

The effect of perioperative allergic conjunctivitis on corneal lymphangiogenesis after corneal transplantation

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ABSTRACT

Introduction Perioperative allergic conjunctivitis accelerates the speed of corneal allograft rejection. This study examines the effect of allergic conjunctivitis, with and without dexamethasone treatment, on the early inflammatory response and lymphangiogenesis in the host cornea following corneal transplantation.

Methods Allogeneic fully MHC-mismatched C57Bl/6 strain donor corneas were transplanted into naive A/J mice and into A/J mice with active allergic conjunctivitis. Further groups of allograft recipients with allergic conjunctivitis were treated post-operatively with twice daily topical dexamethasone 0.1% or phosphate-buffered saline. Mice were killed on days 2 and 6 and corneas were examined by (i) fluorescent immunohistochemistry of frozen sections using anti-CD11b, anti-F4/80 and anti-Gr-1 antibodies, or (ii) whole-mount staining with anti-LYVE-1 antibody. Lymphatic ingrowth and numbers of cells infiltrating the host cornea were compared between groups.

Results There were significantly higher numbers of CD11b⁺ cells and LYVE-1⁺ vessels in the host cornea at day 2 in allergic compared with naive recipients, but no differences between naive and allergic recipients at day 6. In allergic eyes, dexamethasone treatment significantly inhibited LYVE-1 expression at days 2 and 6, and significantly improved allograft survival in recipients with allergic conjunctivitis if maintained for a week.

Conclusions The innate immune response to allogeneic corneal tissue is more vigorous in the presence of allergic conjunctivitis than in naive eyes and is associated with accelerated lymphatic ingrowth to host cornea. Topical dexamethasone inhibits lymphatic ingrowth and this may be one mechanism by which topical steroid enhances graft survival.

INTRODUCTION

Ipsilateral allergic conjunctival inflammation has been shown to accelerate the speed of experimental corneal allograft rejection.^{1,2} As such the presence of perioperative allergic conjunctivitis confers a high-risk status on the graft. The reason(s) for this are not entirely clear. In the better characterised model of high rejection risk corneal transplantation, in which donor cornea is transplanted into a vascularised recipient corneal bed, the reasons for accelerated rejection include facilitated access of allogeneic effector cells to the graft by corneal blood vessels adjacent to the graft–host interface.

Almost 50 years ago Collin demonstrated that vascularised cornea contained both blood and lymphatic vessels.³ This was confirmed in humans

with more modern molecular techniques for identifying lymphatic endothelium⁴ and has led to renewed interest in the role of lymphatic vessels in the immune response to allogeneic cornea.⁵ Corneal lymphatics facilitate egress of antigen presenting cells, an important process in sensitisation of the host to alloantigen. Accordingly, the presence of lymphatic vessels at the time of transplantation may be one explanation for the fact that sensitisation occurs earlier in the vascularised recipient bed than in hosts with a normal avascular cornea.⁶ One recent report suggests that lymphatic vessels are more important than blood vessels in conferring a high risk of rejection in vascularised host cornea.⁷

In unmodified experimental corneal transplantation, ingrowth of lymphatic vessels through the host cornea to the graft–host interface can be demonstrated. That this process is alloantigen-independent is indicated by the similar extent and rate of lymphatic ingrowth in isografts and allografts.⁸ A correlation between corneal inflammation and lymphatic ingrowth in human cornea has been reported.⁴ Studies in animal models have reported that corneal lymphangiogenesis is driven by innate immune cells, particularly CD11b⁺ macrophages which infiltrate the host cornea in response to ocular surface inflammatory stimuli such as suturing,^{9,10} alkali injury¹¹ or dry eye disease.¹²

It is known that infiltration of the conjunctiva by large numbers of CD11b⁺ cells following allergen challenge is a feature of allergic conjunctivitis.^{13,14} The late phase of experimental allergic conjunctivitis is thought to last about 3 days,¹⁵ but the effects of this on post-keratoplasty corneal inflammation and lymphangiogenesis are unknown. The experiments reported here were designed to test the hypothesis that perioperative allergic conjunctivitis alters the development of corneal lymphatic vessels in the first 6 days following corneal allotransplantation.

METHODS

Animals

Female 6–8-week-old A/J strain mice (H-2^k; Harlan UK, Bicester, UK) were used in the allergic conjunctivitis induction protocol and subsequently as corneal allograft recipients. Adult female C57/BL6 strain mice (H-2^b; Harlan), which provide a full MHC mismatch and multiple minor mismatches, were used as corneal donors. Animals were treated in accordance with the UK government regulations for care of experimental animals and with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Induction of allergic conjunctivitis

Mice were sensitised to the allergen short ragweed pollen (SRW) over a 15-day period using a previously described method.² Mice were sensitised by intraperitoneal (i.p.) injection of SRW (Greer Laboratories, Lenoir, North Carolina, USA) pollen, 200 µg with 2 mg aluminium hydroxide (alum; Sigma, St. Louis, Missouri, USA) in 0.4 ml phosphate-buffered saline (PBS) on days 0, 7 and 14. The sensitisation period also involved treatment with eye drops (SRW pollen 500 µg with 25 µg alum in 5 µl PBS) on days 8 and 15. The experimental challenge of 500 µg in 5 µl PBS was administered topically to the right eye on day 27, immediately following corneal transplantation.

Transplantation technique and diagnosis of rejection

Corneal transplantation and diagnosis of graft rejection were performed as previously described.¹⁶ Briefly, recipient mice were anaesthetised with an i.p. injection of fentanyl fluanisone and midazolam. A 2.5 mm donor button was sutured into a 2.0 mm host bed using a continuous 11-0 nylon suture. Animals were examined three times per week and rejection diagnosed by observation of loss of graft clarity as defined by the established grading system.

Immunohistochemistry

On days 2 and 6 mice were killed and whole eyes, including conjunctiva and lids, were exenterated. The eyes were embedded in optimal cutting temperature compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen on a liquid nitrogen-cooled duralumin plate. Specimens were stored at -70°C . Cryostat sections of 8 µm thickness were cut and fixed in a mixture of 30% methanol and 70% acetone at 4°C for 5 min. Slides were washed three times in PBS and some slides were incubated sequentially with 5% skimmed milk and 10% normal donkey serum to block non-specific antibody binding. These slides then underwent primary indirect frozen section immunohistochemical staining using the following primary antibodies: rat anti-mouse LYVE-1, 223322 (R&D Systems, Abington, UK), 1:400 dilution; rat anti-mouse Gr-1, NIMP-R14 (AbCam, Cambridge, UK); donkey anti-rat IgG Alexa 488, A-21208 (Molecular Probes; Invitrogen, Paisley, UK) at a dilution of 1:1000 was used to detect LYVE-1 and Gr-1 staining. Other slides were incubated with a 1:100 dilution of 5 µg/ml anti-mouse CD16/CD32 to block non-specific antibody binding and primary direct frozen section immunohistochemical staining was performed using rat anti-mouse F4/80-FITC, CI:A3-1 (Serotec, Kidlington, UK), 1:100 dilution. Secondary direct immunohistochemical staining was performed on all slides using rat anti-mouse CD11b-PE, M1/70 (BD Biosciences, Oxford, UK), 1:100 dilution. Mouse anti-rat IgG_{2a} and IgG_{2b} isotype controls (all from Serotec) were used at appropriate dilutions as isotype controls. Sections were imaged on a fluorescent microscope (Olympus, Hamburg, Germany) and/or a confocal microscope (Leica, Wetzlar, Germany). Positive-staining cells in each section of a $\times 400$ image of the host cornea were counted by a masked observer using image analysis software (Soft Imaging System, Munster, Germany). Cells were counted in three sections per eye and mean values calculated. At least four eyes per time point were examined in each group. Results are presented as the mean $\pm$ SEM.

Processing of whole-mounted corneas

Following removal of eyes at days 2 and 6, the sclera was punctured and eyes were fixed in acetone for 1 h at room temperature. Corneas were excised. Non-specific antibody

binding was blocked by incubating corneal specimens with 2% BSA and with 10% normal donkey serum, following which whole-mounts were then incubated overnight with rat anti-mouse LYVE-1. After six washes in PBS, specimens were incubated for 1 h at room temperature with Alexa 488-conjugated donkey anti-rat antibody, followed by washing and mounting. Whole-mounts were imaged by fluorescent microscopy (Olympus). The cumulative length of LYVE-1⁺ vessels growing centripetally from the limbus was measured in each corneal quadrant by a masked observer using image analysis software (Soft Imaging System). The system was first calibrated by photographing a 1 mm scale at the same magnification ($\times 100$). Using a freehand drawing software tool, a line was traced manually over each lymphatic vessel and its branches. The length of each line automatically calculated by the software was recorded and the combined total length of lymphatic vessels in each quadrant was determined. The totals for each quadrant were added together to give a measure of lymphatic ingrowth for each cornea. At least four corneas were analysed per group per time point. Mean values were calculated and results are presented as the mean $\pm$ SEM.

Dexamethasone treatment

Female A/J mice were sensitised with repeated i.p. and topical administration of SRW and received corneal allografts as above. Graft recipient eyes were then treated topically twice daily for the first post-operative week with either PBS (n=8) or dexamethasone 0.1% (n=8).

Statistical analysis

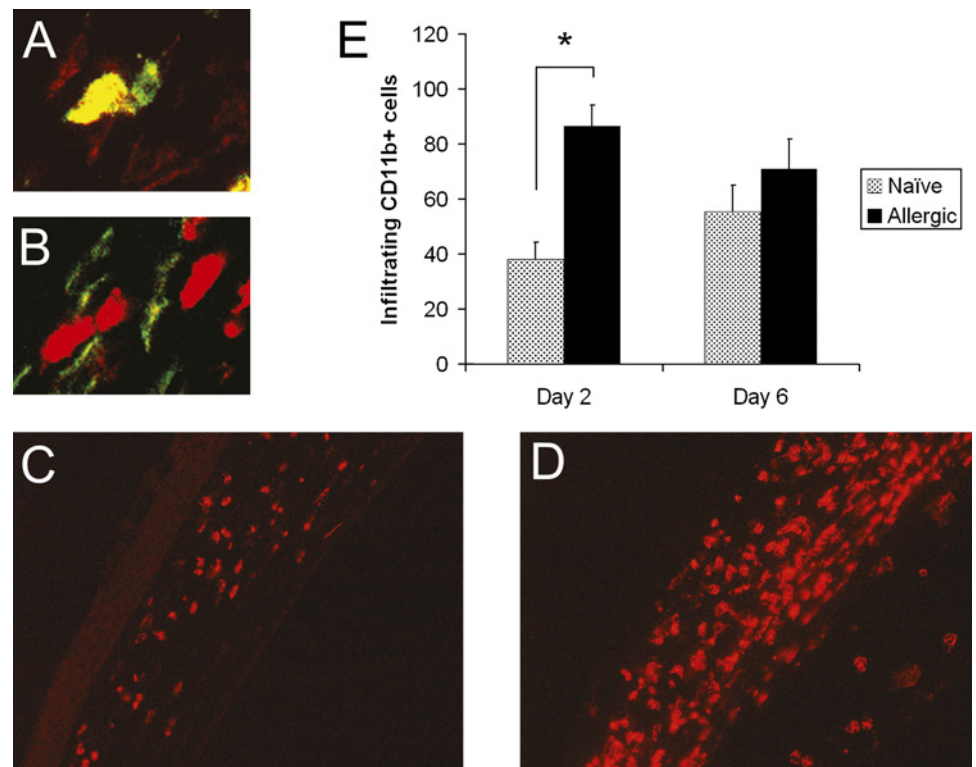
Median graft survival time was calculated for each group and Kaplan–Meier survival curves were constructed. Survival was compared using the log-rank test. The unpaired Student t test was used to compare lymphangiogenesis and numbers of graft-infiltrating leucocytes between groups. For each statistical test values of $p < 0.05$ were defined as statistically significant.

RESULTS

Effect of allergy on innate immune cell infiltration of cornea after transplantation

Inflammatory cells are known to enter the cornea from the conjunctiva via the limbus and accumulations of CD11b⁺ cells were seen at the limbus of both naive and allergic recipients of allografts at 48 h. At this post-transplant interval, inflammatory cell infiltration of the cornea was largely restricted to the host cornea, with relatively few cells seen in donor cornea. Two morphologically and immunohistochemically distinct subsets of CD11b⁺ cells were seen in the host cornea: spindle-shaped CD11b⁺ F4/80⁺ cells and ovoid CD11b⁺ Gr-1⁺ cells (figure 1A,B). Significantly more CD11b⁺ cells were seen in the host cornea at 48 h after corneal transplantation in allergic eyes than in naive eyes (84.46 ± 14.92 vs 38.14 ± 5.97 ; $p = 0.029$; figure 1C–E). In both naive and allergic graft recipients, the majority of CD11b⁺ cells in the host cornea at day 2 were Gr-1⁺ neutrophils. Allergic graft recipients contained more neutrophils (Gr-1⁺) and more macrophages (F4/80⁺) than naive recipients. The difference in macrophage number was statistically significant (mean cell count of 20.13 ± 1.77 in allergic graft recipients vs 7.38 ± 1.95 in naive recipients; $p = 0.003$). The difference in neutrophil number was not statistically significant (mean cell count of 49.9 ± 1.66 in allergic recipients vs 36.3 ± 13.44 in naive recipients; $p = 0.29$). By day 6 after transplantation, large numbers of inflammatory cells could be seen to have migrated beyond the wound into donor cornea. At day 6 after

Figure 1 CD11b⁺ cells in host cornea following transplantation. Two morphologically and immunohistochemically distinct subsets of CD11b⁺ cells were seen in the host cornea: (A) ovoid CD11b⁺ Gr-1⁺ cells (CD11b, red; Gr-1, green; double-positive, yellow), and (B) elongated, spindle shaped CD11b⁺ F4/80⁺ cells (CD11b, red; F4/80, green; double-positive, yellow). At day 2 after transplantation, significantly greater numbers of CD11b⁺ cells were seen in the host cornea of allergic (D; CD11b, red; n=5) allograft recipients than naive (C; CD11b, red; n=5) recipients (E; *p=0.029). There was no significant difference at day 6 (n=4 in both groups).



transplantation, the host cornea in allergic recipients contained slightly more CD11b⁺ cells than naive recipients, but this difference was not statistically significant (figure 1E).

Effect of allergy on lymphangiogenesis in cornea after transplantation

Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) is a membrane glycoprotein which acts as a cell surface receptor on lymphatic endothelial cells. Its physiological role remains unclear, but it is used as a marker of lymphatic endothelium. In the days following corneal transplantation, LYVE-1⁺ structures, presumed to be new lymphatic vessels, are seen to enter the host cornea from the limbus. A small number of the CD11b⁺ cells entering the cornea following corneal transplantation also expressed LYVE-1. Linear and circular LYVE-1⁺ structures were

seen in sections of the host cornea of both naive and allergic recipients (figure 2). The circular structure seen in cross-section at the limbus (figure 2A) represents a large annular lymphatic vessel which is also visible on whole-mount (figure 3A; large arrowhead). The linear structures visible on cross-section (figure 2B) represent smaller lymphatic vessels growing centripetally into the host cornea. These structures were LYVE-1⁺ CD11b⁻. Corneal LYVE-1⁺ CD11b⁻ linear structures are in the superficial stroma/subepithelial plane (figure 2). At day 2 after transplantation, significantly more LYVE-1⁺ radial vessel ingrowth was detected in allergic than in naive allograft recipients (mean cumulative length 2564±361.4 µm vs 1189±217.8 µm; p=0.014; figure 3). At day 6 after transplantation, the lymphatic ingrowth approached the graft–host interface and there was no significant difference between allergic and naive recipients of corneal

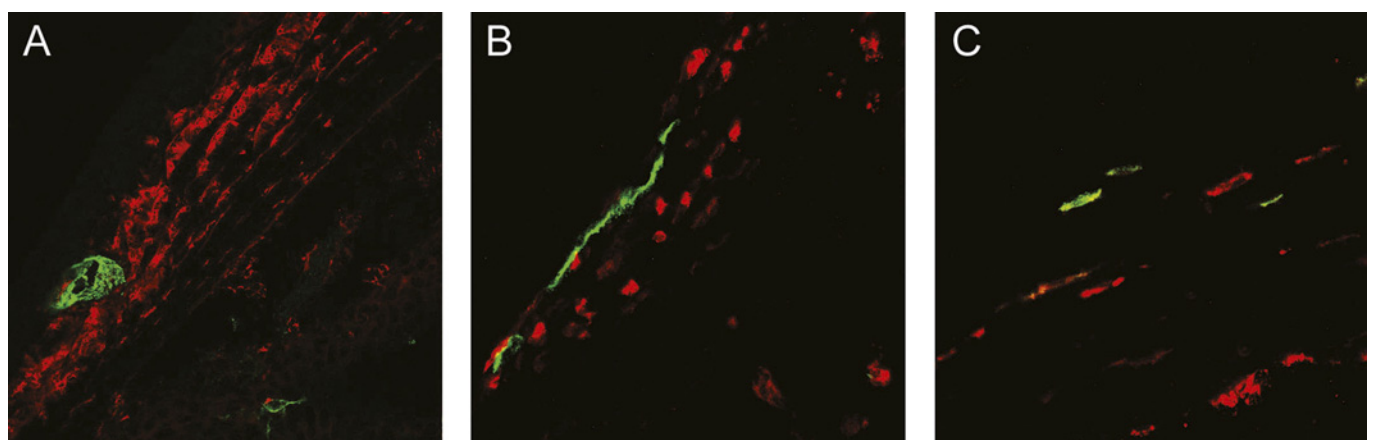
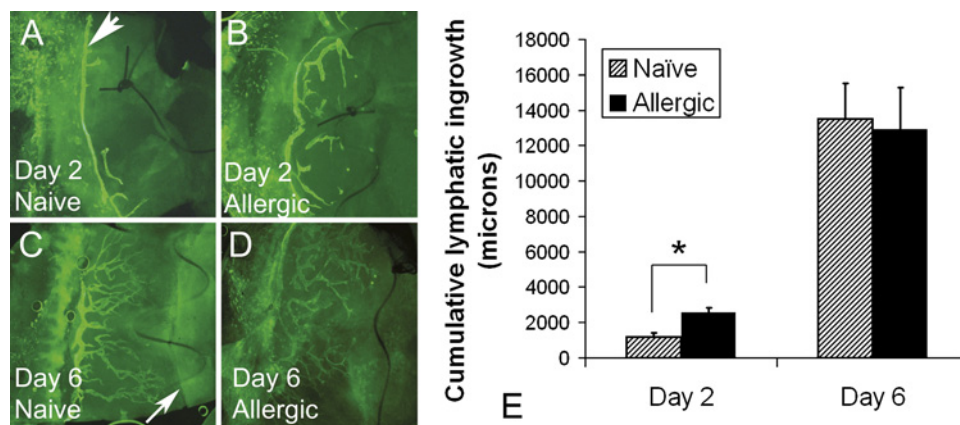


Figure 2 LYVE-1 expression in host cornea. Accompanying the influx of CD11b⁺ cells into the host cornea of both naive and allergic recipients of corneal allograft were ovoid (A) and linear (B) structures which stained with LYVE-1 (green) but not CD11b (red) markers. Occasional CD11b⁺ LYVE-1⁺ double positive cells (yellow) were found among the infiltrating cells (C).

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Figure 3 Corneal lymphangiogenesis after corneal allotransplantation. Panels A–D are images of whole-mounted cornea with an allograft in situ, removed at day 2 or day 6 after transplantation and stained with anti-LYVE-1. At day 2 after transplantation, significantly more LYVE-1⁺ radial vessel ingrowth (green) was detected in allergic (n=6) than in naive (n=6) allograft recipients (A, B, E; *p<0.05). The large white arrowhead indicates the circumferential annular lymph vessel seen at the limbus (A). The smaller arrow indicates the graft–host interface with transplant suture in situ (C). There was no statistically significant difference in lymphatic ingrowth between groups at day 6 (C, D, E; n=4 in both groups).



grafts (mean cumulative length of $12908 \pm 2372 \mu\text{m}$ vs $13527 \pm 1991 \mu\text{m}$; $p=0.886$). Lymphatic ingrowth beyond the graft–host interface was not seen in any specimen, although some CD11b⁺ LYVE-1⁺ cells were seen to enter the graft.

Effect of treatment on corneal lymphangiogenesis

At days 2 and 6 after transplantation, radial ingrowth of LYVE-1⁺ vessels was significantly reduced in dexamethasone-treated allergic recipients of allograft compared with untreated recipients (mean cumulative length at day 2 of $2564 \pm 361.4 \mu\text{m}$ vs $1141 \pm 185.5 \mu\text{m}$; $p=0.029$, mean cumulative length at day 6 of $12908 \pm 2372 \mu\text{m}$ vs $3387 \pm 839.9 \mu\text{m}$; $p=0.009$) (figure 4A,B).

Effect of short-term topical treatment on graft survival in allergic recipients

Eyes with perioperative allergic conjunctivitis received corneal allografts and were subsequently treated with twice daily dexamethasone 0.1% or PBS drops. Allograft survival in dexamethasone-treated animals was significantly prolonged compared with that in the PBS-treated group (median graft survival time 30 days vs 21 days; $p=0.0077$; figure 4C).

DISCUSSION

In the high rejection risk model of corneal transplantation into prevascularised recipient cornea, characterised by pre-existing blood and lymphatic vessels, enhanced early infiltration with neutrophils and macrophages has been reported.¹⁷ A vascularised cornea allows more rapid ingress and egress of immune cells. Corneal transplantation in the setting of allergic conjunctivitis is accompanied by increased conjunctival inflammation, but what this report describes is more rapid ingress of inflammatory cells to the donor cornea accompanied by earlier lymphangiogenesis. We have previously shown that the experimental allergic conjunctivitis induction protocol, which involved conjunctival exposure to allergen, did not induce either corneal infiltration by inflammatory cells or corneal lymphangiogenesis,² indicating that the allergy-associated increase in perioperative corneal inflammation is seen only in the presence of an inflammatory stimulus in the cornea itself, that is, a suture.

There appears to be a relationship between suture-induced infiltration of the cornea with CD11b⁺ cells and the development of new lymphatic vessels. This has been attributed mostly to the macrophage cohort of CD11b⁺ cells, although the exact mechanism has not been established.^{9–10} One theory suggests that these cells enter the cornea in response to inflammation and incorporate themselves into developing lymphatics. Dana and

co-workers found that the typical response to a corneal suture was a decrease in LYVE-1⁺ cells in conjunctiva and an increase in LYVE-1⁺ cells in cornea, suggesting that the conjunctival

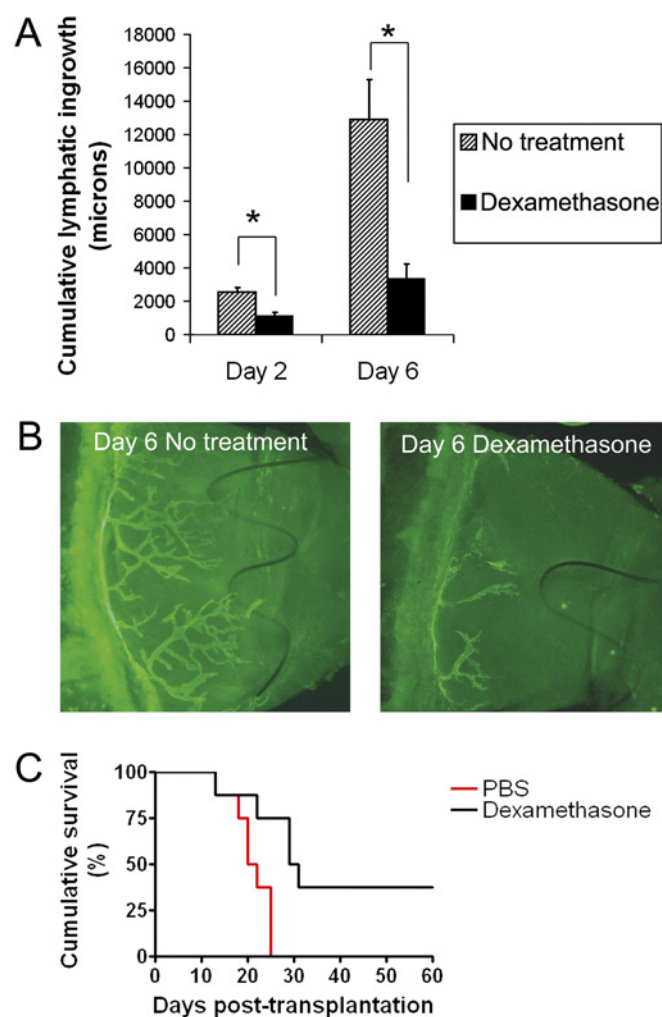


Figure 4 Eyes with perioperative allergic conjunctivitis received corneal allografts and were subsequently treated with twice daily drops of dexamethasone 0.1% or PBS for 7 days. Treatment with twice-daily topical dexamethasone significantly inhibited corneal lymphangiogenesis at days 2 and 6 after transplantation (A, B; *p<0.05) and prolonged corneal allograft survival (C; $p=0.0077$). Median survival time in dexamethasone-treated grafts (n=8) was 30 days compared to 21 days in PBS-treated grafts (n=8).

LYVE-1⁺ cells enter the cornea and contribute to lymphangiogenesis, at least in that experimental setting.¹⁸ The finding in our experiments of increased numbers of CD11b⁺ cells in host cornea at day 2 after transplantation in allergic recipients of corneal grafts is a novel finding, but we found that very few CD11b⁺ cells infiltrating the cornea were LYVE-1⁺, suggesting that only a proportion of the infiltrating cells have the capacity to contribute directly to lymphangiogenesis. Lymphatic vessels in the cornea were CD11b⁻ on immunohistochemistry and this appears to refute the idea that CD11b⁺ graft-infiltrating cells incorporate directly into lymphatic endothelium. However, Kerjaschki *et al*, in a study of de novo lymphangiogenesis in human renal transplantation, found that endothelial progenitor cells did not retain the CD11b marker once incorporated into new lymphatics.¹⁹ Maruyama has described LYVE-1⁺ CD11b⁺ double positive vessels in the cornea on whole-mount.¹⁰ As we performed staining only with anti-LYVE-1 antibodies on corneal whole-mounts, we cannot comment on CD11b expression in these vessels using this technique. It is possible that the discrepancy in CD11b staining of these LYVE-1⁺ vessels is due to differences in the specimen processing prior to immunohistochemistry.

One further finding in these experiments was that CD11b⁺ cells in host cornea at day 2 after transplantation in allergic recipients of corneal grafts expressed markers not only for macrophages (F4/80) but also for neutrophils (Gr-1). Another theory regarding the role of CD11b⁺ macrophages in corneal lymphangiogenesis is that they release VEGF-C, a potent pro-lymphangiogenic cytokine.²⁰ The possible role of neutrophils (which are also CD11b⁺) in this process has not been explored, even though neutrophils have been reported as the dominant CD11b⁺ cells in host cornea after transplantation,⁹ a finding confirmed by our results.

As earlier studies demonstrated that inhibition of CD11b⁺ influx was associated with reduced lymphangiogenesis, is it the case that increasing CD11b⁺ influx is associated with increased lymphangiogenesis? The answer appears to be yes but only slightly. The difference between allergic and naive eyes was statistically significant at day 2, but there was no difference at all at day 6, by which time lymphangiogenesis had reached the graft–host interface in both groups. The difference therefore between eyes with and without active allergic inflammation was the speed, rather than the extent, of lymphangiogenesis. It is interesting that at day 6 CD11b⁺ cells were observed to have entered the graft, but no lymphatic vessels had done so. It may be that this time point is too early to identify graft lymphatics lagging behind the CD11b⁺ infiltrate. More likely explanations are that CD11b⁺ cell infiltration is necessary but not sufficient for corneal lymphangiogenesis and/or that the wound at the graft–host interface provides a relative barrier to lymphatic ingrowth.

Investigators have reported inhibition of corneal lymphangiogenesis using inhibitors of VEGF-A or VEGFR-3 with associated improvements in graft survival.^{21–22} Post-operative topical corticosteroids are the established anti-inflammatory treatment in human corneal transplant recipients, but until recently we have known relatively little about their effect on corneal lymphangiogenesis. Boneham described inhibition of limbal lymphatic growth by topical corticosteroid²³ and we have confirmed this finding in the setting of corneal allograft transplantation. There is experimental evidence that topical corticosteroids inhibit influx of innate immune cells to the cornea and corneal angiogenesis.^{24–25} Inhibition of innate immune cell infiltration of the host cornea may be one indirect

mechanism by which topical corticosteroid inhibits lymphangiogenesis. Subcutaneous dexamethasone injections have been shown to inhibit lymphangiogenesis in xenografted human prostate tumours in mice and this has been attributed to an observed decrease in transcription levels of VEGF-C mRNA in dexamethasone-treated tumour cells.²⁶ That survival of rodent allografts improves with topical corticosteroid treatment has been previously established.^{27–28} This means that it is difficult to say to what extent the improved graft survival in dexamethasone-treated animals in our study is due specifically to suppression of allergic conjunctivitis and to what extent it is due to broader immunosuppressive effects of steroid. Inhibition of lymphangiogenesis is not the sole reason for the improvement in graft survival seen, but it is likely to be a contributory factor.

Antigen from the anterior chamber is known to drain to preauricular and sub-mandibular lymph nodes.²⁹ Experimental lymphadenectomy prolongs corneal graft survival.^{30–31} It is plausible that the route for some antigen-presenting cells from the cornea is via corneal lymphatics to large annular limbal lymphatic vessels and from there via conjunctival lymphatics to conjunctiva-associated lymphoid tissue (CALT) and/or to regional lymph nodes via skin lymphatics. The conjunctiva is known to contain CALT, but the role of this tissue in the response to corneal alloantigen is unknown. Matsuda *et al* have recently demonstrated lymphoid neogenesis with associated LYVE-1⁺ vessels in giant papillae in chronic allergic conjunctivitis.³² There are very few published data on conjunctival lymphatic vessels either in health or disease. Forrester has demonstrated LYVE-1⁺ cells 'lining up' in ocular tissues and hypothesised that these cells may connect to form new lymphatic vessels in response to inflammation/antigen challenge.^{33–34} Such a response might facilitate transit of alloantigen-presenting cells from the cornea.

The ingrowth of new corneal lymphatics following keratoplasty is an important step in the immune response to allogeneic tissue and this response appears to be modified by ocular surface allergic inflammation and topical corticosteroid use.

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