Prolongation of rat renal allograft survival by $CD4^+CD25^-$ T cells induced by recipient dendritic cells transfected with IKK2dn

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Abstract. Previous studies have demonstrated that recipient-derived immature dendritic cells transfected by recombinant adenovirus-mediated IKK2dn (Adv-IKK2dn) and loaded with donor splenocyte lysate generate $CD4^+CD25^-$ T cells (Adv-IKK2dn-$CD4^+CD25^-$ T cells). These cells may inhibit T cell responses *in vitro*. In the present study, Lewis (LW) rats were administered with an intravenous injection of naive $CD4^+$ T cells, empty adenovirus (Adv-0)-dendritic cell-generated $CD4^+CD25^-$ T cells (Adv-0-$CD4^+CD25^-$ T cells), Adv-IKK2dn-$CD4^+CD25^-$ T cells or an equal volume of normal saline, seven days prior to transplantation. The potency and the mechanism of action of Adv-IKK2dn-$CD4^+CD25^-$ T cells was analyzed, as well as an investigation of their tolerogenic properties *in vivo*. Administration of Adv-IKK2dn-$CD4^+CD25^-$ T cells *in vivo* to LW rats was observed to markedly prolong the survival of a kidney allograft from Brown Norway rats. Furthermore, the Adv-IKK2dn-$CD4^+CD25^-$ T cell-treated group exhibited significantly reduced levels of interleukin (Il)-2 and interferon-γ production and increased Il-10 and transforming growth factor-β (TGF-β) secretion. The serum creatinine levels remained at low levels in the Adv-IKK2dn-$CD4^+CD25^-$ T cell-treated group. Their ability to induce allogeneic T cell proliferation was markedly reduced compared with the other groups. These observations indicated that Adv-IKK2dn-$CD4^+CD25^-$ T cells induce prolongation of kidney allograft survival *in vivo*, which is hypothesized to be due to the high expression levels of Il-10 and TGF-β.

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Introduction

Dendritic cells (DCs) are the most important antigen presenting cells, their predominant feature is the ability to stimulate the proliferation of naive T cells and they are also important in the immune response. Mature DCs express high levels of cell surface class II major histocompatibility complex (MHC-II) and co-stimulatory molecules. On account of their capability of presenting alloantigens to T cells, DCs stimulate T cell proliferation to induce an immune response. In contrast, immature dendritic cells (imDCs), characterized by low expression of both MHC-II antigens and co-stimulatory molecules, can be instrumental in the induction of peripheral tolerance. Previous studies have shown that the capacity of DCs to modulate immune responses relates to their state of functional maturation (1-3). Immature dendritic cells (imDCs) are able to capture and process antigens. Studies have previously indicated that imDCs induce peripheral tolerance via T cell anergy, immune deviation, promotion of activated T cell apoptosis and formation of regulatory T cells (Tregs) (2-6).

To date, studies have shown that nuclear factor-κB (NF-κB) is important in DC maturation and tolerance induction (7-9). Moreover, NF-κB activation requires the action of multiple kinases (7,10), including IKK2, which has been shown to be essential for DC maturation. Recipient or donor bone marrow-derived DCs transfected with IKK2dn to block NF-κB have been observed to prevent DC maturation (11,12). In addition, recombinant adenovirus-mediated IKK2dn (Adv-IKK2dn)-DCs prolonged the survival time of kidney transplants in rats by inducing Treg generation (11-13). In addition, a number of observations indicated that donor-derived imDCs transfected with IKK2dn induced the formation of a unique population of $CD4^+CD25^-$ Tregs. These cells are capable of potently inhibiting naive and activated T cell responses *in vitro* and inducing prolongation of kidney allograft survival *in vivo* (11,13).

Our previous study demonstrated that recipient-derived DCs transfected with Adv-IKK2dn inhibit NF-κB

activation and impair DC maturation. In addition, an Adv-IKK2dn-DC-treated group was demonstrated to exhibit markedly prolonged renal allograft survival (12). Furthermore, recipient-derived imDCs transfected by Adv-IKK2dn were shown to generate $CD4^+CD25^-$ T cells, which exert immune tolerance *in vitro* (14,15). In the current study, Adv-IKK2dn-$CD4^+CD25^-$ T cells were administered to Lewis (LW) rats and their ability to induce anti-allotolerance in a rat renal transplantation model was investigated. The results demonstrated that Adv-IKK2dn-$CD4^+CD25^-$ T cells may prolong renal allograft survival in a donor-specific manner and this was hypothesized to result from high expression levels of interleukin (Il)-10 and TGF-β.

Materials and methods

Animals and reagents. Male LW (CrlBR), Brown Norway (BN/CrlBR) and Wistar (WI/CrlBR) rats, 8-10-weeks-old and ~180-200 g, were purchased from Vital River Laboratories (Beijing, China) and maintained in the Soochow University animal facility. Procedures involving animals and their care were conducted in accordance with the institutional guidelines that were in compliance with Regulations for the Administration of Affairs Concerning Experimental Animals and Measures of Jiangsu Province on Administration of Affairs Concerning Experimental Animals. The recombinant rat granulocyte macrophage-colony stimulating factor and recombinant rat Il-4 were purchased from Peprotech, Inc. (Rocky Hill, NJ, USA). Il-2, Il-10, transforming growth factor-β (TGF-β), interferon-γ (IFN-γ) and enzyme-linked immunosorbent assay kits were purchased from R&D Systems (Minneapolis, MN, USA). The $CD4^+CD25^-$ Treg isolation kit and MiniMACS separator were purchased from Miltenyi Biotech (Bergisch Gladbach, Germany). The replication-deficient Adv encoding a kinase-defective dominant negative form of human IKK2 plasmid, pACCMVpLpASR(+)-IKK2dn, was provided by Dr Rain D Martin (University of Vienna, Vienna, Austria). PAdxsi-GFP-IKK2dn and pAdxsi-GFP-0 were constructed by SinoGenoMax Co., Ltd (Beijing, China).

DCs transfected with Adv-IKK2dn and loaded with BN antigen. Bone marrow-derived DCs (from LW rats) were obtained as described previously (12,14,15). Cells were harvested at day five of culture and transfected with Adv-IKK2dn or an empty adenovirus (Adv-0) at a multiplicity of infection of 50 (12). Cells were subsequently cultured for a further two days and pulsed with BN antigens. BN spleen cell lysate (antigen) was prepared by 6 repetitions of freezing (5 min in a dry ice-ethanol bath) and thawing (10 min in a 37˚C bath) and added at a ratio of 1:5, DC:Spleen cells (used to prepare lysate) for the final 48 h of DC culture (Adv-IKK2dn-DC loaded with BN antigen). Subsequently, cells were harvested and used as stimulators for the mixed lymphocyte reaction.

Primary mixed lymphocyte reaction (MLR) and separated T cells. Following 48 h, cells were harvested and used as stimulators for the primary MLR, and the LW spleen T cells were used as responders. The DC:T cell ratio was 1:100. Cultures were prepared in triplicate in 24-well round-bottom microculture plates (200 µl/well with $1x10^6$ T cells) and maintained for 72 h in 5% CO_2 at 37˚C. Uninfected and Adv-0-DC groups served as controls. Following 72 h T cells were separated by magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Separation was achieved by removing $CD4^+$ T cells using an LD separation column (composed of ferromagnetic spheres covered with a cell-friendly coating) by negative selection. CD25 expression was measured by flow cytometry (Beckman-Coulter, Fullerton, CA, USA). The $CD4^+CD25^+$ T cells were then removed by positive selection using an MS separation column (necessary for the isolation of cells which are only minimally labeled with MACS MicroBeads, while leaving enough epitopes free for concurrent antibody staining), which yielded $CD4^+CD25^-$ T cells. Following this, $CD4^+CD25^-$ T cells were collected for subsequent experiments.

Renal transplantation. Renal transplantation was performed as previously described (16). Male BN and LW rats were used as donors and recipients, respectively. LW rats were administered with an intravenous injection of naive $CD4^+$ T cells ($CD4^+$ T cell group), Adv0-DC-generated $CD4^+CD25^-$ T cells (Adv-0-$CD4^+CD25^-$T cell group), Adv-IKK2dn-DC-generated $CD4^+CD25^-$ T cells (Adv-IKK2dn-$CD4^+CD25^-$ T cell group) or an equal volume of normal saline (control group) seven days prior to allotransplantation. In the third party donor group (Wistar donor group) Wistar rats as donors were treated the same as the Adv-IKK2dn-$CD4^+CD25^-$T cell group prior to transplantation. Following transplantation, the survival time of recipients was observed, the T lymphocyte proliferation in recipients was measured, the levels of serum Il-2, Il-10, IFN-γ and TGF-β were detected, and the serum creatinine levels were monitored.

Statistical analysis. Data are presented as the mean ± SD and were analyzed by one-way analysis of variance. Survival curves were established using the Kaplan-Meier method. Graft survival between groups of transplanted animals was analyzed with a log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Adv-IKK2dn-DC induced Tregs. $CD4^+$ T cells were isolated by the negative selection method and CD25 expression was analyzed using flow cytometry. Results showed that the Adv-IKK2dn-DC group contained a markedly lower percentage of CD25 (19.1±4.8%, n=6), compared with the control (77.2±4.8%, n=6) and Adv-0-DC (63.9±2.9%, n=6) groups; the difference was statistically significant ($P<0.05$; Fig. 1). The results indicted that the majority of $CD4^+$ T cells were $CD25^-$ upon completion of MLR with Adv-IKK2dn-DC. These observations indicate that Adv-IKK2dn-$CD4^+CD25^-$ T cells may be distinguished from $CD4^+CD25^+$T cells, which express high levels of CD25 (17).

Prolonged kidney graft survival in Adv-IKK2dn-$CD4^+CD25^-$ T cell-treated rats. To investigate whether Adv-IKK2dn-$CD4^+CD25^-$ T cells exhibited an immunoregulatory function *in vivo*, $1x10^7$ $CD4^+$T cells,

Table I. Rat groupings and individual survival time of kidney transplanted rats.

Group no.	Group	No. of rats	Survival time, days (no. of rats)	Mean survival time, days
1	Control	6	6, 7 (3), 8 (2)	7.2±0.31
2	$CD4^+$ T cells	7	9, 10, 12, 13, 15, 16, 19	13.4±1.33[a]
3	Adv-0-$CD4^+CD25^-$ T cells	6	4 (2), 5 (3), 6	4.8±0.31[b]
4	Adv-IKK2dn-$CD4^+CD25^-$ T cells	8	21 (2), 24, 29 (2), 30, 33, 41	8.5±2.36[a,b,c]
5	Wistar donor	6	6 (2), 7 (2), 8, 9	7.2±0.48[b]

[a]P<0.01, vs. group 1; [b]P<0.01, vs. group 2; [c]P<0.01, vs. groups 3 and 5. Adv-0, empty adenovirus; Adv, adenovirus.

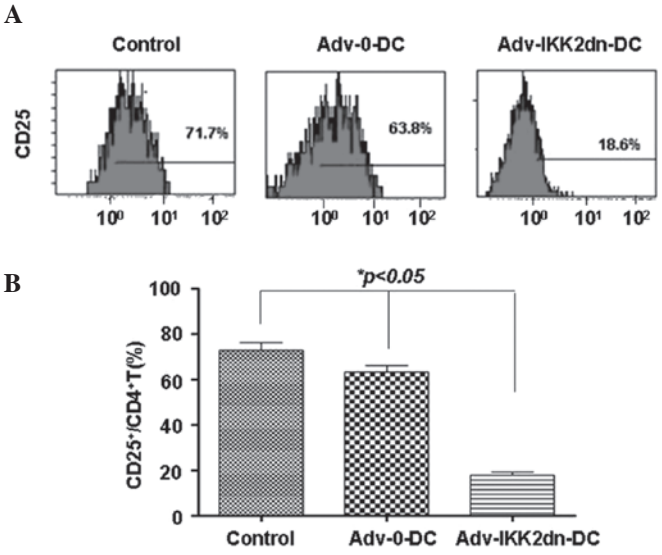

Figure 1. (A) FACs analysis for CD25 expression. Staining was performed with FITC-conjugated anti-CD25 on naive $CD4^+$ T cells, Adv-0-DC-generated $CD4^+$ T cells and Adv-IKK2dn-DC-generated $CD4^+$ Tregs. Negative controls were performed with control isotype FITC-conjugated IgG. Percentages of positive cells are indicated. Representative results from one experiment are shown. (B) Percentages of $CD25^+$ cells were calculated and are presented in the bar graph. Values are representative of data from three independent experiments. P<0.05, vs. control. FACs, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; Adv, adenovirus; Adv-0-DV, empty adenovirus dendritic cell; mAb, monoclonal antibodies; IgG, immunoglobulin G.

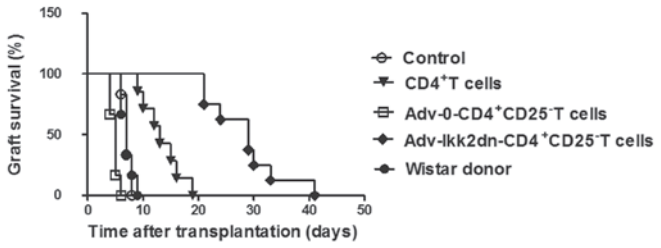

Figure 2. Treatment with Adv-IKK2dn-$CD4^+CD25^-$ T cells prolongs transplanted allo-kidney survival. Five groups were prepared (control, $CD4^+$ T cells, empty adenovirus-$CD4^+CD25^-$ T cells, Adv-IKK2dn-$CD4^+CD25^-$ T cells and Wistar donor). Data are presented as a survival curve. Adv, adenovirus; ADV-0, empty adenovirus.

Adv-0-$CD4^+CD25^-$ T cells, Adv-IKK2dn-$CD4^+CD25^-$ T cells or an equal volume of normal saline were intravenously infused in LW rats seven days prior to kidney transplantation. No immunosuppressive therapy was administered prior to or following transplantation. Rat survival was monitored daily following transplantation.

Results indicated that allograft survival in the Adv-IKK2dn-$CD4^+CD25^-$ T cell-treated group was prolonged significantly in comparison with the $CD4^+$ T cell, control, Adv-0-$CD4^+CD25^-$ T cells and WI donor groups (Fig. 2). In addition, compared with the control group (7.2±0.31 days), the survival time in the WI donor group was not prolonged (7.2±0.48 days; P>0.05; Table I). Previous results indicated that IKK2dn-transfected DCs are capable of inducing tolerance and significantly prolonged transplanted allograft survival (12). The present results supported the hypothesis that imDCs generate or activate Tregs ($CD4^+CD25^-$ T cells) which are important in the induction and maintenance of immune tolerance. Rat numbers and survival time in each group are presented in Table I.

Adv-IKK2dn-$CD4^+CD25^-$ T cells act via the release of cytokines. To detect the mechanism by which Adv-IKK2dn-$CD4^+CD25^-$ T cells significantly prolonged transplanted allograft survival, the serum levels of Il-2, IFN-γ, TGF-β and Il-10 were tested in different groups on days 5 and 14 following renal transplantation. On day 5, in

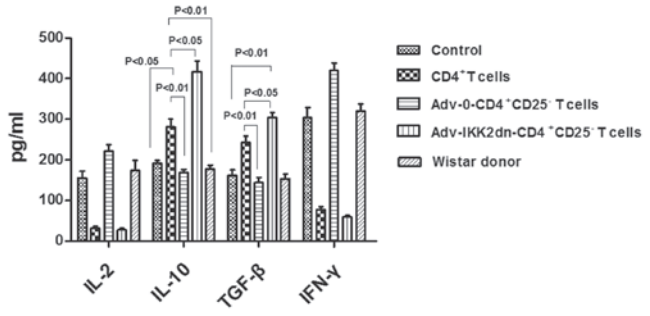

Figure 3. Adv-IKK2dn-CD4+CD25- T cell treatment suppresses Th1 cytokine and increases Th2 cytokine production *in vivo*. Rat serum from allo-kidney transplanted groups was collected five days following transplantation. The serum levels of Il-2, TGF-β, Il-10 and IFN-γ were measured by an enzyme-linked immunosorbent assay. Adv, adenovirus; Th1, T helper; IL, interleukin; TGF-β, transforming growth factor-β; IFN-γ, interferon-γ. AVD-0, empty adenovirus.

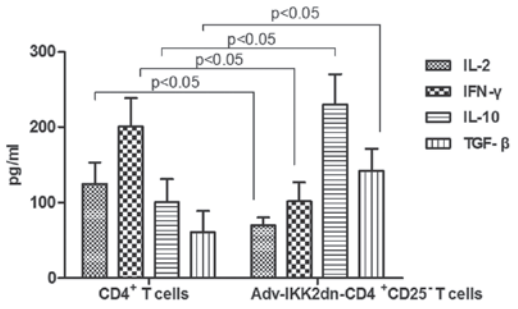

Figure 4. Rat serum from allo-kidney transplanted groups was collected on day 14 following transplantation. Serum levels of Il-2, TGF-β, Il-10 and IFN-γ were measured by enzyme-linked immunosorbent assay. Il, interleukin; TGF-β, transforming growth factor-β, INF-γ interferon-γ; Adv, adenovirus.

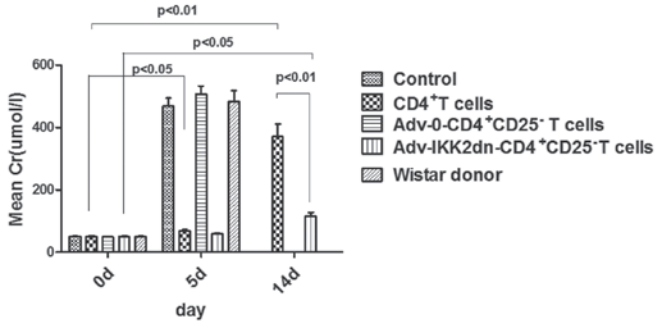

Figure 5. Serum creatinine levels following transplantation. The levels of serum creatinine on days 5 and 14 following transplantation were measured. The Adv-IKK2dn-CD4+CD25- T cell group maintained a low level of serum creatinine. Adv, adenovirus; Adv-0, empty adenovirus.

control, Adv-0-CD4+CD25- T cell and the WI donor kidney transplanted groups, Il-2 and IFN-γ levels were significantly increased compared with the Adv-IKK2dn-CD4+CD25- T cell-treated group and CD4+ T cell-treated group (Fig. 3). Notably, Il-10 and TGF-β production were significantly higher in the Adv-IKK2dn-CD4+CD25- T cell and CD4+ T cell groups compared with the control, Adv-0-CD4+CD25- T cell and the WI donor groups, respectively (P<0.05 or P<0.01). Furthermore, the levels of TGF-β and Il-10 were significantly different between the Adv-IKK2dn-CD4+CD25- T cell and CD4+ T cell groups (Fig. 3; P<0.05).

On postoperative day 14 (Fig. 4), the production of Il-2 and IFN-γ was markedly increased in the CD4+ T cell group, compared with the Adv-IKK2dn-CD4+CD25- T cell group, the differences were statistically significant (P<0.05). In addition, the levels of Il-10 and TGF-β decreased in the Adv-IKK2dn-CD4+CD25- T cell group (Fig. 4), compared with the CD4+ T cell group, the difference between the two groups was statistically significant (P<0.05). Thus, Adv-IKK2dn-CD4+CD25- T cell treatment reduced Il-2 and IFN-γ production and increased Il-10 and TGF-β secretion in the serum of allo-kidney transplanted rats. It also indicated that Adv-IKK2dn-CD4+CD25- T cells significantly prolonged transplanted allograft survival by suppressing the anti-allograft T helper (Th) 1 immune response and enhancing the Th2 response *in vivo*.

Serum creatinine levels. Following transplantation, serum creatinine levels were measured on days 5 and 14. On day 5, the serum creatinine level was markedly increased in the control, Adv-0-CD4+CD25- T cell and WI donor groups. However, in Adv-IKK2dn-CD4+CD25- T cell-treated and CD4+ T cell-treated groups, serum creatine remained at a low level (Fig. 5). On day 14, the serum creatinine level was markedly elevated in the CD4+ T cell-treated group. However, the Adv-IKK2dn-CD4+CD25- T cell-treated group remained at a low level (Fig. 5). There were statistically significant differences between these two groups (P<0.01). Thus, Adv-IKK2dn-CD4+CD25- T cells are important in maintaining stable renal function. These observations indicate that Adv-IKK2dn-CD4+CD25- T cells may extend the length of rat renal allograft survival.

Co-culture MLR. A three-day MLR was performed with syngeneic (LW) or allogeneic (alloantigen-specific BN or third-party WI) irradiated splenocytes (antigen) and T cells obtained from the lymph nodes of rats that had undergone transplantation following different treatments. T cells in each group remained in a low response state to the LW antigen stimulation. T lymphocyte proliferation stimulated by the BN antigen in the Adv-IKK2dn-CD4+CD25- T cell group was significantly lower compared with that in the control, Adv-0-CD4+CD25- T cells and the WI donor groups (Fig. 6; P<0.01). Compared with the control group, the ability of T lymphocyte proliferation in the WI donor group was not reduced (Fig. 6; P>0.05). Each group maintained a high T cell proliferation response to the WI antigen stimulation (Fig. 6). The results indicate that Adv-IKK2dn-CD4+CD25- T cells are capable of suppressing the proliferative responses of naive syngeneic T cells towards donor-specific BN antigens.

Discussion

imDCs are hypothesized to generate or activate Tregs (6,18-20). Tregs are important in the induction and maintenance of immune tolerance (17,21-23). However, the mechanisms underlying their ability to suppress immunity remain to be fully defined and are commonly disputed. The current study demonstrated that regulatory cells generated by Adv-IKK2dn-DC

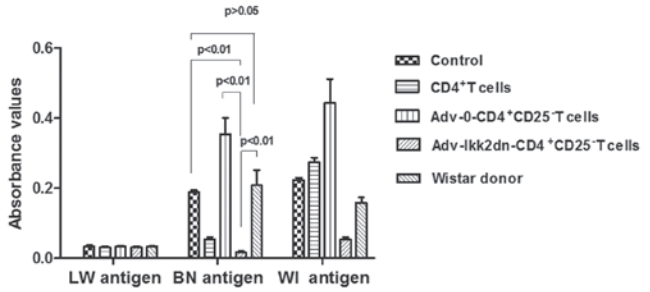

Figure 6. A three-day MLR was performed with irradiated splenocytes (LW, BN or WI) and T cells obtained from the lymph nodes of transplantation rats with various treatments. T cell proliferation was measured by an MTT assay and the results are presented as absorbance values. Absorbance values were read at 490 nm wavelength using an automated microplate reader. MLR, mixed lymphocyte reaction; LW, Lewis; BN, Brown Norway; WI, Wistar; Adv, adenovirus; Adv-0, empty adenovirus.

are a unique population of $CD4^+CD25^-$ T cells, unlike the $CD4^+CD25^+$ Tregs. A previous study observed that imDCs may form a Treg subset that is different from $CD4^+CD25^+$T Tregs *in vitro*. These cells are termed type 1 T regulatory cells (Tr1). At variance with $CD4^+CD25^+$Treg cells, Tr1 cells exert suppressor activity cytokine-independently, but are mainly dependent on direct cell-cell contact and through the T cell receptor to activate inhibitory cells and the membrane surface molecule CTLA-4 is important role in this process (24). A previous study indicated that imDCs transfected by IKK2dn induced the $CD4^+CD25^-$ Tregs and these cells were capable of inducing prolongation of kidney allograft survival *in vivo* (13).

In the present study, recipient DCs transfected by IKK2dn were observed to guide naive T cells to differentiate into $CD4^+CD25^-$ Tregs (Adv-IKK2dn-$CD4^+CD25^-$ T cells) *in vitro*. Notably, Adv-IKK2dn-$CD4^+CD25^-$ T cells administered *in vivo* to syngeneic naive recipient rats prolonged the survival of LW kidney allograft (Fig. 2; Table I), without the requirement for immunosuppression. However, Adv-IKK2dn-$CD4^+CD25^-$ T cells exhibited no effect on the survival of a third-party (WI) kidney allograft (Fig. 2; Table I). Co-culture MLR was used to investigate whether the suppression effector function of Adv-IKK2dn-$CD4^+CD25^-$ T cells is antigen-specific (Fig. 6). T cells from Adv-IKK2dn-$CD4^+CD25^-$ T cell-treated transplanted rats were unresponsive to donor allo-antigens (BN) and partially responsive to third-party (WI) antigens (Fig. 6). Therefore, the regulation of suppression by Adv-IKK2dn-$CD4^+CD25^-$ T cells was hypothesized to be antigen-specific. These results are concordant with the hypothesis of Aiello *et al* (13).

It is also important to determine the mechanisms underlying the suppressive function of Adv-IKK2dn-$CD4^+CD25^-$ T cells. There are two possible types of suppression mechanisms by which Tregs regulate the immune system, via cell contact or cytokine and/or other soluble factors, including TGF-β and Il-10 (25-27). It has been established that Tregs secrete two suppressive cytokines, Il-10 and TGF-β, and may also secrete Il-4. Il-10 inhibits Th1 cells and Th1 type factor proliferation (28), particularly IFN-γ synthesis. TGF-β inhibits immune molecules, macrophage activation and the Th1-type inflammatory response (29). In the current study, Il-2, IFN-γ, TGF-β and Il-10 serum levels in different groups were investigated on days 5 and 14. *In vivo* studies indicated that the Adv-IKK2dn-$CD4^+CD25^-$ T cell-treated group exhibited significantly reduced Il-2 and IFN-γ production and increased Il-10 and TGF-β expression in the serum of allo-kidney transplanted rats (Figs. 3 and 4). These observations indicated that Adv-IKK2dn-$CD4^+CD25^-$ T cells prolong renal allograft survival and it is hypothesized that this occurs due to high expression levels of Il-10 and TGF-β, which is concordant with previous studies (30-31). In addition to the cytokine pathway, it has been hypothesized that DnIKK2-Tregs expressing high levels of inducible nitric oxide synthase are immunoregulatory (13).

In organ transplantation immunity, Th1 cells induce acute rejection, causing graft inactivation, while Th2 cells have a protective effect on the graft (32). The Th1-type factors, Il-2 and IFN-γ, increase the risk of rejection by promoting the immune response between the recipient and the transplanted kidney. Type Th2 factors, particularly Il-4 and Il-10 may inhibit the transplantation immune response and promote the formation of tolerance between the recipient and the transplanted kidney. Previous studies have shown that large amounts of effect cytokines are detected in the acute rejection of transplanted organs, while immune tolerance of transplanted organs mainly produces regulatory cytokines (33,34). The current study demonstrated that renal transplanted rats in the Adv-IKK2dn-$CD4^+CD25^-$ T cell group did not survive long-term. It was hypothesized that the reason for this was due to a time-dependent decrease in the Th2 cytokine secretion of Adv-IKK2dn-$CD4^+CD25^-$ T cells, leading to increased Th1 cytokine secretion and finally inducing rejection. These observations indicate that the inhibitory effect may be mediated by the release of soluble mediators.

The results of the present study indicate that recipient-derived imDCs transfected by IKK2dn induced $CD4^+CD25^-$ T cells prolong renal allograft survival, secrete high levels of cytokine Il-10 and TGF-β. Adv-IKK2dn-$CD4^+CD25^-$ T cells inhibited the transformation of recipient T cells to effector T cells and promoted differentiation into Th2 and Th3 cells. The results indicate that Tregs are important in immune regulation and act by releasing regulatory cytokines. The present study revealed mechanisms underlying the involvement of DCs transfected by IKK2dn in inducing immune tolerance. The current study hypothesizes a clinical use for recipient DCs in cadaveric renal transplantation to induce tolerance.

Acknowledgements

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