

Prolongation of corneal allograft survival by CTLA4-FasL in a murine model

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Abstract

Background To investigate the therapeutic effect of CTLA4-FasL—B7 costimulatory pathway blockage—on graft survival in a murine model of corneal transplantation. **Methods** Orthotopic penetrating keratoplasty was performed on BALB/c mice. The mice were randomized into four groups: the isograft group, untreated allograft group, cyclosporine A drug delivery system (CsA DDS)-anterior chamber implanted group, and 10 µg/mL CTLA4-FasL-treated group. Allografts were from C57BL/6 mice. Survival time of corneal grafts was evaluated. Immunohistological method and TdT-mediated dUTP Nick End Labeling (TUNEL) were applied for the detection of CD4+ T cells and apoptotic cells in corneal transplants. To assess whether peripheral immune tolerance appeared after the treatment of CTLA4-FasL, CsA DDS-implanted- and CTLA4-FasL-treated BALB/c mice with clear grafts received skin allografts at 4 weeks after keratoplasty, and the status of corneal transplants were observed when skin grafts were rejected.

Results Allografts in the CTLA4-FasL group (median survival time [MST]=106 days, $p=0.0042$) and the CsA

DDS group (MST=60 days, $p=0.0037$) revealed extending survival time, compared with that in the untreated allograft group (MST=14 days). There were significantly fewer CD4-positive T cells in both the isograft group and the CsA DDS group. In the untreated allograft group, the number of CD4+ T cells gradually increased from day 1 until the final day of observation (day 21). By contrast, it reached a peak on day 7 and then absolutely reduced in the CTLA4-FasL group. Many apoptotic cells were detected on day 7 in the CTLA4-FasL group, but very few were seen in the other groups. Within 30 days of skin-graft rejection, previously healthy and long-standing corneal grafts became rejected in the CsA DDS group but remained clear in the CTLA4-FasL group.

Conclusions CTLA4-FasL can prolong the survival time of corneal allografts in mice, exerting a negative regulation on T-cell activation simultaneously by blocking B7 costimulatory signals and inducing Fas-FasL apoptotic pathway. Due to the adjunctive role of FasL, it also appears to be a potential activity of tolerance induction through T-cell apoptotic pathways.

Keywords Corneal transplantation · Immune rejection · CTLA4-FasL · Mice

Corneal transplantation appears to have a high and spontaneous rate of success, since cornea is considered immunologically privileged. As with other tissue or organ transplantations, including skin, heart, and liver, immune rejection is also a leading cause of corneal transplant failure, which is mediated by the activation of alloreactive T lymphocytes [1, 2]. T-cell activation requires at least double signal pathways. The first signal results from T-cell receptor recognition of the MHC: peptide complex presented by the APCs; the second requires the binding of B7

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ligand on the APCs with its receptor, CD28, on T cells. Cytotoxic T lymphocyte antigen, also known as CTLA4 [3], is a second receptor with high affinity for both B7 ligands, B7-1 (CD80) and B7-2 (CD80), as unlike CD28 it is able to deliver a negative signal and abort T-cell proliferation by competitively blocking the CD28/B7 pathway. Researchers from several groups found that the second signal, also called the costimulatory signal, played a crucial role in the activation of T cells; blocking this signal with CTLA4-Ig significantly extended the graft survival time in mouse, rabbit, and rat models of corneal transplantation [4–6]. Fas ligand (FasL) is a member of the TNF family that is capable of inducing apoptosis and conferring immune privilege of the eye. Previous studies showed that FasL-positive corneal grafts were accepted at a rate of 45%–50%, whereas FasL-negative corneal grafts were rejected nearly 100% in mice [7, 8]. Consequently, the constitutive expression of FasL on the cornea plays a significant role in corneal allograft survival. A fusion protein consisting of CTLA4 and FasL (CTLA4-FasL) was found to act as both a competitive inhibitor of CD28-mediated costimulation and a stimulator of Fas-mediated apoptosis, and thereby to convert a stimulatory trans signal into an inhibitory one [9, 10]. In this study, we evaluated the therapeutic effects of CTLA4-FasL on the suppression of immune rejection after corneal transplantation in a murine model.

Materials and methods

Animals

Six- to 12-week-old BALB/c and C57BL/6 male mice were obtained from the Beijing Animal Laboratories (Beijing, China), and treated in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Ophthalmic and Vision Research.

The BALB/C mice were randomized into four groups: isograft group, untreated allograft group, cyclosporine A drug delivery system (CsA DDS)-anterior chamber implanted group, and 10 µg/ml CTLA4-FasL-treated group. Allografts were from C57BL/6 mice.

Drug delivery system

Cyclosporine powder (Novartis, Basel, Switzerland) was formulated into polylactide-co-glycolide (PLG) by the Chinese Academy of Sciences Institute of Chemistry (Beijing, China). The polymer was shaped into cylinders 0.5 mm in length and 0.2 mm in diameter, and the ratio of cyclosporine to PLG was 6:4. Each cylinder contained 0.3 mg of cyclosporine.

Allograft pretreatment by CTLA4-FasL

Corneal allografts were incubated in Optisol corneal storage medium with 10 µg/ml of CTLA4-FasL for 18 hours at 4°C, and then washed with Optisol solution to remove unbound reagent immediately before transplantation.

Orthotopic corneal transplantation

Orthotopic penetrating keratoplasty (PKP) was performed in the eyes of BALB/c mice as previously described, with some minor modifications [11]. Briefly, a 2.0-mm diameter donor corneal graft (treated or untreated) was sutured onto the same size recipient graft bed with eight or 12 interrupted 11-0 nylon sutures (Mani, Tochigi, Japan). At the end of the procedure, the CsA DDS was implanted into anterior chamber in the CsA DDS group, which is a positive control group. Attention should be given when fitting the DDS into the inferior part of the anterior chamber to maximally avoid irritation and damage to the iris and the corneal endothelium before the last stitch. Sterile air was refilled into the anterior chamber in the 10 µg/ml CTLA4-FasL-treated group. Antibiotic ointment was applied to the corneal surface, and eyelids were closed for 48 hours with tarsorrhaphy. Sutures were removed on postoperative day 7.

Assessment of graft rejection

Corneal grafts were examined by slit-lamp microscopy every day, and immune rejection was monitored as previously described [11]. A semiquantitative grading scale was used for the extent of corneal opacity: 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; 5+, maximal corneal opacity with total obscuration of anterior chamber. Grafts with an opacity score of 2+ or greater after suture removal were defined as rejection.

Immunohistochemistry

The whole eye globes were excised, made into 6 µm-thick cryostat sections, and prepared for immunohistochemistry on days 7, 14, and 21 respectively (three eyes in each group at each time point). Sections were incubated with 1 µg/ml rabbit anti-mouse CD4 (diluted 1:100 BD Pharmingen), washed, and then incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG secondary antibody. Appropriate positive and negative controls were given. Positive CD4+ T lymphocytes were scored in each group on a scale of 0 to 4+ per 400× high power field (HPF): 1+,

one to ten cells; 2+, 11 to 20 cells; 3+, 21 to 30 cells; and 4+, ≥ 31 cells. Counting and grading were limited to the center of the stained slides and did not involve the host-graft junction area.

TdT-mediated dUTP nick end labeling (TUNEL)

TUNEL assays (TUNEL Kit, Roche, Basel, Switzerland) were performed on 6 μm -thick whole eye globe cryostat sections on postoperative day 7. Apoptotic cells in situ were identified using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to the strand breaks of cleaved DNA. The biotin-labeled cleavage sites were then detected by reaction with horse-radish peroxidase (HRP) conjugated streptavidin and visualized by DAB, showing brown color under a light microscope. Corneal specimens incubated with DNase I (Roche Molecular Biochemicals) were used as a positive control; fluorescein-tagged dNTP without TdT enzyme served as a negative control. Apoptosis cells were scored on a scale of 0 to 4+ per 400 \times high power field (HPF): 1+, one to ten cells; 2+, 11 to 20 cells; 3+, 21 to 30 cells; and 4+, ≥ 31 cells.

According to the positive staining of serial frozen sections in different parts of the cytoarchitecture, CD4 immunohistochemistry (cellular membrane stained by FITC), and TUNEL for cell apoptosis (cellular nucleus stained by DAB), merged images were made under the light microscope to distinguish the correlation of apoptosis with inflammatory cells.

Skin transplantation

To observe whether CsA DDS or CTLA4-FasL-treated recipients with long-term survival had acquired tolerance of donor alloantigen, randomly selected CsA DDS-implanted

($n=5$) and CTLA4-FasL-treated BALB/c mice ($n=5$) with clear grafts accepted orthotopic skin allografts 4 weeks after original corneal transplantation. Briefly, 10 mm \times 10 mm thoracic skin grafts from C57BL/6 mice were placed on graft beds and fixed with 10-0 nylon sutures. Antibacterial ointment was administered every day, whereas no immunosuppressive agents were given to prevent the immune rejection response of skin transplants. Skin grafts were considered rejected when they appeared dried, wrinkled, shed off graft beds, and devoid of normal skin color and luster.

Statistical analysis

Survival time of corneal allografts and skin transplants was analyzed using the Kaplan-Meier survival method. A value of $p < 0.05$ was considered significant.

Results

Corneal allograft survival

Six mice were excluded from this study due to surgical complications. Survival time of corneal transplants was over 3 months in the isograft group ($n=8$). Compared with the untreated allograft group ($n=8$, median survival time [MST]=14 days), allografts incubated in the 10 $\mu\text{g}/\text{ml}$ CTLA4-FasL had the longest survival time ($n=9$, MST=106 days, $p=0.0042$). Those with CsA DDS in the anterior chamber also revealed extended survival time ($n=9$, MST=60 days, $p=0.0037$) (Fig. 1). Opacity scores of corneal transplants were evaluated postoperatively (Fig. 2). The

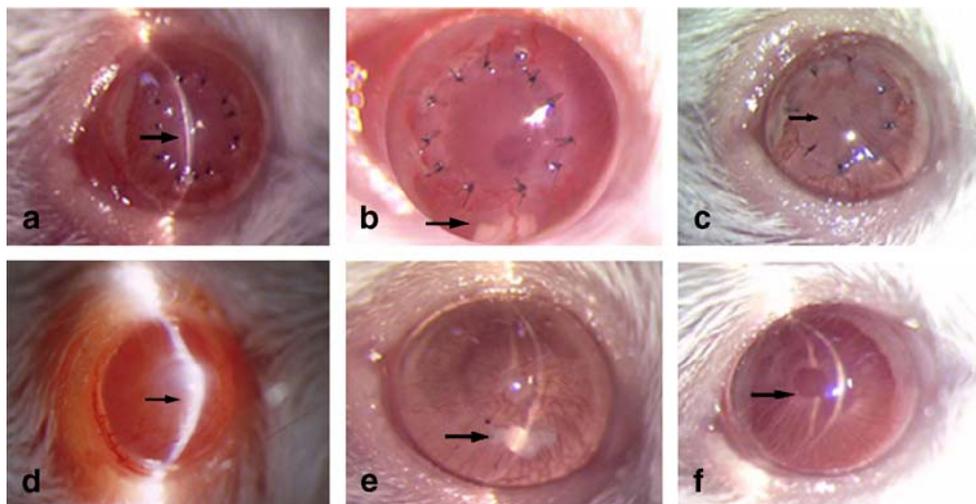


Fig. 1 The status of corneal allografts in each group. In the untreated allograft group (arrow showing opacified allografts): **a** moderate edema of corneal allograft on postoperative day 7; **d** bullous keratopathy with neovascularization on postoperative day 30. In the CsA DDS group (arrow showing CsA DDS in place): **b** clear corneal

allograft with CsA DDS in the inferior of anterior chamber on postoperative day 7; **e** clear corneal allograft with shrinking CsA DDS on postoperative day 30. In the CTLA4-FasL-treated group (arrow showing visible pupil margin): **c** clear corneal allograft on postoperative day 7; **f** transparent corneal allograft on postoperative day 30

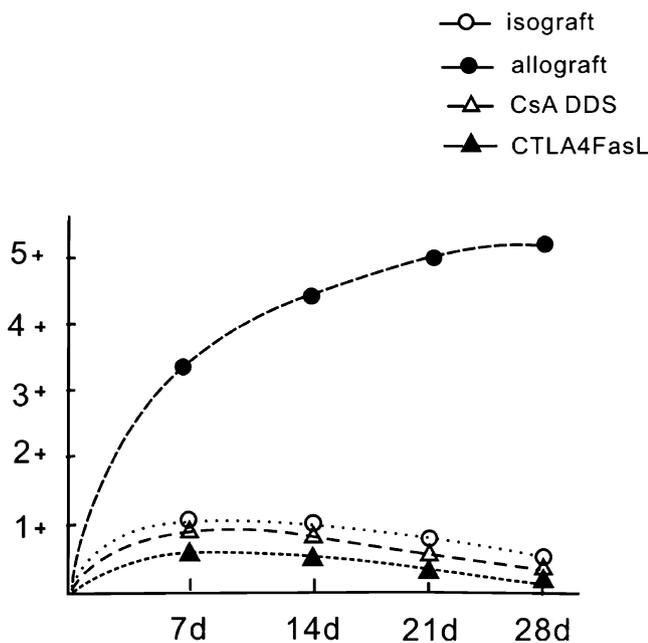


Fig. 2 A grading scale of corneal opacity after PKP in each group. In the untreated allograft group, the opacity scores had an increasing tendency of 2+ to 5+ (considered rejection) from postoperative day 7 to day 28, whereas they maintained 1+ or lower within 28 days in the other groups

onset time of immune rejection ranged from 12 to 16 days in the untreated allograft group, along with resultant corneal opacity. In the CsA DDS group, corneal allografts remained clear for approximately 60 days due to the existence of CsA DDS in anterior chamber and became rejected and completely neovascularized after the DDS was dissolved. Prolonged survival time (approaching 3 months) of allografts was achieved in the CTLA4-FasL-treated group.

CD4 + T cells infiltration and apoptosis

There were significantly few CD4-positive T cells in both the isograft and CsA DDS groups. In the untreated allograft group, the number of CD4+ T cells gradually increased from day 1 until the final day of observation (day 21). By contrast, it reached a peak on day 7 and then absolutely reduced in the CTLA4-FasL-treated group. Changing tendency of CD4+ T cells was shown in Fig. 3. Many apoptotic cells were detected on day 7 in the CTLA4-FasL group, whereas very few were seen in the other groups. Based on the analysis of merged images, CD4+ T cells constituted the majority of apoptotic cells excluding other sporadic inflammatory cells. Judged by cellular morphology and distribution, massive apoptosis was destined to infiltrating cells rather than resident keratocytes in the CTLA4-FasL-treated corneal allografts. Details of apoptosis cells in each group were included in Fig. 4.

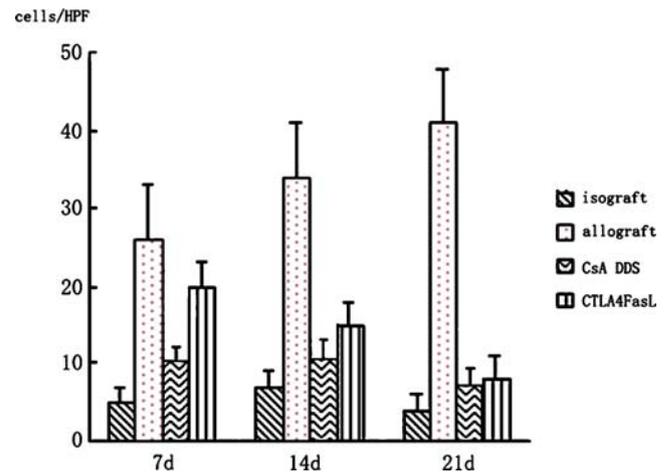


Fig. 3 The number of CD4⁺ T lymphocytes represented a contradictory tendency postoperatively in the untreated allograft and CTLA4-FasL groups. Over the time, the untreated grafts showed an increase in the number of CD4⁺ T lymphocytes in contrast to a decrease in those treated with CTLA4-FasL. There was no obvious change in the isograft and CsA DDS groups

Skin allografts

The fate of skin allografts and corneal allografts was evaluated in the CsA DDS-implanted and CTLA4-FasL-treated BALB/c mice (Fig. 5). The median survival time of skin allografts in the BALB/c mice was 10.2 ± 1.7 days in both the CsA DDS and CTLA4-FasL groups. But for corneal allografts in the two groups, it appeared totally distinct. Within 30 days of skin graft rejection, previously healthy and transparent corneal allografts in the CsA DDS group were rejected, whereas the corneal grafts survived in the CTLA4-FasL group.

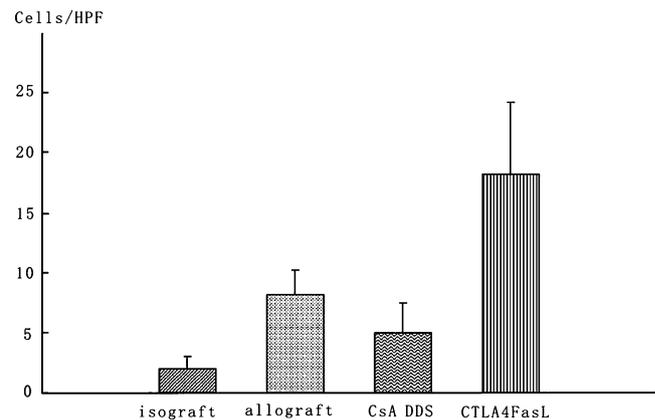


Fig. 4 Apoptosis cells were detected on postoperative day 7 in each group. Median number of apoptosis cells was 2 ± 1 cells/HPF in the isograft group, 8 ± 2 cells/HPF in the untreated allograft group, 5 ± 3 cells/HPF in the CsA DDS group, and 19 ± 6 cells/HPF in the CTLA4-FasL group

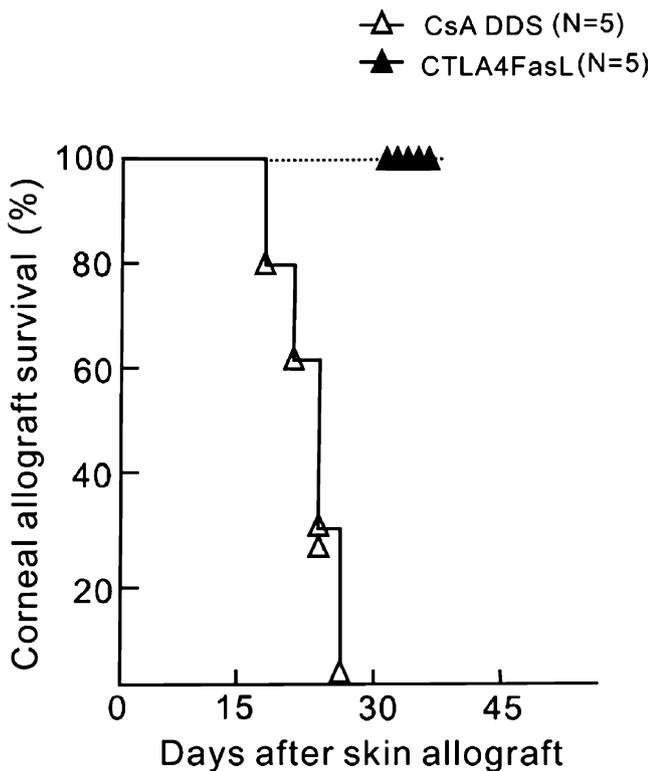


Fig. 5 Fate of long-standing corneal allografts in the CsA DDS-implanted and CTLA4FasL-treated BALB/c mice challenged at 4 weeks with secondary orthotopic skin transplantation. Corneal allograft survival patterns are presented. Originally clear allografts in the CsA DDS group (*white triangles*) were rejected within 30 days of skin allograft rejection, whereas those in the CTLA4-FasL group (*black triangles*) survived

Discussion

CD4⁺ T lymphocytes play a primary role in mediating rejection of orthotopic corneal transplantation in mice [12–14]. Cyclosporine has been found effective in suppressing T-cell-induced immune reactions [15, 16] and widely administered in corneal transplantation [17–19]. However, there were problems of drug insolubility and systemic side effects. We developed biodegradable CsA DDS, implanted it into the anterior chamber for the prevention of immune rejection and uveitis, and achieved satisfactory outcomes [20–22]. The polymer implant in the anterior chamber can deliver a high enough level of cyclosporine to effectively suppress the response of T lymphocytes to antigen presenting cells, aborting the initial recognition of graft antigens and the initiation of an antigraft response. We applied CsA DDS as a positive control to observe the therapeutic effect on the prophylaxis of immune rejection in comparison with the CTLA4-FasL protein.

It has previously been reported that the CTLA4-FasL protein can inhibit mixed T lymphocyte response more effectively than CTLA4-Ig and induce long-term survival of cardiac allografts by gene transfer, with no discussion of toxic effects [9, 23, 24]. In this study, CTLA4-FasL remarkably prolonged the survival of corneal allografts in a fully MHC-mismatched mouse keratoplasty model. Yamada et al. [14] demonstrated that acute rejection was mediated almost exclusively by CD4⁺ T cells rather than CD8⁺ T cells in corneal allografts. Simultaneously, CD4⁺ T cells were more sensitive to Fas-mediated apoptosis than CD8⁺ T cells [25, 26]. Therefore, we focused on the detection of the number of CD4⁺ T cells after the treatment of CTLA4-FasL, and found that there was an absolute reduction tendency. How did CTLA4-FasL interact with other elements of the inflammatory response, arousing a decrease in the number of CD4⁺ T cells in the face of potentially damaging immune reactions? Due to the existence of FasL molecules in CTLA4-FasL, TUNEL was used to observe whether there was an apoptotic phenomenon. We found that massive apoptosis was directed to infiltrating cells rather than resident keratocytes in the CTLA4-FasL-treated corneal allografts, which is similar to the report of Griffith et al. [27] that the Fas-FasL system played a pivotal role in preventing inflammation within the eye by triggering apoptosis of invading Fas-positive inflammatory cells. Since the decrease in the number of CD4⁺ T cells coincided with the incidence of apoptosis at almost the same time point, we presume that CTLA4-FasL delivers an inhibitory and possible death signal to the T cells via the engagement of FasL with Fas expressed by the T cells, which facilitate the deletion of alloresponsive T-cell content.

Immune tolerance

In the case of tolerance induction, the role of CTLA4 remains controversial [28]. However, CTLA4-FasL proteins displayed a potential function of tolerance induction for the utility of FasL in transplantation and conferring immune privilege [7, 29]. Healthy mouse corneal allografts surviving beyond 8 weeks, reported by Yamada et al. [14], was evidence of donor-specific tolerance. In the current study, the survival time of CTLA4-FasL-treated corneal allografts was longer than 3 months, which means a potential development of tolerance. We performed secondary orthotopic skin transplantation in the randomly selected CsA DDS-implanted and CTLA4-FasL-treated BALB/c mice with clear grafts. When the skin allografts were rejected in both groups, immune rejection also occurred in the originally clear corneal allografts in the CsA DDS group but not in the CTLA4-FasL-treated group. It may be presumed that immunologic tolerance developed in corneal

allografts pretreated with CTLA4-FasL rather than CsA DDS. Wells et al. [30, 31] reported that mice with defective passive or active T-cell apoptotic pathways were resistant to induction of transplantation tolerance, which implied that induction of stable peripheral tolerance required apoptosis of host alloreactive T cells. Thus, CTLA4-FasL presented a potential activity of tolerance induction through T-cell apoptotic pathways by engaging FasL with Fas expressed by the T cells. It can induce transplantation tolerance of the alloreactivity, which is, at least in part, dependent on the apoptosis of host alloantigen-specific CD4⁺ T cells.

We previously reported that CTLA4-Ig was able to prolong corneal allograft survival by pretreatment of donor transplants [32]. CTLA4-Ig performs immune suppression only by reversible T cell anergy, whereas CTLA4-FasL does it by irreversible T-cell deletion. As for the construction principle of two kinds of proteins, CTLA4-FasL seems superior to CTLA4-Ig in immunoregulatory function due to the adjunctive role of FasL. Actually, CTLA4-Ig lacks the additive effect of CTLA4-FasL on the prevention of immune rejection in a murine corneal transplantation, which is presumably related to the pro-inflammatory role of FasL. The exact mechanism by which CTLA4-FasL augments tolerance is uncertain, but provision of a strong combination of CTLA4 with FasL for blocking costimulatory signals and inducing Fas-FasL apoptotic pathway of T-cell activation may enhance development of anergy and promote apoptosis, thereby prolonging the survival time of corneal allografts in mice.

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