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Title Expression of an anti-CD4 single chain antibody fragment from the donor cornea can prolong corneal allograft survival in inbred rats

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Key Words: corneal transplantation, gene therapy, anti-CD4 scFv, antigen presentation, inbred rats

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Abstract

Aim To investigate whether expression of an anti-CD4 antibody fragment (scFv) by a lentivector-transduced donor cornea can prolong rat corneal allograft survival. **Methods** Inbred Fischer 344 rats received penetrating corneal allografts from Wistar-Furth donors after a 3h transduction of the donor cornea with a lentivector carrying anti-CD4scFv cDNA (Lv-CD4scFv), a lentivector carrying the reporter gene eYFP (LV-eYFP) or an adenoviral vector carrying anti-CD4 scFv cDNA (Ad-CD4scFv). Unmodified controls were also performed. Graft survival was assessed by corneal clarity and rejection was confirmed histologically. **Results** In organ-cultured corneas, expression of anti-CD4 scFv was detected at 2 days post-transduction with the adenoviral vector, compared with 5 days post-transduction with the lentivector, and was 10-fold higher from the former. More inflammation was observed in Ad-CD4scFv-modified allografts than in Lv-CD4scFv-modified grafts at 15 days post-surgery ($p=0.01$). The median time to rejection for unmodified, LV-eYFP and Ad-CD4scFv grafts was day 17, compared with day 22 for Lv-CD4scFv grafts ($p\leq0.018$). **Conclusion** Donor corneas transduced with a lentiviral vector carrying anti-CD4scFv cDNA showed a modest but significant prolongation in graft survival compared with unmodified, Lv-eYFP and Ad-CD4scFv grafts. However, rejection still occurred in all Lv-CD4scFv grafts, indicating that sensitization may have been delayed but was not prevented.

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Introduction

Despite topical application of glucocorticosteroids, approximately 40% of corneal allografts fail after 10 years, most commonly caused from irreversible immunological rejection mediated primarily by CD4+ T cells.[1] Strategies that have greatly improved the outcomes for vascular organ transplants, such as human leucocyte antigen (HLA)-matching and systemic immunosuppression have shown limited success when applied to corneal transplantation.

Monoclonal antibodies (mAb) have shown some promise as therapeutic drugs for corneal transplantation. In humans, systemic delivery of CAMPATH-1H (a humanised mAb against CD52), can reduce ocular inflammation in patients including those with corneal grafts.[2] Furthermore, systemic delivery of anti-CD4 mAb can prolong corneal graft survival in mice[3 4] and rats.[5 6] Cytotoxic T lymphocyte antigen-4 (CTLA-4), a competitive inhibitor of CD28 signalling, binds with high affinity to CD80/CD86 co-stimulatory molecules expressed on antigen-presenting cells (APCs). Delivery of a soluble CTLA-4 construct or an anti-CD28 mAb can prolong corneal graft survival in mice,[7-9] rats[10 11] and rabbits.[12] In addition, systemic delivery of anti-CD40L mAb can prolong corneal allograft survival in mice.[7]

With the exception of one study,[13] the topical application of whole antibodies to the cornea has shown limited success at prolonging corneal allograft survival in animal models. This is most likely due to the structure of the cornea, which forms a barrier to many therapeutics, including whole antibodies.[14]

Single chain fragment variables (scFv) consist of the variable heavy and the variable light regions from a whole antibody, connected using a linker peptide. ScFv can penetrate pig corneas *in vitro*, but are rapidly cleared from the eye.[14] Gene transfer vectors can be used to produce sustained expression of a scFv within ocular tissue. The cornea is highly amenable to such a therapy as it is

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feasible to transduce donor corneas *ex vivo* with a small dose of vector prior to transplantation in a single intervention.

Anti-CD4 scFv has been shown previously to inhibit alloproliferation in a mixed lymphocyte reaction (MLR).[15] However, despite robust expression of anti-CD4 scFv from rat corneas 4-5 days after transduction with an adenoviral vector carrying the cDNA for anti-CD4 scFv (Ad-CD4scFv) during *in vitro* culture, Ad-CD4scFv allografts rejected at the same tempo as unmodified allografts.[15] Adenovirus is known to be immunogenic and to produce only transient transgene expression and both of these factors may have contributed to the null effect of the Ad-CD4scFv therapy.

The aims of this study were to determine whether sustained expression of anti-CD4 scFv by the rat cornea could be achieved after lentiviral transduction and whether this could modulate corneal allograft survival in a rat model of corneal transplantation.

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Methods

Approval was obtained from the institutional Animal Welfare Committee. Adult (12-week) male inbred Fischer 344 (F344, RTl^{vl}) and Wistar-Furth (WF, RT^{lu}) rats were bred within our facility. Rats were housed at 21°C in 50-55% humidity under a 12h light/12h dark cycle, and were allowed access ad libitum to water and dry rations (Ridley AgriProducts, Melbourne, Australia). The genetic integrity of inbred strains was maintained by lineage records and tested yearly by allozyme electrophoresis.

Viral vector construction, titration and purification

A replication-deficient E1-, E3-deleted serotype 5 adenovirus (Adv) encoding anti-CD4 scFv with a factor H secretory sequence (fHSS) and 6-histidine tag (6-his) under individual CMV promoter control (Ad-CD4scFv) was purified as previously described.[15] This construct also encoded enhanced green fluorescence protein (eGFP) which was driven under a separate CMV promoter. Adv titre was determined by the tissue culture infectious dose method ($TCID_{50}$) on HEK-293A cells and was expressed as plaque forming units (pfu)/ml. Adv-CD4scFv had a titre of 4 x 10^8 pfu/ml.

Anti-CD4 scFv cDNA (with fHSS and 6-his) was amplified from the Ad-CD4scFv shuttle plasmid and cloned into pHIV1-SDmSV-DLTR within the same open reading frame as a 2A self-processing sequence (an 18 amino acid sequence which cleaves at its C-terminus through a ribosomal 'skip' mechanism, and allows expression of multiple transgenes within a single open reading frame) and enhanced yellow fluorescence protein (eYFP) cDNA to form the construct pHIV-1SDmSV-CD4scFv_F2A_eYFP-DLTR, which was used to generate the lentiviral vector (Lv), Lv-CD4scFv. The pHIV-1SDmSV-eYFP-DLTR plasmid was used to generate the Lv, Lv-eYFP.[16] Lv production was performed as described previously.[16] All vector stocks were tested for replication-competent lentivirus by assaying expression of HIV-1 p24 (HIV-1 p24 ELISA kit, PerkinElmer Inc, Boston, MA,

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USA) in transduced cells over 3 weeks and found to be negative. Lv titration was performed using methods modified from a previously described protocol.[17] Lv titre was determined through proviral integration using quantitative real-time PCR (qPCR) on genomic DNA (gDNA) from transduced A549 cells (CCL-185, American Type Culture Collection), after 4 weeks of culture. A gDNA standard made from A549 cells containing one copy of the Lv genome and two copies of the transferrin gene per cell was used for calibration. Primers were designed to detect the gag sequence, forward 5'AGCTAGAACGATTTCGCAGTTGAT3' and reverse '5 CCAGTATTTGTCTACAGCCTTCTGA3' and the human transferrin sequence (reference gene), forward 5'GCCCTGCCTGCCTACA3' and reverse 5'CAGGTTGTGCTTCTGACTCACT3'. qPCR was performed using Quantitect™ SYBR Green PCR master mix (Qiagen, Hilden, Germany) in a Rotor-Gene™ 6000 real time thermal cycler (Corbett Life Science, Mortlake, NSW, Australia). Cycle threshold (Ct) values were determined using the Rotor-Gene™ 6000 Series Software 1.7 (Corbett Life Science, Mortlake, NSW, Australia) and the ΔΔ-Ct method was used to calculate the copy number of *gag* per cell. Titres were expressed as transducing units/ml. The titre of Lv-eYFP was 2.38 x 10^9 TU/ml and the titre of Lv-CD4scFv was 1.25 x 10^9 TU/ml.

In vitro transduction of rat corneas

Globes were removed and decontaminated in 10% w/v povidone iodine (Faulding Pharmaceuticals, Salisbury, SA). Corneas were dissected and the iris detached. Corneas were placed in HEPES-buffered RPMI(Gibco GRL, Gaithersburg, MD, USA) containing 2% FCS, then transferred to a round-bottom 96 well plate (Nunc, Roskilde, Denmark), endothelium up. HEPES-buffered RPMI (2% FCS) containing either Lv (2.5 x 10^7 TU/cornea; multiplicity of infection (MOI = 400)) or Adv (2 x 10^7 pfu/ml; MOI = 320) was added dropwise to the cornea and incubated for 3 hours at 37°C, 5% CO_2 in air. Corneas to be used for transplantation were washed twice in HEPES-buffered RPMI (without serum or L-glutamine) and rinsed in ophthalmic BSS ≤ 10 min prior to surgery. Corneas that were used for culture were placed in 2ml HEPES-buffered RPMI (10% FCS) supplemented with 2.5 µg/ml

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amphotericin B (Amphostat, Thermo Electron, Melbourne, Vic, Australia) in a 24 well plate and incubated for up to 11 days at 37°C, 5% CO_2 in air, with a medium change every 48-72 h.

Reporter gene expression from transduced corneas

Detection of eYFP from transduced rat corneas was performed using methods described previously.[16]

Detection of functional anti-CD4 scFv by flow cytometry

Functional anti-CD4scFv was detected by flow cytometry on CD4-positive rat thymocytes as described previously.[14]

Orthotopic corneal transplantation in the rat

Recipient male F344 rats received penetrating 3 mm diameter corneal allografts from male WF donors under general anaesthesia, as described previously.[18] The WF/F344 strain combination represents multiple major and minor histocompatibility barrier differences. Donor corneas were secured with eight interrupted 10-0 nylon sutures. Grafts were examined daily under the operating microscope and grafted eyes were scored separately for clarity, corneal neovascularisation and inflammation on a 0-4 numerical scale with 0.1 increments. Corneal graft clarity was used to determine graft failure. Grafts were deemed failures when clarity scores ≥2.0 (graft obscuring observation of iris vessels). After rejection or after 60 days, rats were euthanised by isoflurane overdose. Globes were removed and processed for histology.

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Histology

Tissue samples were fixed in buffered formalin for at least 24 h, dehydrated and embedded in paraffin wax. Sections (5 mm thick) were cut at the microtome and mounted on chrome alum-subbed slides prior to staining with H&E and mounting in DePeX mounting medium (BDH Laboratory Supplies, Poole, UK).

Statistical analyses

Unpaired two-tailed Student t-tests were performed using Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA, USA) to determine statistical differences for *in vitro* assays. Corneal graft survival was analysed by non-parametric statistical analysis using SPSS statistical software (SPSS Inc., Chicago, IL, USA). For comparisons amongst three or more groups, a Kruskal-Wallis test (corrected for ties and with Bonferroni correction where appropriate) was performed, and to further identify which groups were statistically different from one another, pair-wise Mann Whitney U-tests (corrected for ties) was used. For comparisons between two groups, Mann Whitney U-tests (corrected for ties) were performed. Significance was set at $p<0.05$.

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Results

Lv-eYFP-transduced rat corneas showed transduction of 49% of endothelial cells after a 3h transduction with Lv-eYFP (MOI=400). Transduced corneas showed minimal oedema after the 3h transduction and the mean corneal endothelial cell density of the Lv-eYFP-transduced corneas was comparable to unmodified corneas (1346 ± 189 cells/mm^2 and 2103 ± 445 cells/mm^2 respectively; n=3; $p > 0.05$).

Next, we assessed expression of anti-CD4 scFv from rat corneas transduced with either Lv-CD4scFv (MOI=400) or Ad-CD4scFv (MOI=320) after a 3h transduction *in vitro*. Untransduced corneas were treated in the same manner as the transduced corneas, excluding addition of virus. After the 3h transduction, corneas were washed and cultured for 11 days. Secretion of functional anti-CD4 scFv into the culture supernatant was detected via the 6-his tag using flow cytometry on rat CD4+ thymocytes (Figure 1).

Significantly higher levels of anti-CD4 scFv were detected in the culture supernatant of corneas transduced with Ad-CD4scFv, compared to the Lv-CD4scFv from days 2-11 ($p<0.05$), with a 10-fold difference seen at day 5 (Figure 1). However, even from the Lv-CD4scFv-transduced corneas, 33-37 ng of scFv was secreted every 3 days. Anti-rat CD4 scFv expression was detected from 2 days after transduction in Ad-CD4scFv corneas, whilst Lv-CD4scFv corneas did not express detectable levels of anti-rat CD4 scFv until day 5 post transduction. Untransduced corneas produced no detectable expression of the anti-rat CD4 scFv (Figure 1).

Next, corneal allografts were either left unmodified or transduced with Lv-CD4scFv, Lv-eYFP or Ad-CD4scFv prior to transplantation (Table 1). All but one allograft underwent rejection. The median time to rejection for unmodified (n=10), Lv-eYFP (n=10) and Ad-CD4scFv (n=8) allografts was 17 days. Lv-CD4scFv allografts (n=8) experienced a modest, but significant, prolongation in survival compared with other allografts with a median day to rejection of 22 days. Immunological rejection of Lv-

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CD4scFv grafts was similar both clinically and histologically to unmodified and Lv-eYFP allografts and there was no difference in the day of vessel infiltration into the graft post-operatively. However, significantly more inflammation was observed in the Ad-CD4scFv-transduced allografts compared to the Lv-CD4scFv-transduced allografts at day 15 post-operatively (Table 2).

Table 1: Corneal graft survival following *ex vivo* transduction of donor corneas with viral vectors.

Treatment	**Graft**	**N**	**Median day of host vessel crossing into graft**	**Day to rejection**	**Median day to rejection**
Unmodified	allograft	10	10	11, 12, 14, 15, 16, , 18, 19, 19, 20, >60	17
Lv-eYFP	allograft	10	10	10, 11, 14, 15, 16, 18, 19, 19, 27, 30	17
Lv-CD4scFv	allograft	8	10	17, 20, 20, 21, 22, 25, 27, 38	22*
Ad-CD4scFv	allograft	8	11	13, 13, 14, 16, 17, 18, 21, 22	17

* p=0.004 compared with the unmodified and LV-eYFP allografts, (Kruskal-Wallis test, corrected for ties, with Bonferroni adjustment) and p=0.018 compared with Ad-CD4scFv (Mann Whitney U-test, corrected for ties).

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Table 2: Ocular inflammation in corneal allografts after *ex vivo* transduction of the donor cornea with viral vectors prior to corneal transplantation.

Treatment		**Median inflammation score**			
	n	**Day 1**	**Day 5**	**Day 10**	**Day 15**
Unmodified	10	0.5	0.6	0.4	0.1
Lv-eYFP	10	0.8	0.9	0.2	0.5
Lv-CD4scFv	8	0.7	0.8	0.3	0.05
Ad-CD4scFv	8	0.7	0.7	0.2	0.9*

*p=0.01 compared with Lv-CD4scFv (Mann Whitney U-test, corrected for ties).

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Discussion

Lv-CD4scFv allografts showed a modest but significant prolongation in survival compared with unmodified, Lv-eYFP or Ad-CD4scFv allografts. However, all Lv-CD4scFv allografts did undergo rejection, and the clinical and histological assessments of rejection were similar to the unmodified and Lv-eYFP allografts. As reported previously,[15] Ad-CD4scFv-transduction of corneal allografts did not modulate the tempo of rejection.

Why was the tempo of rejection modified after lentiviral but not adenoviral transduction? First, the immunogenicity of the Adv may have offset the immunosuppressive abilities of the anti-CD4 scFv, and prevented any modulation in allograft survival. Adenoviral vectors are well-established to be immunogenic in the eye.[19] We noted significantly more inflammation in Ad-CD4scFv-treated allografts than in Lv-CD4scFv-treated allografts at 15 days post-graft. We surmise that the pro-inflammatory nature of the adenoviral vector may have counteracted the potential immunomodulatory effects of anti-CD4 scFv secretion into the anterior chamber and limited transgene expression. Second, expression of the anti-CD4 scFv from the adenoviral vector may have been too transient.[15] We have previously reported long-term expression of the reporter gene eYFP (>60 days post-graft) in rat corneal isografts after transfection of the donor cornea with the same lentiviral vector used herein.[16] We hypothesize that the anti-CD4 scFv was expressed for a similar time, as its expression was driven by the same internal promoter (SV40) within the same lentivector. The eGFP reporter protein is detectable as early as one day after transduction of the rat cornea with an adenoviral vector analogous to the one used in this study,[15] and two days after transduction with the lentiviral vector.[16] In vivo, expression of eGFP is high at 1 and 3 days post-transduced with the adenoviral vector,[15] compared with the lentivector, but is low by day 14 and virtually undetectable by day 30 (data not shown). Thus, compared with the lentivector, adenovirus-mediated gene transfer resulted in expression of greater amounts of transgenic protein, but for a shorter period of time and at the expense of more ocular inflammation.

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Corneal allografts expressing LV-CD4scFv exhibited a significant delay in their tempo of graft rejection, compared with controls, but all grafts were eventually rejected. The evidence described herein (and from previously-published work) supports the contention that in vitro, more scFv is produced from an adenovirus-transfected rat cornea than from a lentivirus-transduced cornea, and that the kinetics of expression may be a little faster in the former. We also have evidence to show that in vivo as distinct from in vitro, expression from the adenoviral vector is transient whereas expression from the lentiviral vector is more long-lasting. We found that 33-37 ng of scFv protein was secreted from a Lv-transduced rat cornea every 3 days. We have previously reported that 26 ng purified anti-CD4 scFv at a concentration of 0.13 ng/μl can inhibit proliferation in a MLR,[15] however an insufficient amount of transgenic protein at the site of action in vivo is a possible reason for the delayed rejection response, rather than its abolition.

It should be emphasized that the site at which sensitisation to ocular alloantigens occurs in rats is unclear. In the mouse, bilateral removal of the cervical lymph nodes induces indefinite corneal graft survival across a weak histocompatibility barrier.[20] However, we have reported that the bilateral removal of some or all of the superficial cervical, facial, internal jugular and posterior cervical lymph nodes prior to corneal transplantation has no effect on the tempo or incidence of immunological rejection in the inbred WF/F344 combination.[21] T cell sensitisation may occur within the eye, its environs, in other lymph nodes, or the spleen. Soluble antigen introduced into the rat anterior chamber has been tracked through the venous circulation into the cervical lymph nodes,[22] but molecules of different molecular mass have been shown to partition differently after they leave the eye.[23] Thus, whereas over 50% of 40 kDa dextran-Texas red appears subsequently in facial and cervical lymph nodes, smaller (5 kDa) and larger (500 kDa) dextran molecules cannot be detected in these nodes and may be taken up by terminal lymphatics, drain into the venous circulation,[23] or be taken up by antigen-presenting cells in the anterior segment.[24] The destination of our 28 kDa scFv, after it leaves the eye is unclear, but T cells in the loco-regional area or at a distance may be

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targeted. We suggest the data support the contention that the anti-CD4 scFv is not affecting naïve CD4+ T cells in the cornea, but rather at a distance following drainage of the scFv from the aqueous. Alternatively, the scFv may be affecting CD4+ effector cells that take a little time to generate.

In conclusion, our results indicate that lentivector-mediated, but not adenoviral vector-mediated, expression of anti-CD4 scFv from donor corneal endothelium is able to modulate allograft rejection, but the site of action of the transgenic protein is unknown. The fact that all of the corneal allografts did eventually reject indicates that sensitization and generation of an effector cell response did occur, but with delayed kinetics. Transfection of donor corneas *ex vivo* with both adenoviral and lentiviral vectors encoding a variety of transgenes has previously been shown to extend corneal allograft survival significantly.[25] The decision as to which vector is best to use will depend upon the function of the transgenic protein being expressed: in some contexts, high levels of relatively short-term expression may be warranted, whereas in others, sustained long-term gene expression may be necessary to achieve the desired effect.

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Competing interests None

Contributorship

SLA performed experimental work, analysis and interpretation of data and writing of the manuscript. CJF, LFM, KK and CKT each performed some experimental work, and analysis and interpretation of data. HMB and DJC were involved in the design of the experiment, and the drafting and revision of the manuscript for important intellectual content. KAW was responsible for the conception and design of the study and the drafting and revision of the manuscript for important intellectual content. SLA takes overall responsibility for this work.

Figure legends

Figure 1: Anti-CD4 scFv expression from rat corneas transduced with LV-CD4scFv and Ad-CD4scFv. Rat corneas were transduced with Lv (MOI of 400) and Adv (MOI of 320) for 3h and cultured for 11 days in a total volume of 2 ml. Culture supernatant was collected and replaced every 3 days from day 2. Flow cytometry on rat thymocytes using culture supernatants was performed to detect anti-rat CD4 scFv via the his-6 tag. Error bars represent one standard deviation from the mean, n=3 corneas. Comparisons were made between corneas transduced with Ad-CD4scFv and Lv-CD4scFv: ****$p<0.0001$; ***$p=0.0006$; **$p=0.004$; *$p=0.02$ (unpaired two-tailed Student t-test).

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References

1. Coster DJ, Williams KA. The impact of corneal allograft rejection on the long-term outcome of corneal transplantation. *Am J Ophthalmol* 2005;**140**:1112-22.

2. Dick AD, Meyer P, James T, et al. Campath-1H therapy in refractory ocular inflammatory disease. *Br J Ophthalmol* 2000;**84**:107-9.

3. He YG, Ross J, Niederkorn JY. Promotion of murine orthotopic corneal allograft survival by systemic administration of anti-CD4 monoclonal antibody. *Invest Ophthalmol Vis Sci* 1991;**32**:2723-8.

4. Vitova A, Filipec M, Zajicova A, et al. Prevention of corneal allograft rejection in a mouse model of high risk recipients. *Br J Ophthalmol* 2004;**88**:1338-42.

5. Coupland SE, Krause L, Karow AC, et al. Delay in corneal allograft rejection due to anti-CD4 antibody given alone and in combination with cyclosporin A and leflunomide. *Ger J Ophthalmol* 1995;**4**:294-301.

6. Ayliffe W, Alam Y, Bell EB, et al. Prolongation of rat corneal graft survival by treatment with anti-CD4 monoclonal antibody. *Br J Ophthalmol* 1992;**76**:602-6.

7. Ardjomand N, McAlister JC, Rogers NJ, et al. Modulation of costimulation by CD28 and CD154 alters the kinetics and cellular characteristics of corneal allograft rejection. *Invest Ophthalmol Vis Sci* 2003;**44**:3899-905.

8. Konig Merediz SA, Zhang EP, Wittig B, et al. Ballistic transfer of minimalistic immunologically defined expression constructs for IL4 and CTLA4 into the corneal epithelium in mice after orthotopic corneal allograft transplantation. *Graefes Arch Clin Exp Ophthalmol* 2000;**238**:701-7.

9. Zhang EP, Franke J, Schroff M, et al. Ballistic CTLA4 and IL-4 gene transfer into the lower lid prolongs orthotopic corneal graft survival in mice. *Graefes Arch Clin Exp Ophthalmol* 2003;**241**:921-6.

Archived at Flinders University: dspace.flinders.edu.au

10. Comer RM, King WJ, Ardjomand N, et al. Effect of administration of CTLA4-Ig as protein or cDNA on corneal allograft survival. *Invest Ophthalmol Vis Sci* 2002;**43**:1095-103.

11. Thiel MA, Steiger JU, O'Connell PJ, et al. Local or short-term systemic costimulatory molecule blockade prolongs rat corneal allograft survival. *Clin Experiment Ophthalmol* 2005;**33**:176-80.

12. Gebhardt BM, Hodkin M, Varnell ED, et al. Protection of corneal allografts by CTLA4-Ig. *Cornea* 1999;**18**:314-20.

13. Pleyer U, Milani JK, Dukes A, et al. Effect of topically applied anti-CD4 monoclonal antibodies on orthotopic corneal allografts in a rat model. *Invest Ophthalmol Vis Sci* 1995;**36**:52-61.

14. Thiel MA, Coster DJ, Standfield SD, et al. Penetration of engineered antibody fragments into the eye. *Clin Exp Immunol* 2002;**128**:67-74.

15. Jessup CF, Brereton HM, Sykes PJ, et al. Local gene transfer to modulate rat corneal allograft rejection. *Invest Ophthalmol Vis Sci* 2005;**46**:1675-81.

16. Parker DG, Kaufmann C, Brereton HM, et al. Lentivirus-mediated gene transfer to the rat, ovine and human cornea. *Gene Ther* 2007;**14**:760-7.

17. Parker DG, Coster DJ, Brereton HM, et al. Lentivirus-mediated gene transfer of interleukin 10 to the ovine and human cornea. *Clin Experiment Ophthalmol* 2010;**38**:405-13.

18. Williams KA, Coster DJ. Penetrating corneal transplantation in the inbred rat: a new model. *Invest Ophthalmol Vis Sci* 1985;**26**:23-30.

19. Bennett J. Immune response following intraocular delivery of recombinant viral vectors. *Gene Ther* 2003;**10**:977-82.

20. Yamagami S, Dana MR. The critical role of lymph nodes in corneal alloimmunization and graft rejection. *Invest Ophthalmol Vis Sci* 2001;**42**:1293-8.

21. Brice SL, Kirk K, Brereton HM, et al. The influence of cervical and thoracic lymphadenectomy on corneal allograft rejection in inbred rats. *Br J Ophthalmol* 2012;**96**:448-50.

Archived at Flinders University: dspace.flinders.edu.au

22. McMenamin PG, Kezic J, Camelo S. Characterisation of rat corneal cells that take up soluble antigen: an in vivo and in vitro study. *Exp Eye Res* 2006;**83**:1268-80.

23. Lindsey JD, Hofer A, Wright KN, Weinreb RN. Partitioning of the aqueous outflow in rat eyes. *Invest Ophthalmol Vis Sci* 2009;**50**:5754-8.

24. McMenamin PG, Crewe J, Morrison S, et al. Immunomorphologic studies of macrophages and MHC class II-positive dendritic cells in the iris and ciliary body of the rat, mouse, and human eye. *Invest Ophthalmol Vis Sci* 1994;**35**:3234-50.

25. Parker DG, Brereton HM, Coster DJ, Williams KA. The potential of viral vector-mediated gene transfer to prolong corneal allograft survival. *Current Gene Ther* 2009;**9**:33-44.

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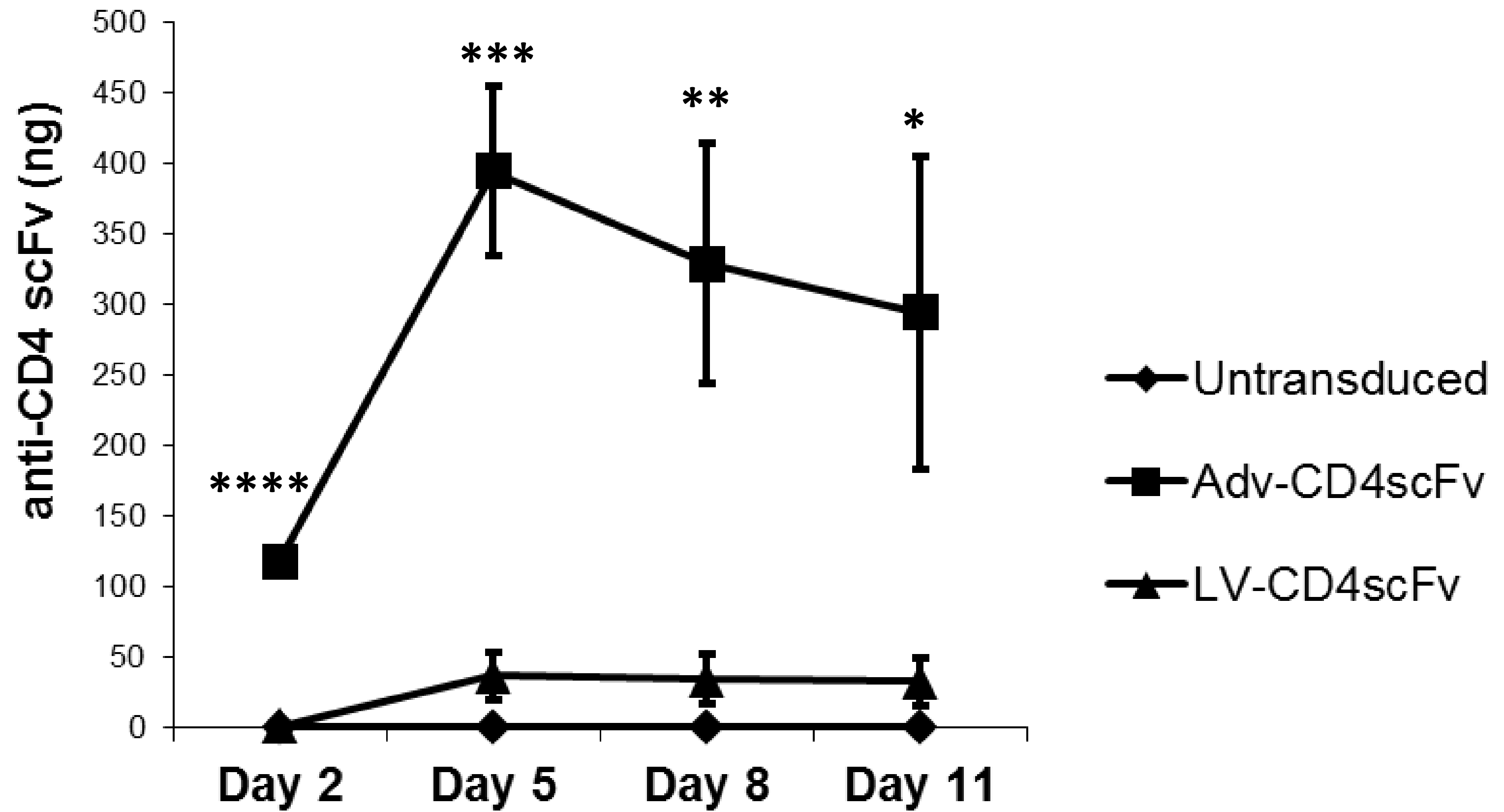
Fig 1



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