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Full Length Research Paper

Further study on molecular biological effects of CD4 D1 inhibitor J2 on allografts following corneal transplantation in mice

Da-Jiang Wang[1], Han Zhang[1], Guo-Jiang Chen[2], Li-Qiang Wang[2], Ruo-Nan Xu[2], He Xiao[2], Ren-Xi Wang[2], Ming Yu[2], Yan Li[2], Song Li[3] and Yi-Fei Huang[1*]

[1]Department of Ophthalmology, Chinese PLA General Hospital, Beijing 100853, China.
[2]Department of Molecular Immunology, Institute of Basic Medical Sciences, Beijing 100850, China.
[3]Department of Molecular Drug Design, Institute of Pharmacology and Toxicology Sciences, Beijing100850, China.

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Our previous experiments have revealed that a non-peptide organic ligand of CD4 D1-J2 as a potential inhibitor of CD4 D1 can inhibit corneal allograft injection and prolong the corneal allograft survival. The present study aimed to further investigate molecular biological effects of J2 treatment on allografts following corneal transplantation in mice. Allogeneic orthotopic penetrating keratoplasty was performed using C57BL/6 mice as donors and BALB/c mice as recipients. Allograft recipient mice were randomly divided into J2-treated group, CsA-treated group (positive control) and DMSO treatment group (negative control). After treatment for consecutive 12 days, T cell phenotype analysis, CD4, CD8 immunohistochemical staining, T-cell proliferation and mRNA expression analysis of cytokines were performed. The subsequent molecular biological assays revealed that the immunosuppressive activity of J2 was associated with its inhibitory effects on the $CD4^+$ T cells and cells-mediated responses. J2 is as effective as CsA in prevention of allograft rejection following corneal transplantation.

Key words: $CD4^+$ T cells, immunosuppressive agent, CD4 D1 inhibitor, CsA, corneal transplantation.

INTRODUCTION

Because of the unique immunological property, the success rate of human corneal transplantation, compared with other types of vascularized organ transplants, is high in normal corneal beds (Price et al., 1991). However, allograft rejection of corneal transplantation also remains a critical unresolvable problem, particularly in high-risk patients with vascularized corneas, previously failed corneal grafts, or both (Price et al., 1991; Williams et al., 1992). The precise immune mechanisms that mediate corneal graft rejection remain poorly understood even after 50 years of research in laboratory animals. As known, $CD4^+$ T cells participate in the pathogenesis of multiple immune-based human conditions, including autoimmune diseases, allogenic organ transplant rejection, and graft versus host diseases following allogenic bone marrow transplantation (Hafler and Weiner, 1987; Traugott et al., 1983). Many experiments have revealed that $CD4^+$ Th1-cell mediated delayed-type hypersensitivity (DTH) was the most important mechanism of corneal allograft rejection (He et al., 1991; Ayliffe et al., 1992; Yamada et al., 1999; Haskova et al., 2000).

CD4 is a transmembrane glycoprotein expressed on the surface of thymocytes and mature T lymphocytes (Janeway, 1992). The extracellular part of CD4 consists of four immunoglobulin-like domains (D1–D4) in a rod-like structure (Lange et al., 1994; Wu et al., 1997). Mutagenesis studies have suggested that residues in the membrane distal D1–D2 module bind predominantly non-polymorphic residues of (major histocompatibility complex) MHC class II molecules (Fleury et al., 1991; Moebius et al., 1993). Recent studies using site-directed mutagenesis further identify that the FG loop and the CC' loop within the membrane-distal CD4 D1 are essential for binding MHC class II proteins (Li et al., 1997). The interaction between CD4 and MHC class II molecules plays an

*Corresponding author. E-mail: yi_yi0809@126.com. Tel: +8610-66936837. Fax: +8610-68226682.

important role in the development and activation of CD4+ T cells by increasing the affinity of TCR for the peptide/ class II molecule of MHC (Miceli and Parnes, 1993). Once activated, $CD4^+$ T cells stimulate B-cell antibody production and cytolytic T cell generation, thereby regulating the induction of immune responses. Therefore, small molecular inhibitors of the CD4-MHC class II interaction could potentially block the undesirable activation of $CD4^+$ T cells and thus serve as effective immunosuppressive agents.

In our previous work, we introduced a new small molecular compound named J2 targeting CD4 D1 as a potential antagonist for CD4 T-cell function. It was obtained through a computer-assisted screening approach that targeted CD4 D1 functional sites for potential inhibitors of the interaction between CD4 and MHC class II proteins. It was shown to block stable CD4-MHC class II molecule binding, inhibit CD4-depedent T cell proliferation and IL-2 secretion *in vitro* and possess immunosuppressive activity in mouse models of skin and corneal transplantation rejection *in vivo* (Xiao et al., 2007a, 2007b). It was also confirmed that 15 mg/kg intragastrically was a suitable dose for immunosuppressive activity. However, the molecular biological effects of CD4 D1-J2 on allografts following corneal transplantation in mice still remains unclear, and whether J2 is as effective as CsA is not reported. Here in this paper, we further studied the molecular biological effect of J2 compared with CsA on corneal allografts in murine eyes.

MATERIAL AND METHODS

Animal and grouping

C57BL/6 inbred mice were used as donors and BALB/c inbred mice as recipients. Female donors and recipients were 8 weeks of age, weighing 20-22 g. All animals were purchased from the Animal Institute of Chinese Medical Academy (Beijing, China) and treated according to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. Allograft recipient mice were randomly divided into J2 treatment group, CsA treatment group (positive control) and DMSO treatment group (negative control). Autografts were also performed as controls for assessment of graft rejection.

Orthotopic corneal transplantation

As described previously (Dana et al., 1997), Mydriasis in the eyes of the recipients was achieved by local application of tropicamide phenylephrine eye drops (Santen, Janpan). The donor button was excised with Vannas scissors and placed in Optisol corneal preservation solution. The recipient graft bed was prepared by excision of the equal size of the cornea. The donor button was then secured with eight to ten interrupted 11-0 nylon sutures (Sharpoint, 100 Dennis Drive, PA, USA). To protect the transplant, blepharorrhaphy was performed by interrupted suture twice (Prolene 10.0), which remained in place for 24 h, and antibiotic ointment was applied in the palpebral fissure. All grafted eyes were examined after 24 h, and transplant sutures were removed in all cases on day 7. Transplanted grafts that exhibited opacity due to operative complications, such as bleeding, suture dehiscence, or lens opacification within the first 3 days after surgery, were excluded from the analysis. Recipient mice were pretreated with J2 (15 mg/kg) 3 h before transplantation intragastrically, and went on for 12 consecutive days. Recipients that received cyclosporin A (CsA) treatment (10 mg/kg) served as positive controls and recipients treated by DMSO served as negative controls.

Assessment of graft survival

Grafts were evaluated by microscope once a day for two weeks. At each time point grafts were scored for opacification. A scoring system previously described (Sonoda and Streilein, 1992) was used to measure the degree of opacification between 0–5+: 0 = clear and compact graft; 1+ = minimal superficial opacity; 2+ = mild deep (stromal) opacity with pupil margin and iris vessels (iris structure) visible; 3+ = moderate stromal opacity with only pupil margin visible; 4+ = intense stromal opacity with the anterior chamber visible; and 5+ = maximal corneal opacity with total obscuration of the anterior chamber. Grafts with an opacity score of 2+ or greater after 2 wk were considered as rejected (immunologic failure). Median survival time was calculated and statistical comparisons were performed.

Histopathological examination

Twenty-one days after transplantation, three mice from each group were sacrificed, and the eye globes were fixed in 10% formaldehyde solution, paraffin-imbedded, sliced, and stained with hematoxylin and eosin (H-E) for light microscopy.

Immunohistochemical staining of $CD4^+$ and $CD8^+$ cells

Twelve recipient mice were selected for immunohistological evaluation (n=3 per group), and sacrificed on day 21. Thin frozen sections (5 μm) were cut, air-dried, and fixed in 100% aceton at -20˚C for 10 min. After inhibition of endogenous peroxidase activity and blockade of endogenous biotin, monoclonal rat anti-mouse antibodies against CD4 (clone: H129.19) and CD8 (clone:53-6.7) were used as the primary antibodies (BD, U.S.A) with Anti-Rat Ig HRP (BD, USA) as the second antibody. Chromogen DAB was applied to the tissue for 5 min, followed by counterstaining with Mayer's hematoxylin for 1 min. Positive cells were stained brown. Negative controls were treated with PBS instead of the primary antibodies. Photographs were taken in the same anatomic area-the central zone of each graft of each slide. Cells were counted on the photographs to quantify the degree of graft infiltration by each cell type.

RT-PCR

Eighty mice were selected for RT-PCR assay (n=20 per group). Donor and the surrounding recipient cornea were excised on day 7, 14, 21, and 28 after transplantation, dissected apart, snap-frozen in liquid nitrogen, and stored at -70℃ until required for RNA isolation and cDNA synthesis. Corneal tissue removed from ungrafted BALB/c animals acted as controls. Tissues were pooled and total RNA was extracted with TRIZOL (InvitrogenTM life technologies, Inc. Carlsbad, CA, USA) according to the manufacturer's instructtions. Total RNA was reversely transcribed to cDNA by reverse transcriptase using superscript Ⅱ Rnasefree reverse transcriptase (InvitrogenTM, USA) according to the manufacturer's instructions. Then samples were incubated at 42℃ for 50 min and the reaction was ceased by heating at 70℃ for 15 min. l μl of cDNAs of each sample was mixed and amplified with PCR mixture, which contained 1.6 μl of 10 mM dNTP mixture, 1.2 μl of 25 mM $MgCl_2$,

1U of Taq polymerase (TaKaRa BIO INC, Shiga, Japan) together with 0.2 µl of 10 mM of each primer in total 20 µl volume. The primer sequences were: IL-2 (740-bp) sense: 5'–CACTCCTCACAGTGACCT, antisense: 5' –ATAATAGATCA-TTTCAGAT-3'; IL-10 (300-bp) sense: 5' –ACCTGGTAGAAGTGA-TGC-3', antisense: 5'–GTCC AGCAGACTCAATAC-3'; IFN-γ (270-bp) sense:5'–CGAGTGGTCCA CCAGCTG-3', antisense: 5'–GTTGAGGAGACAG ACATT-3'. The constitutively expressed gene, β-actin, served as the internal positive control to confirm that each sample had equal amounts of RNA. β-Actin (540-bp product) sense was: 5'AGTGGGCCGCTCTAGGCACCAA-3'; antisense: 5'A CTCTTTGATGTCA CGCACGATTTC-3'. The reaction conditions consisted of denaturing at 95 ℃ for 30 s, annealing at 54~56 ℃ for 30 s with different primers, followed by extension at 72 ℃ for 40 s, with 26 cycles. RNA-free distilled water served as negative controls. PCR products were separated by electrophoresis on 2.0% agarose gels and visualized using ethidium bromide. Densitometric analysis was performed using Tanon Gel image system (Tanon, shanghai, china). Relative expression of total IL-2 mRNA, IFN-γ mRNA and IL-10 mRNA was recorded by the optical density and contrasted with the signal obtained of β-actin. Each experiment was repeated at least three times.

Flow cytometry

Mouse T lymphocyte subset antibody cocktail (PE-Cy7-labeled CD3ε, PE-labeled CD4 and FITC-labeled CD8a) was used for flow cytometry analysis (BD Biosciences Phamingen, CA, USA). Cells were stained in PBS with 2% heat-inactivated FCS and 0.2% sodium azide, and fixed using PBS with 1% paraformaldehyde. Peripheral blood cells were collected from vena caudalis at 1w, 2w, 3w and 4w after operation and were detected respectively. Whole blood was treated with FACS lysing solution (Becton Dickinson, Japan) to eliminate red blood cells after staining. Data collection and analysis were performed on a FACS Calibur flow cytometry using CellQuest software (Becton Dickinson, USA).

In vitro T-cell activation assays

Right cervical lymph nodes ipsilateral to the allograft-containing eye were removed from each group (n=3) 3 weeks after grafting. Single-cell suspensions prepared from lymph nodes of individual animals were used as responders (5×10^5 cells/well) and added to x-irradiated (2000 R) C57BL/6 spleen cells (5×10^5 cells/well) in a final volume of 200 µL of culture medium. Triplicate cultures were prepared in 96-well flat-bottomed micro-culture plates (Corning, Corning, NY). The cultures were incubated at 37℃ in an atmosphere of 5% CO_2 for 3 days. 50 µL of the Mammalian Cell Lysis Solution was added to each well in the culture plates. The plates were shaken for 10 min, and 50 µL of the Luciferase/Luciferin solution was added to each well. The plates were shaken again and sealed using TopSeal A™-self adhesive transparent adhesive seal. The plates were loaded in the stacker cassette of the MicroBeta and were allowed to dark adapt for a period of at least 30 min and luminescence was subsequently counted. Interleukin-2 (IL-2) and interferon (IFN)-γ levels in culture supernatants were measured by ELISA methods (R and D Systems, Minneapolis, MN) following the manufacturer's instructions. These experiments were repeated twice and similar results were obtained.

Statistical analysis

Time to rejection was calculated as the time to the event from the date of transplant and evaluated with the Kaplan-Meier method. The number of cells stained positively by immunohistochemistry, survival times and clinical scoring data were compared among various groups by one-way ANOVA on computer (SPSS 10.0).

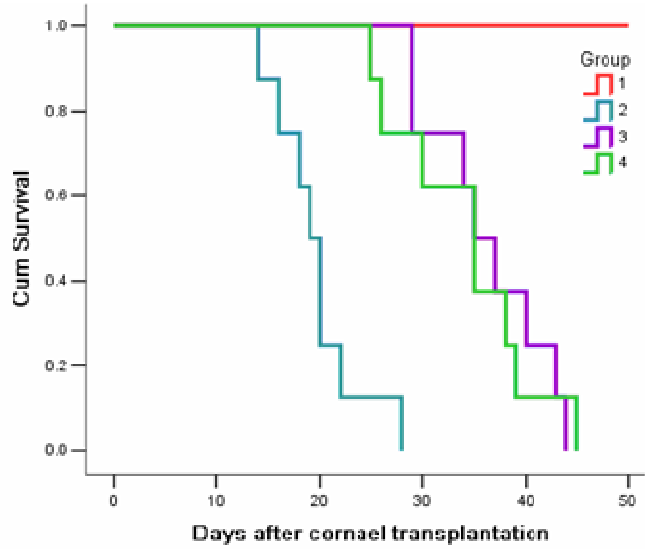

Figure 1. Prolonged survival of corneal allografts by administration of J2. Group 1, autograft control; Group 2, DMSO-treated control; Group 3, 15 mg/kg J2-treated recipients; Group 4, 10 mg/kg CsA-treated recipients.

RESULTS

Incidence and timing of graft rejection

Corneal transplantation in the autograft group led to slight perioperative stromal transplant oedema which was no longer detectable from the sutures removed day onwards. Throughout the entire period of the examination the transplants remained clear. The mean survival time of allografts in the control group was 18.63±4.21 days; for the CsA, 35.38±5.76 days and for J2, 33.13±6.81 days (Figure 1). Therapy with J2 (15 mg/kg) produced a statistically significant prolongation of transplant survival ($P<0.05$) compared with the allogeneic control.

From the fourteenth day after transplantation, DMSO-treated control group grafts exhibited severe opacity and edema; blood vessels had entered the transplanted grafts showing that they were all rejected (Figure 2A). In the CsA and J2 treatment group, the grafts showed no rejection (Figures 2B and 2C). They were clear with clearly visible details of the iris. Pathological examination showed the rejected allografts to become heavily edemous and infiltrated with a great deal of inflammatory cells with newly formed blood vessels entering the allografts (Figure 2D). In the unrejected allografts, only minimal anterior stromal edema and no inflammation cell was present (Figures 2E and 2F). Immunohistochemical staining showed abundant $CD4^+$ and $CD8^+$ cells infiltrating the allografts of the control group, however, corneas

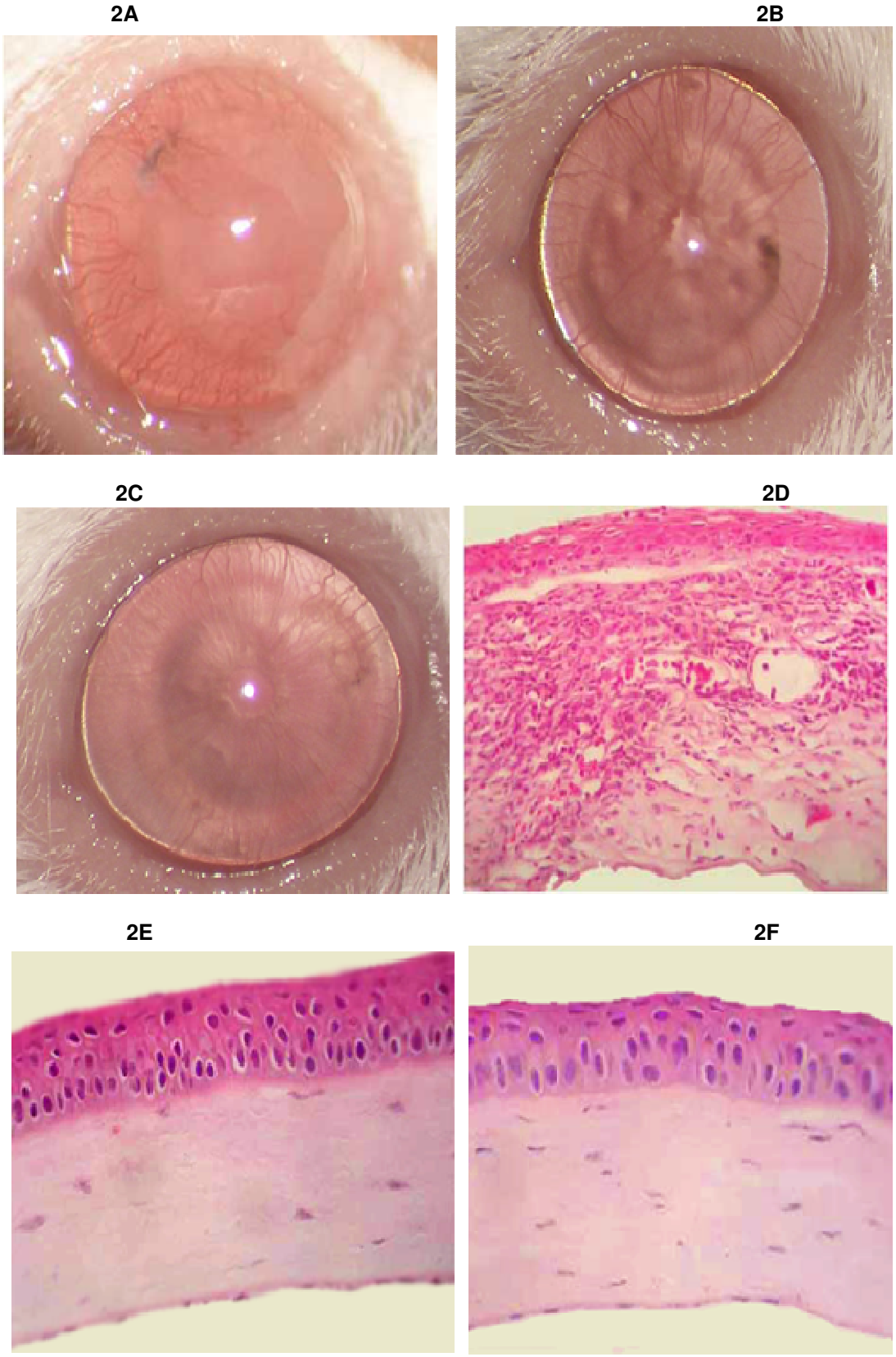

Figure 2. Histopathological examination of the mouse allografts (×100).
A and C: The DMSO-treated control allografts on postoperative day 21. B and D: The J2- treated allografts on postoperative day 21. E and F: The CsA- treated allografts on postoperative day 21.

Table 1. Pattern of inflammatory cells in the different therapy groups.

Inflammatory cells	Autograft control	DMSO-treated control	15 mg/kg J2-treated	10 mg/kg CsA-treated
CD4	0.00±0.00	35.89±6.35	10.78±3.35**	9.22±3.07**
CD8	0.00±0.00	19.78±5.19	7.33±3.43**	8.89±3.18**

**P<0.01 compared with DMSO-treated control.

Table 2. Analysis of cytokine mRNA expression after corneal transplantation by semi-quantitative RT-PCR.

cytokine	1w				2w				3w				4w			
	Gp1	Gp2	Gp3	Gp4	Gp1	Gp2	Gp3	Gp4	Gp1	Gp2	Gp3	Gp4	Gp1	Gp2	Gp3	Gp4
IL-2	±	+	+	±	-	+	±	±	-	+++	±	+	-	++	++	+++
IFN-γ	-	+	±	+	-	++	-	-	-	+++	-	-	-	++	+	+
IL-10	+	+	+	+	+	++	+	+	-	++	±	+	-	++	+	+

-, No detectable product; ±, +, ++, +++, increasing amounts of detectable product. Gp 1, autograft control; Gp 2, DMSO-treated control; Gp 3, 15 mg/kg J2-treated recipients; Gp 4, 10 mg/kg CsA-treated recipients.

taken from animals treated with either J2 or CsA had fewer infiltrating cells, almost normal corneal thickness and less neovascularisation. Table 1 shows the pattern of inflammatory cells in the different therapy groups.

RT-PCR studies of cytokine mRNA

IL-2, IFN-γ or IL-10 mRNA was not amplified by RT-PCR in contralateral non-grafted eyes. Transplantation induced a different cytokine mRNA expression pattern in DMSO-treated allografts, as compared to autografts (Table 2). J2 or CsA addition decreased the cytokine mRNA expression of Th1 cells at wk 2 and wk 3 after allogeneic corneal transplantation. But increasing IL-2 and IFN-γ mRNA was found at wk 4 after transplantation which was in correspondence with the histopathological observation and immunohistochemical examination. In autogrfts, weak IL-2 and IFN-γ mRNA expression was found only at wk 1 after transplantation, and they were not found at other time points. IL-10 mRNA was constantly expressed at all the time points in allografts, however, there were no significant quantitative differences among three allografts groups. While in autografts a decrease after 2 weeks was observed.

Subpopulation of lymphocytes in peripheral blood mononuclear cells

Three weeks after keratoplasty, J2 significantly decreesed the percentage of $CD4^+$ T cells in the recipient mice compared to the DMSO-treated controls (P<0.01) (Figure 3A). However, there were no significant quantitative differences of the $CD8^+$ T cells between J2 treated recipient mice and the DMSO-treated controls (Figure 3B). And 4 weeks after the corneal allotrans-plantation, the percentage of $CD4^+$ T cells in peripheral blood of the J2-treated mice recovered to the level comparable to those DMSO-treated animals. There was no significant difference between them (P>0.05). Still, the $CD8^+$ T cells were not affected by J2 addition. These data demonstrated that, J2 addition did not cause the depletion of the $CD4^+$ T subset but resulted in a temporary diminution.

Lymphocyte proliferation assay

Nineteen days after transplantation, the significantly decreased reactions to alloantigen of lymphocytes in the draining lymph nodes were observed in J2 and CsA-treated recipient mice compared with the DMSO-treated control. But there were no statistical differences between the J2-treated and CsA-treated groups (Table 3).

Effect of J2 on alloantigen-induced production of cytokines in splenocytes

The effect of J2 on the CD4 Th1 cell cytokine production, including IL-2 and IFN-γ, in the draining lymph nodes of the recipient BALB/c mice engrafted with allogeneic C57BL/6 corneas was examined by using ELISA assays. Strong secretion of both IL-2 and IFN-γ was observed in the DMSO treated allografted recipient mice. With J2 and CsA treatment, both the secretions of IL-2 and IFN-γ from the allografted recipient spleen lymphoid cells were reduced to the level comparable to those from autografted control mice. As shown in Figure 4A, there were significant quantitative differences of the IL-2-producing between T cells in the spleen of the J2-treated allografted recipient mice and that of DMSO-treated hosts (P<0.05).

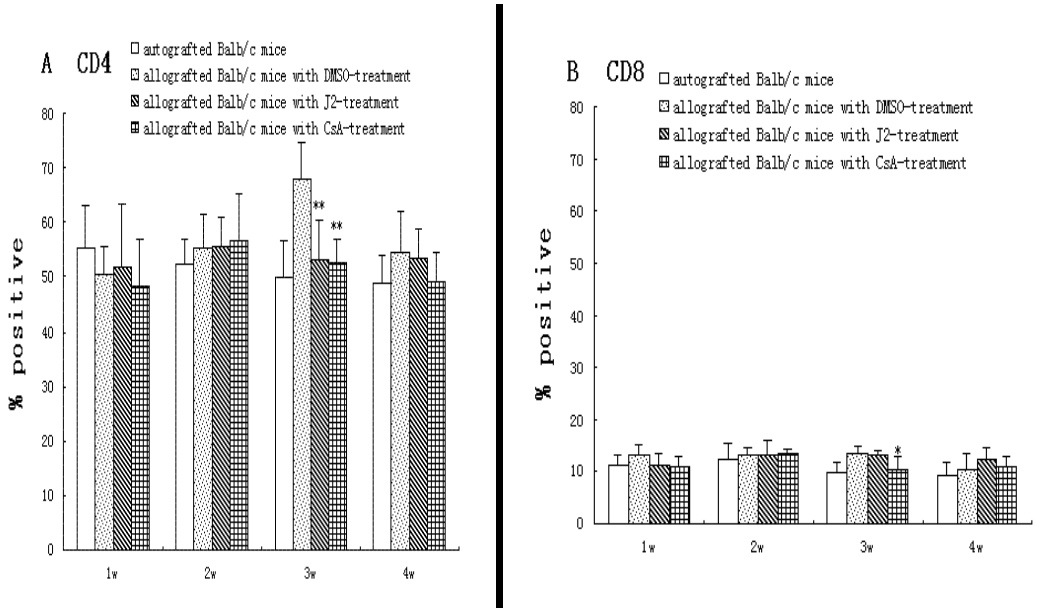

Figure 3. Phenotype analyses of the T cells from peripheral blood of the different groups. 1, 2, 3 or 4 weeks after corneal transplantation, mononuclear cells were isolated and analyzed by flow cytometry for lineage-specific surface markers: (A) CD4 and (B) CD8. The values expressed are the mean fluorescent-positive cells in each group (%, n=5). $^{**}P<0.01$ compared with DMSO-treated controls, $^{*}P<0.05$ compared with DMSO-treated controls, n=5 mice/group.

Table 3. Effect of J2 on alloantigen-induced cell proliferation.

Group	N	Cell proliferation index
autograft	3	10.22±3.21
allograft+DMSO	3	19.26±4.71
allograft+J2	3	9.11±0.95**
allograft+CsA	3	7.71±4.14**

$^{**}P<0.01$ compared with DMSO-treated controls.

Similarly, J2 was shown to dramatically reduce the quantity of the IFN-γ-secreting recipient cells in the spleen of allografted mice compared with the DMSO-treated control group ($P<0.01$) (Figure 4B).

DISCUSSION

Immune rejection of corneal transplantation is a complex process that involves many factors. Immunosuppressants such as CyclosporineA and FK506 are now used for prevention of allograft rejection in clinical corneal transplantation. However, both of them are nonspecific. And their therapeutic applications are seriously limited by severe associated toxicities and unfavorable side effects (Sigal et al., 1991). $CD4^+$ T cells are known to play a prominent role in the pathogenesis of corneal transplantation reactions (Niederkorn, 2002). The interaction between CD4 and MHC class II molecules is critical for the activation of $CD4^+$ T cells. Thus, inhibitors of the CD4-MHC class II interactions are believed to be able to potently block the undesirable activation of $CD4^+$ T cells and consequent T cell responses, and then serve as effective immunosuppressive agents. Current therapeutic reagents for CD4-mediated immunological diseases include the monoclonal antibodies (mAbs), small peptide-based therapeutics and small non-peptidic organic inhibitors. In comparison with mAbs, small non-peptidic organic inhibitors are less immunogenic (Satoh et al., 1997; Koch and Korngold, 1997). And in contrast to peptides, non-peptidic organic structures are normally more stable and also more amenable for chemical synthesis and modifications (Huang et al., 1997; Edling et al., 2002). Therefore, there is an impending need for more specific, stable and effective therapeutic agents. Development of small non-peptidic organic inhibitors targeted to functional epitopes thus becomes a promising alternative immunotherapeutic strategy. Previous studies have identified a surface pocket of CD4 D1 domain as the critical epitope involved in stable CD4-MHC class II interaction. This CD4 surface pocket is constructed by the FG loop and the CC' loop (Li et al., 1997; Doyle and Strominger, 1987; Clayton et al., 1989). During recent years, efforts are underway to develop small molecular mimics or inhibitors of this surface-binding pocket, in pursuit of potential therapeutic agents (Li et al., 1997; Satoh et al., 1997; Jameson et al., 1994; Friedman et al., 1996). J2 is a novel chemical inhibitor of CD4 D1 via a

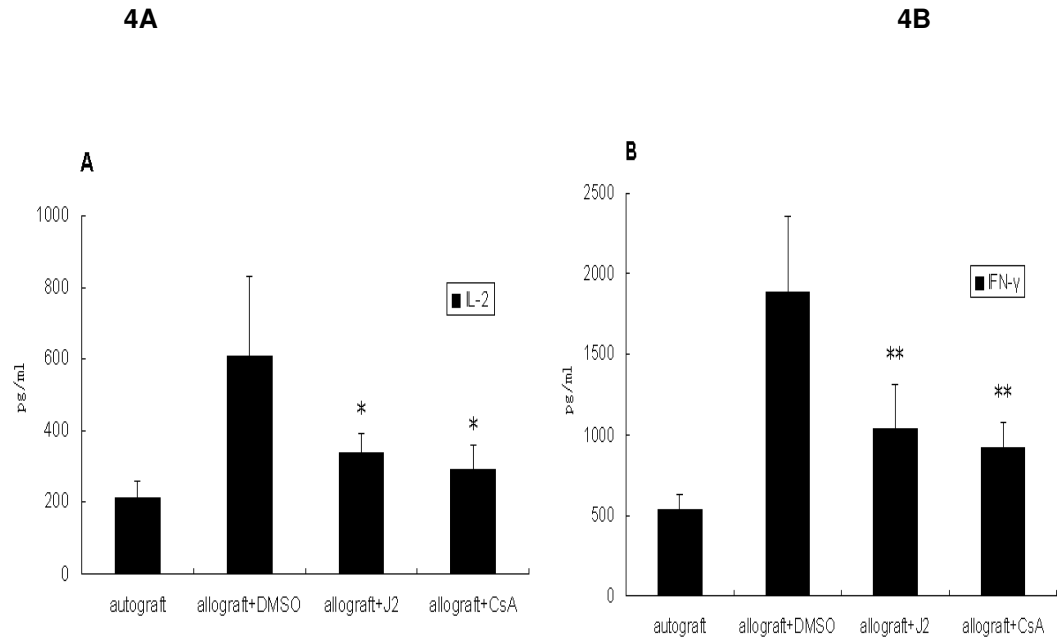

Figure 4. Cytokine secretion by alloantigen-stimulated T cells. 19 days after corneal transplantation, donors' splenocytes (5×10^6)in the absence or presence of alloantigen were incubated in a 96-well plate for 72 h, and the cytokine levels of IL-2(A) and IFN-γ(B) in the culture supernatant were determined by ELISA. IL-2 and IFN-γ levels in splenocytes of the allograting group in the absence of alloantigen were 72.32 ± 22.51 and 760 ± 110.26 pg ml^{-1}, respectively. Results were presented as mean ± SEM, n=3. $^{*}P$ <0.05, $^{**}P$ <0.01 versus allografted recipient mice treated by DMSO.

rational drug design approach. In previous studies, we have tested its activities on modulation of CD4-mediated immune responses.

In this study, we were able to prove the immunosuppressive effect of J2 on delayed rejection after allogeneic corneal transplantation. Therapy with J2 (15 mg/kg) produced a statistically significant prolongation of transplant survival (P<0.05) compared with the allogeneic control. J2 was as effective as CsA in the prophylaxis of acute allograft rejection following corneal transplantation. The immunosuppressive efficacy of J2 was confirmed by histology, immunohistochemistry and flow cytometry. Unlike antibody treatment, J2 addition led to the depletion of the target cell and thus resultant long-time immunosuppression. J2 treatment resulted in a temporary diminution of the $CD4^+$ T cells. However, no significant quantitative changes of the $CD8^+$ subset were observed in those mice by J2 treatment, indicating the selectivity of this effect. There was a significant reduction in the number of infiltrating cells of the $CD4^+$ and $CD8^+$ subsets, which were major mediators of acute rejection in this model. And J2 addition decreased the cytokine mRNA expression of Th1 cells 2 and 3 weeks after allogeneic corneal transplantation. In this study, we focused on the action of J2 on T lymphocytes. We investigated alloantigen induced T-cell proliferation and production of cytokines (IL-2 and IFN-γ) *in vitro*. The proliferative response was suppressed by J2. These findings suggested that J2 influenced T-cell activation and inhibited Th1 cells *in vitro*.

We did not see any adverse events in the J2 treated animals. A major problem in clinical transplantation is the nephrotoxicity and neurotoxicity of CsA or FK506 which is mediated via inhibition of phosphatase calcineurin (Ryffel et al., 1994). J2 has no effect on calcineurin and hence may not have potential to induce nephrotoxicity and neurotoxicity in men. Activation of lymphocytes is crucial for inducing immunological tolerance. J2 interferes with the early activation of lymphocytes and probably prevents the development of immunological tolerance in this experimental model. In conclusion, our results show that intragastrically immunosuppression with J2 significantly prolongs corneal allograft survival. We believe that the data warrant further evaluation of J2 as a possible therapeutic agent in the treatment of preclinical and clinical high-risk keratoplasty.

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