generated by additional stimulation with TNF-α (10ng/mL) on day 6 for 2 days.

Phenotypic evaluation of DCs The phenotypic characterization of imDC and mDC were carried out using FACS Calibur and CellQuest software. Cells were washed and incubated with α-FCR (CD16/CD32) (2.4G2) (Pharmingen, San Diego, CA, USA) at 4℃ for 10 minutes to block nonspecific binding of fluorochromes. The following directly conjugated antibodies were incubated with DCs at 4℃ for 30 minutes: OX62-FITC, MHC-Ⅱ-FITC, CD80-PE, CD86-PE (0.25μg at a final volume of 100μL). Cells were washed three times and analyzed using the FACS Caliber (Becton-Dickinson, San Diego, CA, USA).

Scanning electron microscopy DCs were harvested and using the previously described method of Klinkert *et al*[21]. Briefly, DCs were collected and fixed in 2.5% glutaraldehyde and 0.1mol/L cacodylate buffer. After dehydrations with ethanol, the cells were embedded in eponate-12 and reviewed using a JEOL JSM-T300 microscope.

Induction of corneal neovascularisation Corneal neovascularisation model was induced by alkali injury, as described by Ormerod *et al* [22] with some modifications. Briefly, a 3-mm-diamete circular filter disc was incubated with 1mol/L NaOH for 30 seconds. The filter disc was placed in the center of the left corneal surface for 40 seconds in anesthetized rats. The ocular surface was then irrigated with 60mL physiological saline. Fourteen days later, neovessels entered the mid-peripheral zone from the limbus. Only rats with neovessels in all four quadrants served as recipients of orthotropic keratoplasty.

Treatment schedules The corneal neovascularisation rats were randomly divided into three groups of 25 rats per group. In imDC group, donor bone marrow-derived imDC of 1×10^6 were injected into SD rats *via* tail vein before corneal transplantation; the mDC group received mDC of 1×10^6. Control group received the same volume of PBS.

Orthotropic corneal transplantation and definition of graft rejection Orthotropic corneal transplantation was performed as described by Williams and Coster [23] with some modifications. Each animal was deeply anaesthetized with an intramuscular injection of ketamine (75mg/kg), atropine (0.2mg/kg) and xylazine (5mg/kg) before all surgical procedures. The donor eye was excised, and the center cornea was cut with a 3.5mm trephine then stored in sodium hyaluronate. The recipient graft bed was prepared by 3mm excision of the central cornea. The donor button was then secured in place with eight interrupted 10-0 nylon sutures. Antibiotic ointment was applied to the eye after operation. The sutures' ends were cut as short as possible, and the eyelids were shut for 24 hours with tarsorrhaphy. Transplant sutures were kept in place and not removed. From the first day after keratoplasty, grafts were examined and scored by slit lamp microscopy every day till graft rejection occurred. Then the rat corneas were examined every 2 days. A scoring system was used to evaluated graft opacity, edema, and vascularization[24]. Graft opacity was scored from 0 to 4: 0=no opacity; 1= faint opacity with iris details clearly visible; 2= some details of the iris seen with difficulty caused by corneal opacity; 3= extensive opacity, but pupil still could be seen; 4= total opacification. Graft edema was scored from 0 to 2:0 = absence of edema; 1= edema existed but was mild; and 2= very important edema when graft was clearly elevated, more easily observed at its edge. Graft neovessels were scored 0 if no vessel was present in the graft, and from 1 to 3 depending on whether vessels reached only to the periphery (grade 1), the intermediary zone (grade 2), or the central part of the graft (grade 3). Graft rejection was diagnosed on the day at which, in a previously clear graft, a score of 5 was reached and with an opacity grade of at least 3 in all cases.

Specimen preparation On day 3, 7 and 14 after keratoplasty, four rats from each group were sacrificed. The eye globes were taken and fixed in 10% formaldehyde solution. The corneal were cut at 4μm thickness, fixed with 4% paraformaldehyde, and stained by hematoxylin-eosin. The spleens were taken for Western blot assay.

Western blot detection of Foxp3 in spleen Spleen homogenate (100mg) was separated by 15% SDS-PAGE (Invitrogen, Carlsbad, CA, USA) under reducing conditions. After completion of electrophoresis, transfer (2 hours) was performed by semidry transfer cell. Polyvinylidene difluoride (PVDF) membranes were incubated overnight with bevacizumab at a final concentration of 10g/mL in blocking buffer (5% skim milk in PBST). After three washes in 0.2% skim milk in PBST, membranes were then incubated with anti-Foxp3 (1:500) for 120 minutes. Membranes were next incubated with peroxidase-labeled rabbit anti-rat IgG(1:4 000) for 90 minutes. Densitometric analysis of the film was performed using a model GS-710 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA) in transmittance mode and analyzed using Bio-Rad Discovery software.

Statistical Analysis Data are expressed as mean value±SD of all individual experiments. Graft survival was analyzed using the Kaplan-Meier survival method. The log-rank test was used to compare the rates of corneal graft survival in different settings. P <0.05 was defined as statistically significant. Western blot data were analyzed using One-way ANOVA for mean ±SD, and the expression differences between different groups were calculated.

RESULTS

Morphological appearance and flow cytometry profiles of cell surface OX62, MHC-II, CD80, CD86 on imDC, and mDC When alive and viewed by phase-contrast microscopy, imDC show considerable membrane ruffling and motility but do not form prominent dendrites and the cells formed small clusters (Figure 1A). mDCs extend large, delicate, sheet-like processes that can drape around the cell bodies of lymphocytes (Figure 1B). When looked at with a scan electron microscope, The imDCs were small round or oval, the branches sample protuberance short and less(Figure 1C), mDC have long, slender dendrites branches, These branches can extend several times the length of the cell body, and are reminiscent of the typical morphology (Figure 1D). These highly purified imDCs and their mature counterparts were stained with PE-conjugated anti-CD80, anti-CD86, and FITC-conjugated anti-OX62, anti-MHC-Ⅱ and subjected to flow cytometry analyses. As shown in Figure 2, imDC expressed only at low levels MHC-Ⅱ and co-stimulatory molecules (CD80, CD86). mDC expressed high levels of MHC-Ⅱ and co-stimulatory molecules when these cells were stimulated by TNF-α for 48 hours. As seen in Figure 2, at least 90% of the cells positive for OX62, a DC-specific surface marker.

Clinical Evaluation After surgery, mild corneal edema was seen in all animals and disappeared 3 days after transplantation. On postoperative day 14, control and mDC groups exhibited rejection episodes. The grafts exhibited severe edema and infiltration into the epithelium and stroma, and newly formed vessels began to penetrate the transplanted grafts. However, in imDC group, almost all grafts showed no indications of rejection and the graft remained clear until 18 days after surgery (Figure 3).

Incidence and timing of graft rejection The mean survival time of the control group was 9.0 (SD0.46) days. Adoptive transfer of donor derived imDCs, unlike mDCs, significantly prolonged graft survival, the allografts survived more than 18 days. ImDC-treated groups showed high graft survival rates on postoperative day 14. In relation to the rejection onset time, the statistical analysis showed a significant difference between the control group and imDC-treated groups (P<0.05). In contrast, adoptive transfer of mDCs led to graft survival similar to that in recipients treated with PBS alone (Figure 4).

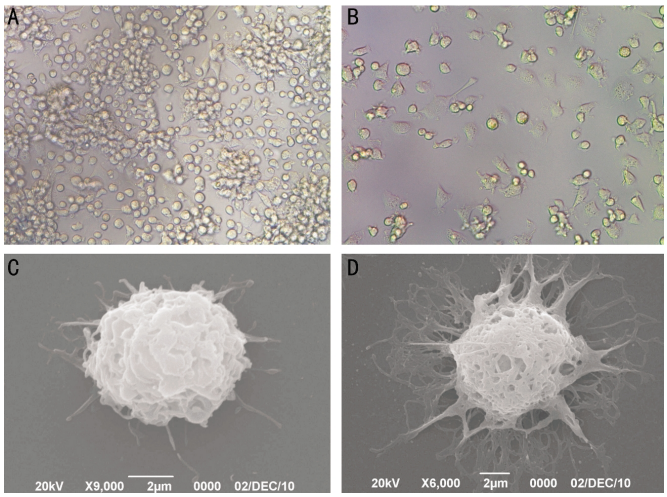

Figure 1 Morphological appearance of imDC and mDC Phase-contrast microscopy showed visible DCs morphological rules. A: The main cell is small round or oval in imDCs, cells formed small clusters composed of four to six; B: The mDCs have long, slender dendrites. These dendrites can extend several times the length of the cell body, and are reminiscent of the typical morphology; C: Scanning electron microscopy showed the branches sample protuberance short and less in imDC; D: mDC form prominent dendrites and have long cytoplasmic veils by scanning electron microscopy.

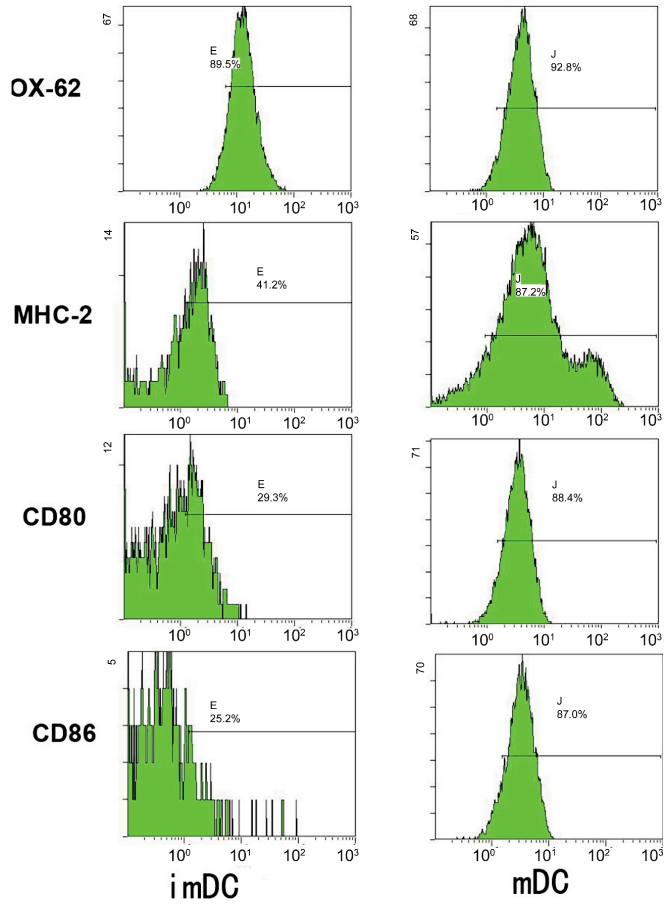

Figure 2 Expression of cell surface molecules on DCs After 6 days in culture in GM-CSF and IL-4-supplemented medium, the loosely attached clusters were collected as imDCs. mDCs were generated from BM cells cultured with rrGM-CSF for 6 days followed by stimulation with TNF-α for 48 hours. DCs were pooled for flow cytometry.

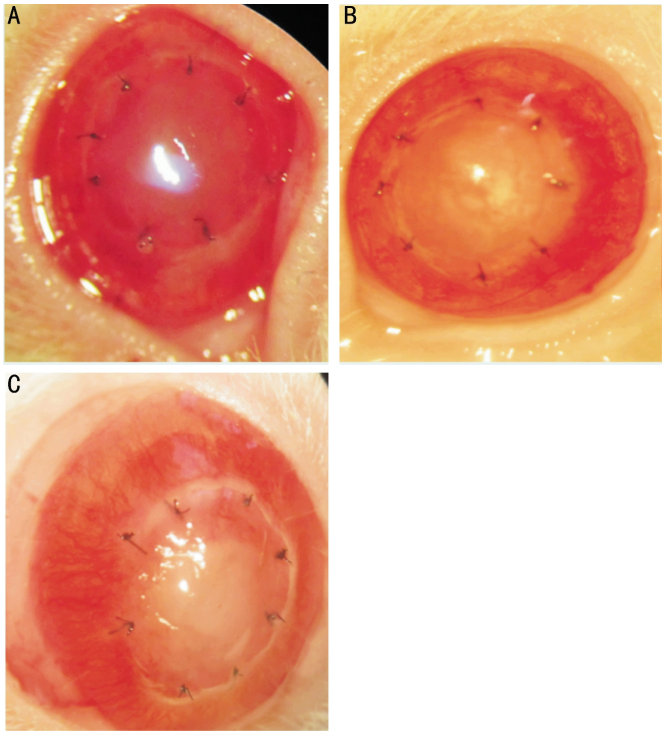

Figure 3 The graft survival in different groups After surgery, heavy corneal edema was seen in control group at the time of postoperative day 14 (A). enlarged blood vessels were seen around the graft in mDC group (C). In imDC group, the graft remained clear, with less neovasculature moving into the grafts (B).

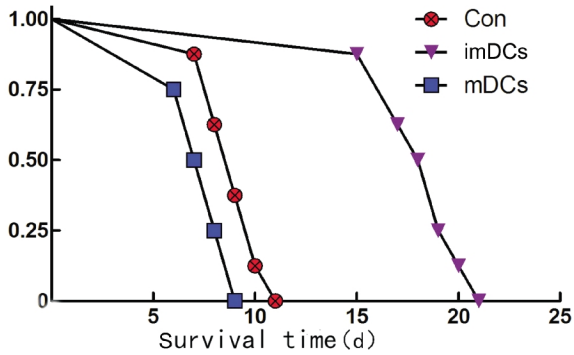

Figure 4 Survival curves for different groups Three experimental groups were distinguished: comparison of allograft survival between the three of the control group (MST, 9.0 ±0.46 days, n=16), imDC group (MST, 18.25 ±0.68 days, n=16) and mDC group (MST, 7.63±0.49 days, n=16).

Hematoxylin–Eosin Staining The allografts of the all rats at the time of postoperative day 3 had mild edema. The allografts of the control and mDC group rats at the time of postoperative day 7 had edema and infiltration of mononuclear cells, and showed a small amount of new blood vessels. The imDC groups showed less in filtration of the immune cells than the control groups and had no new blood vessels. The allografts of the control (Figure 5) and mDC (Figure 5C) group rats at the time of rejection (postoperative day 14) had edema and a heavy infiltration of mononuclear cells. The edema was recognized by the augmentation in the thickness of the total cornea and vacuoies, especially at the basal cell layer of the epithelium. Mononuclear cell

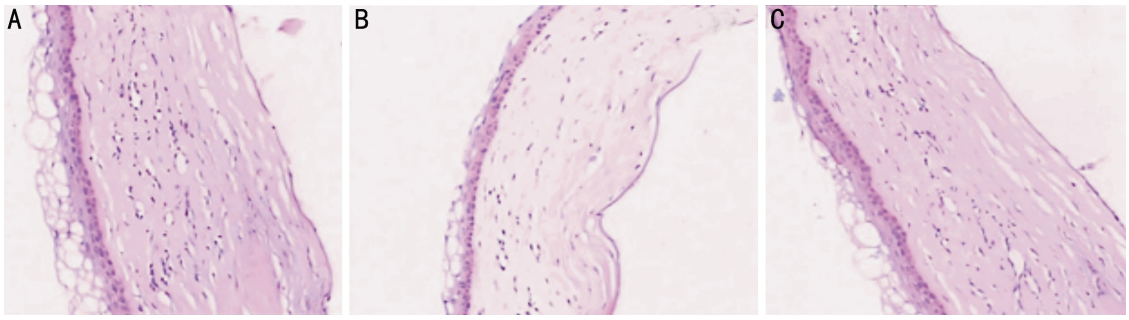

Figure 5 The hematoxylin-eosin staining of allografts from different groups The allografts of the control (A) and mDC (C) group rats at the time of postoperative day 14 had edema and infiltration of cells, especially in the stroma, and there were lot of new blood vessels. The corneal neovascularization was reduced in imDC group (B) and do not have much mononuclear cell infiltration.

infiltration was observed in all layers of the cornea. Cells infiltrated the deep layer of the epithelium, significantly in the stroma, and around the endothelium. There were lot of new blood vessels. Allografts from rats treated with imDC obtained on day 14 did not have such mononuclear cell infiltration (Figure 5B). Corneal neovascularization was reduced strongly by treated with imDC.

Detect of Foxp3 by Western Blot Assay $CD4^+CD25^+$Treg expressed a critical transcription factor, Foxp3. The change of Foxp3 suggests that this action maybe by the induction of $CD4^+CD25^+$Tregs. As shown in Figure 6, Foxp3 were detectable in every group at 3, 7 and 14 days. imDC caused a significant increase in Foxp3 expression compared with mDC mice and PBS control group.

DISCUSSION

The basic paradigm to explain how DCs can both induce immunity and tolerance centers on DCs maturation. We prepared imDCs generated from BM cells obtained from Wistar rat cultured with GM-CSF and IL-4 for 6 days. mDC were generated from BM cells cultured with GM-CSF for 6 days followed stimulation with TNF-α for 2 days. Then we define the developmental morphology and phenotype of DCs to serve as a baseline for investigations of DCs functions in tolerance in the rat. The morphology of DCs was characterized by phase-contrast microscopy and scanning electron microscopy. We detected the expression of different maturation state of DCs phenotype by CD80, CD86 and MHC class-Ⅱ. The OX62 mAb was restricted to DCs [25]. Under steady-state conditions, DCs maintain an immature status until an inflammatory signal promotes their activation, at which time they up-regulate co-stimulatory markers such as CD80, CD83, CD86, and MHC molecules [26]. Our results revealed that both populations showed a high level of OX62. Although both populations expressed the activation of co-stimulatory molecules CD80, CD86 and MHC-Ⅱ, mDCs expressed high levels of CD80, CD86, and MHC molecules, typically with a 2-fold increase over their imDC counterparts whereas imDC had relatively high levels of MHC molecules and extremely low levels of CD80, CD86. Together, these results indicate that imDC are myeloid DC subsets distinct from mDC subsets.

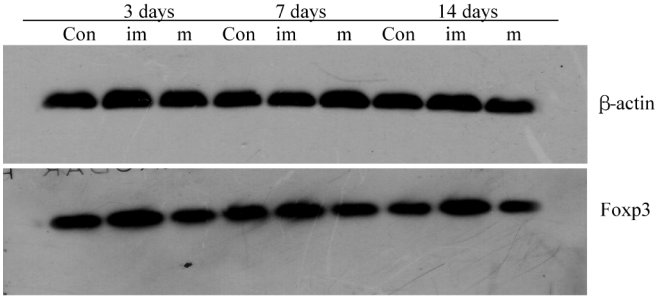

Figure 6 Foxp3 proteins detection by Western blot assay Line 1 to 3 was the expression of Foxp3 on postoperative day 3 (control group; imDC group; mDC group); Line 4 to 6 was the expression of Foxp3 on postoperative day 7 (control group; imDC group; mDC group); Line 7 to 9 was the expression of Foxp3 on postoperative day 14 (control group; imDC group; mDC group), Foxp3 can be detected in every groups and these higher molecular weight bands are only present in the imDC groups.

For the study of the influence of different maturation state of DC to high-risk corneal transplantation immune rejection, we using alkali burn method induced corneal neovascularization, when corneal neovascularization anastomosed penetrating corneal transplantation was performed. A week before transplantation, 1×10^6 different maturation state of donor bone marrow-derived DCs was injected *via* tail vein. Adoptive transfer of Wistar imDCs, unlike mDCs, 7 days before corneal transplantation significantly increased the median survival time from 9 days to 18 days. Corneal allograft survival is prolonged significantly in recipients of imDC cultured with GM-CSF and IL-4, this paradigm has been widely supported by data from experimental transplant models, including observations that imDC of either donor or host origin can promote tolerance induction. For example, a single injection of immature donor-derived DC 7 days before transplant extends [27] or prolongs indefinitely [28] mouse MHC-mismatched heart allograft survival in a donor-specific manner. but all rat occurred transplantation rejection by day 18, this may because the injected normal imDCs are not likely to remain immature *in vivo* after recirculation and home into the damaged tissue where chronic inflammation is always present.

ImDC plays a very important role in the balance of transplantation tolerance, therefore, it's necessary to discuss the mechanism of immune tolerance. Tregs were first described by Sakaguchi *et al* [29]. Recently, Tregs, with their potent suppressive effects on normal responder T cell function, have become the focus of transplant immunology[30,31]. It has been suggested that Tregs play an important role in transplantation tolerance[32-34]. These Tregs represent a unique lineage of $CD4^+$T cells characterized by a constitutively high expression of CD25 ($CD4^+CD25^+$Tregs), in which forkhead box p3 (Foxp3) has been demonstrated as critically important for both their development and function [35,36]. $CD4^+CD25^+$ Tregs have been shown to play a major role in the maintenance of peripheral tolerance [37, 38]. Elegant studies by several investigators have elucidated their vital role in T cell homeostasis and immune regulation [39-42]. Data also indicate that the role of $CD4^+CD25^+$ Tregs is not limited to self-tolerance and the prevention of autoimmunity[43].

The development and function of Tregs are controlled by the X-chromosome-encoded forkhead transcription factor, Foxp3 [35,44,45]. Foxp3 is the most specific current marker for Tregs because, unlike CD25, Foxp3 is not up-regulated in activated effector T cells [36,46]. Foxp3 has been shown to be specifically expressed in murine $CD25^+CD4^+$Treg cells and as such appears to be a "master gene" controlling the development and suppressive function of these cells [44, 45]. To investigate the mechanism of immune hyporesponsiveness induced by donor-derived imDC of corneal grafts in rats. We have now investigated the interaction between different DC populations and $CD25^+CD4^+$Tregs in high-risk corneal transplantation. In trying to understand such issues, we have looked particularly at the role of DCs and attempted to shed some light on the nature of the $CD25^+CD4^+$Tregs-DC interaction. The Western blot was used to detect Foxp3 expression of $CD4^+CD25^+$Tregs. Western blot analysis showed that there was a robust increase in $CD4^+CD25^+$Tregs in the imDC administration rat, whereas $CD4^+CD25^+$Tregs were decreased in number in both the untreated and the mDC treated recipient mice compared with imDC group. In addition, $CD4^+CD25^+$Tregs obtained from the imDC treated recipient rat showed greater suppression of the allogeneic activation of $CD4^+CD25^+$Tregs by recipient, indicating that imDCs had a much more potent ability to induce $CD4^+CD25^+$Tregs as compared with mDCs. Collectively, the protective effect of the donor derived imDC on the high-risk corneal transplantation could involve the generation of alloreactive $CD4^+CD25^+$Tregs.

In summary, we report the therapeutic effect of imDC in high-risk corneal transplantation model. Analysis of the recipient rat suggested that the protective effect of the imDC strongly correlate with the presence of $CD4^+CD25^+$Tregs in the high-risk corneal transplantation models. Our data finally prove the hypothesis proposed by Steinman *et al* [46] that DCs in the steady state are prone to induce tolerance *in vivo*, immunization with immature donor DCs has therapeutic potential for the induction of transplant tolerance. Our findings also demonstrate that the administration of imDC leads to the induction of regulatory T cells that are able to down-modulate immune responses *in vivo*. The immune suppression function of $CD4^+CD25^+$Tregs, mediated by Foxp3 gene, is one of the mechanisms in corneal transplantation tolerance.

Currently, the precise molecular mechanism by which imDC treatment increases naturally occurring $CD4^+CD25^+$Tregs and mediates its immune tolerance effects remains to be elucidated. Understanding the underlying mechanisms of Tregs modulation remains elusive, and further studies are warranted to provide insight as to how Tregs develop and suppress effecter T cells. On the other hand, the mechanism responsible for the generation of $CD4^+CD25^+$Treg from the primed naive $CD4^+$T cells with imDCs remains unknown. Further study will be needed to clarify the mechanism responsible for their inhibitory effect.

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