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Research paper

Alloantigen-specific T-cell hyporesponsiveness induced by dnIKK2 gene-transfected recipient immature dendritic cells

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ABSTRACT

Immature dendritic cells (iDCs) have been shown to be able to induce peripheral T-cell tolerance through distinct pathways. Here, we investigated the tolerogenic property of recipient iDCs whose maturation was arrested by a dominant negative mutant of inhibitor of nuclear factor kappa-B kinase 2 (dnIKK2) gene. We found that dnIKK2-iDCs presented a typical semi-mature morphology and expressed lower levels of CD80 and CD86, slightly higher MHC-II than untransfected iDCs. The expression of these molecules had no significant change even dnIKK2-iDCs were pulsed by donor antigen. In primary mixed leukocyte reaction (MLR), dnIKK2-iDCs exhibited impaired ability to stimulate allogeneic T-cells, but induced $CD4^+CD25^-$ T-cell formation. In co-culture MLR, these $CD4^+CD25^-$ T-cells suppressed T-cell alloreaction in an antigen-specific manner. Besides, $CD4^+CD25^-$ T-cells inhibited IL-2 and IFN-γ release, whereas promoted IL-10 and TGF-β secretion. These data suggested recipient dnIKK2-iDCs could maintain peripheral tolerance through down-regulating costimulatory molecule expressions and inducing $CD4^+CD25^-$ T-cell formation.

1. Introduction

Renal transplantation is the optimal mode of replacement therapy in most patients with end-stage renal disease [1–3]. However, recipients of renal transplants need the lifelong use of immunosuppressive medications to prevent graft rejection [4]. The use of immunosuppressive medications is associated with cumulative side effects, including increased risks of infection, malignancy, cardiovascular disease and diabetes [5,6]. Therefore, elimination of the lifelong need for immunosuppressive drugs and induction of immune tolerance remains an elusive and important goal in the management of renal transplantation.

In the induction of tolerance or immunity, dendritic cells (DCs), the most potent professional antigen-presenting cells (APCs), play key roles. After transplantation, DCs present alloantigen to T-cells through distinct pathways of allorecognition [7,8]. Donor DCs present intact donor MHC molecules to T-cells through the direct pathway; recipient DCs present donor peptides bound on self (recipient) MHC molecules to T-cells through the indirect pathway. Besides, a study has suggested that recipient T-cells can recognize donor MHC molecules transferred, intact on the surface of recipient DCs through the semi-direct pathway [9]. Thus, both donor and recipient DCs have the potential to induce immunity or tolerance during transplantation. However, the potential of DCs to induce immunity or tolerance is largely dependent on the maturation status of DC: immature DCs (iDCs) can induce and maintain peripheral T-cell tolerance, whereas mature DCs (mDCs) induce T-cells immunity [10,11]. The tolerogenic properties of iDCs have prompted considerable interest in exploiting the strategies of iDC-induced peripheral tolerance for the transplantation. These strategies include the use of cytokines and growth factors such as interleukin (IL)-10, transforming growth factor (TGF)-β, and

Abbreviations: dnIKK2, dominant negative mutant of inhibitor of nuclear factor kappa-B kinase 2; DC, dendritic cell; iDCs, immature dendritic cells; dnIKK2-iDCs, immature dendritic cells transfected with dnIKK2 gene; Adv0, empty adenoviral vector; Adv0-iDCs, iDCs transfected with Adv0; MLR, mixed leukocyte reaction; FCM, flow cytometry; TEM, transmission electron microscope; ELISA, *enzyme*-linked immunosorbent assay; LW, Lewis rat; BN, Brown Norway rat; WI, Wistar rat; BN Ag, Brown Norway rat antigen; CD, cluster of differentiation; MHC-II, major histocompatibility complex class II; BM, bone marrow.

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granulocyte macrophage-colony stimulating factor (GM-CSF), or overexpression of costimulatory molecules.

Recently, transcription factor nuclear factor (NF)-κB has been shown to be associated with the maturation and function of DCs [12,13]. Inhibition of NF-κB or activation of its upstream inhibitor of NF-κB kinase 2 (IKK2) can block DC antigen presentation both *in vitro* and *in vivo* [13–16]. In keeping with this, a study demonstrated that treatment of human monocyte-derived iDCs with a recombinant adenovirus-mediated kinase defective dominant-negative mutant of IKK2 (dnIKK2) gene inhibited the allogeneic leukocyte mixed reaction (MLR); and the DCs failed to increase the expression of MHC II and costimulatory molecules in response to CD40 engagement [17]. In addition, *Tomasoni* and his colleagues used an adenoviral vector encoding dnIKK2 to block NF-κB activation of rat bone marrow (BM)-derived iDCs and found that dnIKK2-transfected iDCs were maintained at immature state [18,19]. These iDCs were capable of prolonging kidney allograft survival when infused *in vivo* in rat recipients before transplantation [18,19]. These findings suggested that transfection of iDCs with adenovirus-mediated dnIKK2 gene might be a potent strategy for the induction of transplantation tolerance.

Of note, as mentioned above, the origin of iDCs has important impacts on the mode of alloantigen presentation. Donor iDCs not only are an important source of intact alloantigen for direct recognition pathway, but also indeed provide a source of donor antigen for the stimulation of T-cells with indirect allospecificity [20]. Thus, the use of donor iDCs in transplantation has a potential risk of cross-priming and immunity rather than tolerance [21]. In addition, donor iDCs, administered 7 days before transplantation to prevent allograft rejection, is not applicable for clinical cadaveric renal transplantation. In this study, we transfected recipient BM-derived iDCs with an adenoviral vector encoding dnIKK2 gene to arrest the iDC maturation (dnIKK2-iDCs) and determined the tolerogenic property of dnIKK2-iDCs.

2. Materials and methods

2.1. Reagents

Adenovirus vectors were constructed by SinoGenoMax Co., Ltd (Beijing, China). RPMI 1640, lipopolysaccharides (LPS), dimethyl sulfoxide (DMSO), cell proliferation and cytotoxicity assay kit (MTT) were purchased from Sigma (St Louis, MO). Rat lymphocyte separation medium was purchased from Sino-American Biotechnology Company (Shanghai, China). Fetal bovine serum (FBS) was purchased from Nordic Immunological Laboratory (Tilburg, Netherlands). Magnetic-activated cell sorting (MACS) kit was purchased from Invitrogen Corporation. Recombinant rat IL-4 and granulocyte–macrophage colony-stimulating factor (GM-CSF) were purchased from PeproTech Inc. Phycoerythrin (PE) labeled anti-rat CD80, CD86, and MHC-II antibodies were purchased from Serotec. IL-2, IL-10, TGF-β, and IFN-γ ELISA Kits were purchased from R&D Systems (Minneapolis, MN, USA). The replication-deficient adenovirus encoding human IKK2 plasmid, pACCMVpLpASR(+)-IKK2dn, was a kind gift from Dr. Rain D Martin (University of Vienna, Vienna, Austria). pAdxsi-GFP-dnIKK2 and pAdxsi-GFP-0 were constructed by SinoGenoMax Co., Ltd (Beijing, China).

2.2. Animal preparation

A total of 18 male Lewis (LW/CrlBR), Brown Norway (BN/CrlBR), and Wistar rats (Crl: (WI) BR), weighing 180–200 g at 8–10 weeks of age, were obtained from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). Rats were kept under specific pathogen-free (SPF) conditions in accordance with the NIH guidelines for the care and use of laboratory animals. They were fed a standard rodent chow and maintained in a temperature-controlled (23–25 °C) facility with a strict 12-h light/dark cycle, and given free access to food and water. All procedures involving the rats were approved by Soochow University Animal Management Committee and Beijing City Animal Management Committee.

2.3. Generation of recipient BM-derived iDCs

LW (recipient) BM-derived iDCs were prepared as previously described. Briefly, BM was isolated from recipient tibia and femur by rinsing the bones with serum-free medium. BM cells were passed through a 200-mesh sieve to remove the residue. After being centrifuged at 1500 rpm for 5 min, BM cells were then re-suspended in serum-free medium. Mononuclear cells were isolated from BM cell suspensions by Ficoll/Hypaque density gradient centrifugation. Then the cells were cultured in 6-well plates (Costar, Cambridge, MA) containing RPMI 1640 with 10% heat-inactivated FBS in the presence or absence of rat GM-CSF (5 ng/mL) and IL-4 (5 ng/mL) at a density of 2×10^6/mL per well. After 2 days of culture, half the medium was removed and replaced with fresh medium containing cytokines. Subsequently, all the mediums were removed and replaced with fresh medium every day. At day 5, non-adherent and semi-adherent cells were harvested. iDCs were examined by transmission electron microscope (TEM).

2.4. Generation of dnIKK2-transfected iDCs (dnIKK2-iDCs)

dnIKK2 cDNA was cloned into adenovirus transfer vector pShuttle-CMV-TEMP (Sinogenomax, Beijing, China). Then, pShuttle-CMV-TEMP-dnIKK2 was transferred into pAdxsi-GFP (SinoGenoMax Co., Ltd., Beijing, China) to generate pAdxsi-GFP-dnIKK2. One day before the iDC infection, 293 T-cells were cultured in 6-well plates for 24 h in DMEM containing 10% FBS without antibiotics. When grown to 80–90% confluence, the 293 T-cells were co-transfected with the adeno-vectors containing pAdxsi-GFP-dnIKK2, pHelper 1.0 and pHelper 2.0 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) to produce adenovirus-dnIKK2 (Adv-dnIKK2). Then the culture supernatants containing the adenovirus were harvested. The virus particles (VP) were purified by two-step cesium chloride density gradient ultracentrifugation procedure and determined by optical density (OD, VP/mL = $OD_{260} \times 1.1 \times 10^{12}$).

iDCs were cultured in 6-cell plates containing serum-free RPMI 1640 for 4 h. Subsequently, the iDCs were infected with adenovirus at an MOI of 50 for 3 h. The cultures were then replaced with fresh medium containing GM-CSF and IL-4 and further cultured for 2 days. After 2 days, the iDCs were collected and re-suspended in RPMI 1640 containing GM-CSF and IL-4. The infection efficiency was assessed under an inverted fluorescence microscope and the percentage of cells positive was quantified using flow cytometry (FCM). iDCs were also infected with empty adenoviral vector (Adv0). Untransfected iDCs and the iDCs cultured with LPS (1 ng/L) were used as control.

2.5. Western blotting analysis for dnIKK2 expression in iDCs

Recipient iDC monolayers were prepared and washed quickly 2 times with cold PBS. Then the iDCs were lysed with a lysis buffer and protein concentrations were determined by bicinchoninic acid (BCA) assay kit. Protein samples were mixed with loading buffer and loaded onto a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE). After electrophoresis, the SDS–PAGE separated proteins were transferred to a nitrocellulose membrane

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