Identification of therapeutic targets of inflammatory monocyte recruitment to modulate the allogeneic injury to donor cornea.

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ABSTRACT

Purpose. We sought to test the hypothesis that monocytes contribute to the immunopathogenesis of corneal allograft rejection and identify therapeutic targets to inhibit monocyte recruitment.

Methods. Monocytes and pro-inflammatory mediators within anterior chamber samples during corneal graft rejection were quantified by flow cytometry and multiplex protein assays. Lipopolysaccharide (LPS) or Interferon (IFN)γ stimulation of monocyte derived macrophages (MDM) was used to generate inflammatory conditioned media (CoM). Corneal endothelial viability was tested by nuclear counting, connexin 43 and propidium iodide staining. Chemokine and chemokine receptor expression in monocytes and MDM was assessed in microarray transcriptomic data. The role of chemokine pathways in monocyte migration across microvascular endothelium was tested in vitro by chemokine depletion or chemokine receptor inhibitors.

Results. Inflammatory monocytes were significantly enriched in anterior chamber samples within one week of the onset of symptoms of corneal graft rejection. MDM inflammatory CoM was cytopathic to transformed human corneal endothelia. This effect was also evident in endothelium of excised human cornea and increased by the presence of monocytes. Gene expression microarrays identified monocyte chemokine receptors and cognate chemokines in MDM inflammatory responses, which were also enriched in anterior chamber samples. Depletion of selected chemokines in MDM inflammatory CoM had no effect on monocyte transmigration across an endothelial blood-eye barrier but selective chemokine receptor inhibition reduced monocyte recruitment significantly.

Conclusions. We propose a role for inflammatory monocytes in endothelial cytotoxicity in corneal graft rejection. Therefore targeting monocyte recruitment offers a putative novel strategy to reduce donor endothelial cell injury in survival of human corneal allografts.
INTRODUCTION

Corneal transplantation remains the most commonly performed transplantation worldwide\(^1\) and 25% of all corneal allografts fail within five years primarily as a result of immune mediated rejection\(^2\). The cellular requirements and the sequence of events in the effector component of the allogeneic response leading to endothelial corneal graft rejection are not fully understood and much of our information has been obtained from animal models. It is widely believed that CD4\(^+\) T cells play an critical role in the rejection of rodent orthotopic corneal allografts\(^3-5\) yet rejection can still occur in CD4 and interferon (IFN)\(\gamma\) KO mice\(^3,6\). Furthermore, multiple and redundant effector mechanisms have been implicated in graft rejection\(^7\) and may explain the poor outcomes in respect of rejection in corneal transplant recipients treated only with calcineurin antagonists which block T cell clonal expansion\(^8\). Several lines of evidence in rodent corneal transplantation suggest monocyte and macrophage involvement in the cell-mediated allogeneic response to transplanted cornea. Firstly, large numbers of macrophages are found in tissue sections at onset of corneal rejection in mouse and rat. At the earliest time points following the onset of corneal rejection in the rat, graft-infiltrating macrophages exceed T cells and NK cells\(^9\), and in mice, MOMA-2\(^+\) macrophages were reported among the earliest graft-infiltrating cells, before and after the onset of corneal rejection\(^10\). Secondly, local depletion of macrophages by subconjunctival administration with clodronate liposomes of corneal allotransplant recipients significantly prolonged corneal graft survival in treated rats using two different strain combinations\(^11,12\). Local depletion of macrophages was found to downregulate infiltration of all alloreactive cell types, downregulate local and systemic cytotoxic lymphocyte responses and prevent the generation of antibodies\(^13\). Thirdly, earlier pilot investigation of immune cell populations in aqueous humour samples from the eye in patients at presentation with acute transplant rejection indicated a high proportion and selective recruitment of CD14\(^+\) cells to the anterior chamber of the eye, likely to represent mononuclear phagocytic cells\(^14\), corroborating an earlier report\(^15\). We sought to reconfirm and extend these data, investigate the mechanisms by which these cells may contribute to the mechanism of human corneal graft failure and investigate possible strategies to inhibit their recruitment across the blood-eye barrier, as a potential novel therapeutic intervention.
MATERIALS AND METHODS

Ethics

Ethics approval was provided by designated UK National Research Ethics Service committee for anterior chamber sampling from patients with acute corneal graft rejection and use of human corneal specimens in research (Research Ethics Committee reference: 11/LO/1294). Corneas, with healthy endothelium, were excised at surgery in keratoconus patients. Written informed consent was obtained from all participants. This study adhered to the tenets of the Declaration of Helsinki.

Aqueous humour analysis

Aqueous humour samples (100-200 µL), were obtained from ten patients presenting with corneal allograft rejection (Table 1). Diagnosis was confirmed by the finding in all patients of active anterior chamber inflammation and keratic precipitates on the donor corneal endothelium at slit-lamp biomicroscope examination. Control aqueous samples were obtained from nine patients undergoing routine cataract surgery (five male and four female; median age 57 range 3-85) without any other ocular disease. Samples were centrifuged at 400g for five minutes. The soluble fraction was collected and the cell pellet was resuspended in 100 µL phosphate buffered saline (PBS) with 0.5% bovine serum albumin (Sigma) and 0.01% sodium azide (Sigma). Total cell counts were enumerated with a hemocytometer and the cells were stained with directly conjugated fluorescent antibodies to CD14 (Becton Dickinson, clone M5E2) and CD16 (Becton Dickinson, clone 3G8). Immunostaining was quantified with a FACScalibur flow cytometer (Becton Dickinson) and FlowJo analysis software (version 9.4.3). Chemokine concentrations in these samples were measured using a flow cytometric multiplex bead assay kit (Milliplex, HCYTOMAG-60K).

Preparation of monocyte-derived macrophage conditioned media

Monocyte-derived macrophages (MDM) were prepared from human peripheral blood mononuclear cells (PBMC) as previously described. After being allowed to differentiate for six days, the medium was changed to RPMI medium (Sigma) and 10% foetal calf serum (FCS, Biosera) with or without 100 ng/mL lipopolysaccharide (LPS) for 6 hours or 10 ng/mL IFNγ for 24 hours, using 1 mL media per 10⁶ MDM. Conditioned media (CoM) from these cultures were then centrifuged at 10,000 g for five minutes and stored at -80°C. Residual LPS in LPS stimulated CoM was neutralised by addition of 10 µg/mL polymyxin B as described previously and residual IFNγ was neutralised in IFNγ stimulated CoM by addition of 2 µg/mL blocking antibody to IFNγ. Effective functional neutralisation of LPS and IFNγ under these conditions was confirmed in monocyte transwell migration assays as described below, but without endothelial cells. We confirmed previous reports
that LPS inhibits monocyte migration in transwell assays and found that IFN\(\gamma\) had the same effect, which was reversed by addition of polymyxin B to neutralise LPS or antibody to neutralise IFN\(\gamma\) (Supplementary Figure 1).

Human corneal endothelial cell toxicity

The immortalised human corneal endothelial cell line (HCEC-B4G12, Ref- ACC 647, DCMZ, Germany)\(^1\) was cultured in human endothelial serum free medium (Gibco) and 10 ng/ml FGF-2 (Gibco) in tissue culture plates pre-coated with 10 \(\mu\)g/mL laminin (Sigma Aldrich) and 10 mg/mL chondroitin sulphate (Sigma). At 70% confluence, the media was then changed to 10% CoM from LPS or IFN\(\gamma\) stimulated and unstimulated MDM before being washed with PBS and stained with calcein-AM (Sigma) and propidium iodide (PI, Sigma) as per manufacturer’s instructions. Cellular fluorescence was imaged \textit{in situ} on a Leica SPE inverted confocal microscope. Following removal of epithelium, freshly excised full thickness human cornea specimens were cut into quadrants at surgery with a diamond blade, placed in DME medium (Gibco) with 10% FCS overnight and then incubated for 24 hours with CoM from stimulated and unstimulated MDM. The corneas were then washed in PBS, fixed in 4% paraformaldehyde overnight, incubated in a blocking solution (PBS, 0.1M Lysine, 0.05% Triton X-100) for one hour, before immunostaining with a rabbit antibody for Connexin (Cx)43 (Sigma), fluorophore-conjugated secondary antibody and with a bis-benzamide nuclear counterstain. The stained cornea was washed in PBS and mounted in citifluor solution (Citifluor) and imaged using a Leica SPE confocal microscope. Sample identifiers were blinded for image analysis. Cx43 staining was quantified as previously described\(^1\) and nuclear counting within multiple high power fields was performed manually.

Transwell migration assay across an endothelial barrier

Human cerebral microvascular endothelial cells (hCMEC/D3)\(^1\) cells were obtained as kind gift from Dr PO Couraud (Institut Cochin, Paris, France). These were cultured in endothelial growth medium-2 (EGM-2, Lonza) supplemented with 5% FCS, 1% penicillin-streptomycin (Gibco), 1.4 \(\mu\)M hydrocortisone (Sigma), 5 \(\mu\)g/ml ascorbic acid (Sigma), 1% chemically defined lipid concentrate (Gibco), 10mM HEPES (Gibco) and 1 ng/ml human fibroblast growth factor (Sigma). Cells were seeded at 5x10^4 cells/cm\(^2\) on the apical side of 0.33 cm\(^2\) polycarbonate transwell inserts (Corning No. 3421, 6.5 mm diameter, 5.0 \(\mu\)m pores) pre-coated with Cultrex rat type 1 collagen 50 \(\mu\)g/ml (R&D Systems). To form a monolayer hCMEC/D3 cells were maintained in culture for six days with media changes after three and six days\(^1\) and supplemented with 10mM lithium chloride (Santa Cruz Biotechnology) for the entire culture period to generate tight junctions\(^2\), confirmed by transendothelial electrical resistance (TEER) measurements using an electrical Volt-Ohm-Meter (EVOM-2, 5
World Precision Instruments). After six days, the transwells were then transferred into wells containing 10% stimulated or unstimulated CoM for 24 hours. Cellular transmigration across the endothelial barrier was assessed by addition of 5x10^5 peripheral blood mononuclear cells (PBMC) obtained from healthy volunteers into the upper chamber of the transwell for three hours at 37°C, before collecting cells in the lower chamber. This cell suspension was then stained for CD14 and CD16 and enumerated by flow cytometry using FlowCheck™ polystyrol fluorospheres (Beckman Coulter) to standardise cell counting. Monocytes and lymphocytes were discriminated by light scatter properties and CD14/CD16 staining. Transmigration of cells into the lower chamber was expressed as a proportion of the input.

**Chemokine and chemokine receptor expression data**

Data on normalised chemokine receptor expression in monocytes and chemokine expression in LPS or IFNy-stimulated MDM was obtained from the European Bioinformatics Institute data repository (www.ebi.ac.uk/arrayexpress/) using accession numbers E-TABM-1206 and E-MEXP-2032. A network of interacting chemokines and chemokine receptors was adopted from the KEGG cytokine-cytokine receptor interaction reference pathway (www.genome.jp/kegg/kegg2.html, map0460) and constructed in Gephi graph visualization software (version 0.8.2). The transcriptomes of hCMEC/D3 cells after 24 hour incubation with CoM from unstimulated MDM and LPS-stimulated MDM with PMB were also compared by genome-wide expression arrays. Total RNA was purified from cell lysates collected in RLT buffer (Qiagen) using the RNeasy Mini kit (Qiagen). Samples were processed for Agilent microarrays as previously described and loess normalized data were analysed using the TM4 microarray software suite MeV (version 4.9). Pathway enrichment analysis of differentially expressed gene lists was performed using the online bioinformatics tools InnateDB and Ingenuity Pathway Analysis (http://www.ingenuity.com/). Microarray data are available from the EBI Array Express repository (http://www.ebi.ac.uk/arrayexpress/) under accession no E-MTAB-3692.

**Chemokine depletion and chemokine receptor targeting**

To deplete single or a combination of different monocyte chemotactic chemokines, biotinylated anti-human antibody against CCL2, CCL3, CCL4, and CCL8 were added at a concentration of 5 μg/mL to the different CoM. The biotinylated antibodies were incubated with the CoM for one hour at room temperature before addition of magnetic streptavidin beads (Dynabeads MyOne Streptavidin T1, Life Technologies) using 10^9 beads/mL. These were incubated for one hour with the CoM before removing the beads magnetically. Successful depletion was confirmed by ELISA using paired capture and detection antibodies for each chemokine (eBioscience) according to manufacturer’s instructions and analysed using a Multiskan absorbance plate reader (Thermo Labsystems). For chemokine receptor targeting, 5x10^6 cells/mL PBMC were incubated
with inhibitors of CCR2 (RS 504393 or BMS CCR2 22, both from Tocris), CCR5 (Maraviroc, Tocris), or CXCR4 (AMD 3465 hexahydrobromide, Tocris), used at 10 nM, for 30 minutes.

**Statistical analysis**

Data were analysed using Graphpad Prism software Version 5. The Mann-Whitney U- or t-tests were used to test significance. Values of $p<0.05$ were defined as statistically significant.
RESULTS:

Enrichment of inflammatory monocytes in the aqueous humour of patients with acute corneal graft rejection.

Samples of aqueous humour from the anterior chamber were obtained from ten patients with endothelial corneal allograft rejection. The demographic characteristics, primary diagnosis leading to corneal transplantation, number of previous transplants and previous episodes of allograft rejection, and corticosteroid therapy at time of rejection are summarised in Table 2. Total cell counts in the aqueous humour samples revealed that a cellular infiltrate was only detectable in aqueous humour samples from patients presenting within seven days of the onset of symptoms (Figure 1A), irrespective of concomitant corticosteroid treatment (Supplementary Figure 2). Within these samples, we confirmed our previous observation\(^{14}\) that CD14\(^+\) cells were significantly enriched in the aqueous humour compared to peripheral blood (Figure 1B). We extended these data to show that the enrichment of CD14\(^+\) cells was entirely due to CD14\(^{hi}\)CD16\(^{low}\) classical inflammatory monocytes, known to be recruited to inflammatory foci (Figure 1C).

Monocyte-derived macrophages generate inflammatory mediators that deplete corneal endothelial cells.

Classical inflammatory monocytes are extremely short-lived with a half-life of less than 24 hours\(^{24}\), unless they differentiate into macrophages as a result of environmental signals. Therefore, we reasoned that if monocyte recruitment to the eye contributes to the pathogenesis of corneal transplant rejection, inflammatory mediators generated by monocyte-derived macrophages (MDM) may cause donor corneal endothelial cytotoxicity and depletion. Macrophages produce inflammatory mediators in response to innate immune danger signals or interferon (IFN)\(_{\gamma}\) production by lymphocytes. We therefore modelled macrophage inflammatory responses by stimulating MDM with either lipopolysaccharide (LPS) or recombinant IFN\(_{\gamma}\), and we pooled conditioned media (CoM) from stimulated and unstimulated MDM cultures from multiple experiments in order to minimise the confounding of donor to donor variability. In order to focus on the role of the MDM-derived inflammatory response in downstream experiments with these CoM, we neutralised LPS or IFN\(_{\gamma}\) activity by addition of polymyxin B or blocking antibody to IFN\(_{\gamma}\) to the relevant samples. We first assessed the effect of MDM CoM on survival of an immortalized human endothelial cell line and found that CoM from LPS-stimulated MDM induced significant endothelial cell death, indicated by propidium iodide (PI) staining (Figure 2A). In order to extend these observations further, we then evaluated the effect of MDM CoM on endothelial cells in excised corneal specimens ex vivo by counting the number of nuclei in the endothelial layer and by quantifying
expression of the gap junction protein connexin (Cx)43 as a surrogate for the integrity of the endothelial layer.

In keeping with the effect on the corneal endothelial cell line, we found that CoM from LPS-stimulated MDM induced significant loss of cells of the corneal endothelium and reduction of detectable Cx43 immunostaining (Figure 2B-D). In these experiments, we also found depletion of endothelial cells and loss of Cx43 staining as a result of incubation with CoM from IFN-γ-stimulated MDM, albeit to a lesser degree than LPS-stimulated CoM (Figure 2B-D). In addition, we found that the presence of monocytes in this experimental model significantly increased the cytopathic effect of LPS-stimulated CoM as measured by reduction in Cx43 staining (Figure 3).

Identification of putative targets to modulate monocyte recruitment.

Our data implicate recruitment of functionally active monocytes in the pathogenesis of corneal allograft rejection. Therapeutic targeting of monocyte recruitment may therefore provide a novel strategy to limit injury to donor corneal endothelium. Given the importance of chemokine pathways to mediate cell-specific recruitment, we sought to identify the principal chemokine or chemokine receptors that control human monocyte recruitment to inflammatory foci. To do this we cross-referenced previously published transcriptomic data for chemokine receptor expression in human monocytes25 (Figure 4A), chemokine expression by MDM stimulated with LPS or IFN-γ26 (Figure 4B) with established networks of chemokine and chemokine receptor interactions (Figure 4C). This analysis identified eight chemokines (CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL13 and CCL18) and the most highly expressed chemokine receptors (CCR1, CCR2 and CCR5) which may participate in amplification of monocyte recruitment to putative inflammatory foci. Importantly, using a multiplex protein assay which included reagents for CCL2, CCL3 and CCL4, we found that each of these chemokines were also detectable at significantly greater levels in anterior chamber samples from patients with acute corneal graft rejection within seven days of symptom onset, compared to samples from patients with greater than seven days symptoms or samples from control patients (Figure 5). These data highlight the most likely monocyte chemokine pathways involved in monocyte recruitment in acute corneal graft rejection.

Macrophage inflammatory responses drive monocyte recruitment across a blood-eye barrier

Next, we developed an experimental model for monocyte transmigration across the blood-eye barrier in order to test the hypothesis that the chemokine pathways described above were necessary for monocyte recruitment and could therefore be targeted to effectively reduce monocyte recruitment in corneal allograft rejection. We used a well-characterised human endothelial cell line derived from brain microvasculature to establish an endothelial barrier with tight junctions in transwells (Figure 6A-B). Inflammatory CoM from LPS or IFN-γ-stimulated MDM added to the bottom compartment of the transwell apparatus induced significantly greater monocyte transmigration from top to bottom compartment (Figure 6A and 6C). We used unfractionated PBMCs.
in order to compare monocyte and lymphocyte recruitment. Consistent with our *in vivo* finding that monocytes are enriched in the anterior chamber of patients with acute corneal graft rejection, we found significantly greater transmigration of monocytes compared to lymphocytes in this *in vitro* model (Figure 6C).

**Chemokine receptor targeting to attenuate monocyte transmigration across a blood-eye barrier.**

In order to reduce monocyte recruitment in corneal graft rejection to a functionally significant degree, we reasoned that it might be possible to target either the chemokines or chemokine receptors. To test the effect of chemokine targeting we depleted LPS-stimulated MDM CoM of selected chemokines identified in the experiments above individually, or all of these chemokines together. We found no attenuation of monocyte transmigration in the endothelial blood-eye barrier model (Figure 7A). In response to pro-inflammatory stimuli, endothelial cells upregulate cell adhesion molecules and chemokines that contribute to leukocyte recruitment\(^ {27}\). Accordingly, genome-wide transcriptional responses in hCMEC/D3 cells to CoM from LPS-stimulated MDM revealed upregulation of canonical cell adhesion molecules involved in leukocyte adhesion and diapedesis, and significant enrichment of secreted products with chemotactic activity (Supplementary Figure 3) including CCL2, CCL5, CCL7 and CCL8 (Figure 7B), thereby supporting monocyte recruitment despite depletion of these chemokines in the CoM from LPS-stimulated MDM. Therefore we considered targeting their cognate chemokine receptors instead. A number of small molecules inhibitors of the chemokine receptors CCR2 and CCR5 have already been evaluated in clinical trials. We therefore tested the effect of small molecule inhibitors of these chemokine receptors on monocyte transmigration. We found that targeting CCR2 with inhibitory molecules significantly attenuated monocyte recruitment (Figure 7C). A CCR5 inhibitor also showed the same effect but did not reach statistical significance in four experimental replicates. In contrast a small molecule targeting CXCR4 had no effect on monocyte recruitment (Figure 7C). CXR4 is expressed by monocytes (Figure 4A) but its ligand, CXCL12 (Figure 3C) was not upregulated in LPS or IFN\(\gamma\)-stimulated MDM (Figure 4B). Of note, CCR2 expression and function are known to be downregulated as monocytes are differentiated to macrophages\(^ {30}\), although some subsets of macrophages may retain higher CCR2 expression\(^ {31}\). This is also reflected in our analysis of the transcriptomes of monocytes and MDM (Supplementary Figure 4), even after MDM stimulation with LPS or IFN\(\gamma\), and is consistent with the hypothesis CCR2 has a specific role in tissue recruitment of circulating monocytes.
**DISCUSSION**

In the present manuscript, we show three lines of evidence to support a role for monocytes in corneal rejection. Firstly, classical inflammatory monocytes are specifically enriched in anterior chamber specimens from patients with acute corneal rejection. Secondly, the pro-inflammatory mediators of monocyte derived cells are sufficient to induce cell death of a human corneal endothelial cell line in vitro and death of primary human corneal endothelial cells in corneal buttons ex vivo. Thirdly, the addition of monocytes to proinflammatory mediators significantly enhances corneal endothelial cell death. CD14hi monocytes comprise about 10% of PBMC in health. Therefore, the finding that 40-50% of cells within aqueous humour samples of patients at the time of acute corneal allograft rejection are monocytes clearly indicates selective recruitment, further supported by the finding of elevated levels of chemokines CCL2, CCL3 and CCL4, known to be chemoattractant for monocytes. These were almost entirely CD16low cells, which is the predominant monocyte subset to be recruited to inflammatory foci and suggests that these cells are likely to be functionally active in the allogeneic tissue injury response. It is of note that the cellular infiltrate was only evident in patients with symptoms for less than seven days, indicating that these specimens were examined shortly following the onset of the effector phase of the allogeneic response. Of note, this observation was not confounded by presence or absence of corticosteroid treatment, albeit our sample size was too small for statistically robust subgroup analysis. Additional comparisons in immune correlates of alternative corneal pathologies would be necessary to test the specificity of our findings for acute corneal allograft rejection.

Monocytes typically survive less than 24 hours, or differentiate into tissue resident macrophages and dendritic cells[4]. We speculate that monocyte recruitment to the anterior chamber represents one of the earliest events in the effector phase of corneal allograft rejection and is associated with monocyte differentiation. Hence, the absence of cells later than seven days following the onset of transplant rejection symptoms may reflect the transition to macrophages that migrate from the anterior chamber or adhere to the transplant surface, as suggested by data from rodent models[9]. Our study does not address the question of the initial inflammatory trigger that stimulates monocyte recruitment in the first instance. The intersection of coagulation pathways with inflammation, and inflammatory responses that arise from so-called danger associated molecular patterns suggest tissue injury may lead to innate immune responses that augment adaptive immune responses to allogeneic antigens[32]. In corneal transplantation the mechanical trauma of surgery may cause significant tissue damage, leading to activation of resident antigen-presenting cells and enhanced immunogenicity, in keeping with the danger model proposed by Matzinger[33].
Macrophage infiltration has also long been recognised as a hallmark of acute allograft rejection after heart transplantation\(^3\)\(^4\), and a number of studies suggest that macrophages can promote acute renal allograft rejection\(^3\)\(^5\)\(^-\)\(^7\). The functional consequence of modulating macrophage function in corneal transplantation was most directly shown by prolongation of rat corneal allograft survival following depletion of conjunctival macrophages with clodronate liposomes\(^1\)\(^3\). One component of the present study was to assess whether inflammatory responses from MDM may contribute to loss of donor corneal function in rejection by causing endothelial cell death and thereby compromising the transparency of donor cornea. In two separate models we found CoM from LPS stimulated MDM, and to a lesser extent IFN\(\gamma\)-stimulated MDM caused significant corneal endothelial cell loss. Furthermore, monocytes had an additional direct cytotoxic effect on human corneal endothelium \textit{ex vivo} but only in the context of inflammation. This suggests therefore that the inflammatory cellular microenvironment either drives further pro-inflammatory cytokine release and subsequent cytotoxicity; in keeping with published evidence showing inflammatory cytokines to promote endothelial cell apoptosis\(^3\)\(^8\)\(^-\)\(^{4}\)\(^1\) or that the monocytes themselves become activated and release tissue destructive lysosomal enzymes or free radicals that are directly cytotoxic\(^4\)\(^2\)\(^-\)\(^4\)\(^4\). Further characterisation of the fate and phenotype of the monocyte derived macrophages that accumulate during corneal rejection is necessary to test these hypotheses. Due to the limited availability and volume of human aqueous humour sampling, this was not possible in the present study, but requires renewed assessments of tissue specimens from rejected corneal allografts or further experimental studies in animal models. In addition, the mechanism of corneal endothelial cell death is not addressed in our current experiments, but specific cell death pathways, including apoptosis, pyroptosis and necroptosis all intersect with inflammatory responses and may therefore be implicated.

Elucidating which of these pathways makes the greatest contribution will be important in future studies in order to identify targets to inhibit corneal endothelial cell death.

Our data suggest a rationale for therapeutic targeting of monocyte recruitment and we investigated targeting of the monocyte chemotaxis. Cross-reacting specificities of chemokines and chemokine receptors generate a network with potential for significant functional redundancy that may undermine the use of specific inhibitors. We cross-referenced chemokine receptor expression in monocytes and chemokine production in stimulated MDM with existing databases for chemokine pathways in order to identify those that may contribute to inflammatory monocyte recruitment in our model. Our experimental model simulated changes to chemokine levels associated with acute human corneal graft rejection. We therefore adopted an experimental system in which we could test the role of selected chemokine or chemokine receptors for monocyte migration across endothelial cells which exhibit the tight junction features of the blood-aqueous or blood-brain barrier. Given the
functional redundancy within the chemokine network, we were not surprised to find that depletion of individual chemokines in this system had no significant effect on monocyte migration. It was more surprising that depletion of several chemokines that we predicted may play a role also had no effect on chemokine transmigration. However, we found that the endothelial cells modelling the blood-brain barrier also produced these chemokines and can therefore drive monocyte recruitment in response to paracrine activation by macrophages. Therefore, we tested the effect of targeting key monocyte chemokine receptors instead, using CCR2 and CCR5 small molecule inhibitors which have shown some efficacy in preclinical and clinical models of rheumatoid disease. Blockade of CCR2, which interacts with CCL2, CCL7, CCL8 and CCL13 did result in partial inhibition of monocyte transmigration, suggesting that this receptor at least plays a non-redundant role in recruitment of inflammatory monocytes. Additional sampling and analysis of the humoral and cellular components enriched in acute corneal rejection is needed to overcome the limitations of the small aqueous humour sample size in the present study. Nonetheless, our findings add to the evidence for the role of monocyte recruitment early on in the effector phase of corneal transplant rejection and highlights the potential for chemokine receptor blockade to reduce donor endothelial cell injury in rejection. In vivo studies will be required to examine the efficacy of this novel immunomodulatory approach in attenuating allogenic injury to donor endothelium and prolonging human corneal transplant survival. Of note, experimental corneal rejection was ameliorated in mice with targeted deletions of CCR1, but not in mice deleted for CCR2 and CCL3. Although total mononuclear cell recruitment to the site of rejection was attenuated in CCR1 deficient mice, monocyte recruitment specifically was not evaluated and cellular recruitment in CCR2/CCL3 deficient mice was not reported. Therefore genetic and pharmaceutical targeting of chemokine receptors in mouse models merit further evaluation in order to pave the way for first in man clinical studies.
REFERENCES


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<td>endothelial rejection by one or more of the following:</td>
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**TABLE 2**

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**Figure Legends**

**Figure 1**

(A) Total cell counts in aqueous humour (AH) samples were compared in patients with more or less than seven days symptoms of corneal allograft rejection. In patients for whom a cellular infiltrate was evident (n = 5), the proportion of all CD14+ cells (B) and monocyte subsets discriminated by the combination of CD14 and CD16 staining (C) was compared in AH and contemporaneous PBMC samples. The inset dot plot shows the gating strategy used to quantify each of monocyte subsets indicated. *indicates statistically significant differences (p<0.05, Mann-Whitney U-test). Measurements for individual patient samples are shown in A and summarised as median ±IQR in B and C.

**Figure 2**

(A) Propidium iodide and calcein staining in a human corneal endothelial cell line incubated with CoM from unstimulated (control) and LPS or IFNγ stimulated MDM, was visualised by immunofluorescence microscopy at 30 and 690 minutes. (B) Cx43 and nuclear staining in the endothelial layer of human cornea specimens incubated overnight with CoM from unstimulated and LPS or IFNγ stimulated MDM was visualised by confocal microscopy. Quantitation of numbers of visible nuclei and positive Cx43 staining per high power field is summarised from four separate experiments (C-D). *indicates statistically significant differences (p<0.05, Mann-Whitney U-test). Bars represent median ±IQR. Fluorescence images are representative of four separate experiments in each case.

**Figure 3**

(A) Cx43 and nuclear staining (DAPI) in the endothelial layer of human cornea specimens incubated ex vivo for 24 hours with CoM from unstimulated (Control) and LPS or IFNγ stimulated MDM followed by an overnight incubation with CD14 selected human monocytes (Mo) was visualised by confocal microscopy. Quantitation of numbers of visible nuclei and positive Cx43 staining per high power field is summarised from four separate experiments (B-C). *indicates statistically significant differences (p<0.05, Mann-Whitney U-test). Bars represent mean ± SEM. Fluorescence images are representative of four separate experiments.

**Figure 4**

(A) Relative mRNA expression of chemokine receptors by peripheral blood monocytes from separate healthy volunteer donors and (B) mean fold change of chemokine transcript levels in LPS or IFNγ-stimulated MDM, are derived from previously published data sets. (C) A network diagram of known chemokine receptor-ligand
interactions for all the chemokine receptors expressed in A, above the median normalised expression level of 8 (Log2), in which the size of the nodes is proportional to the number of interactions. Interactions between chemokine receptors (yellow nodes) in this network and chemokines (red nodes) which are upregulated in LPS or IFNγ-stimulated MDM are highlighted.

Figure 5
Comparison of selected chemokine concentrations (CCL2, CCL3 and CCL4) in aqueous humour samples from patients with corneal allograft rejection grouped by duration of symptoms (more or less than one week duration), and samples from control patients undergoing cataract surgery. Measurements from individual patient samples and the median of each group are indicated. *indicates statistically significant differences (p<0.05, Mann-Whitney U-test).

Figure 6
(A) Schematic representation of the transwell model to evaluate PBMC transmigration across a blood-eye barrier. The hCMEC/D3 cell line is cultured in transwell and allowed to form tight junctions, followed by incubation in CoM from stimulated and unstimulated MDM in the lower chamber and addition of unfractionated PBMC in the top chamber. (B) Measurement of trans endothelial electrical resistance (TEER) across hCMEC/D3 cells in culture with and without Lithium supplementation to encourage tight junction formation. (C) Quantiation of CD14+ (monocytes) and CD14- (lymphocytes) fraction of PBMC migrating through the hCMEC/D3 barrier as proportion of the total PBMC loaded into the top chamber, in response to each of the MDM CoM indicated in the lower chamber. In B, bars represent mean ±SEM of at least 10 separate measurements. In C, bars represent mean ±SEM of six separate experiments. *indicates statistically significant differences (p<0.05, t-test).

Figure 7
(A) Monocyte transmigration across hCMEC/D3 cells incubated with CoM from LPS stimulated MDM was compared with and without depletion of selected chemokines from the CoM reflected in the final concentration of the each of the chemokines indicated in the heat map panel. (B) Gene expression heat map of chemokines upregulated in hCMEC/D3 cells incubated with CoM from LPS stimulated MDM compared to CoM from unstimulated MDM in four independent experiments. (C) Monocyte transmigration across hCMEC/D3 cells incubated with CoM from LPS stimulated MDM was compared in the presence and absence of small molecule inhibitors of the chemokine receptors indicated. Data bars represent mean±SEM of four separate experiments in each case. *indicates statistically significant differences (p<0.05, t-test).
Figure 1

A. Cell count per AC sample

B. Percentage of monocytes

C. CD16 and CD14 expression in classical, intermediate, and non-classical monocytes.
Figure 2

A

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<th>Control</th>
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<th>IFNγ CoM</th>
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Calcein-AM
Propidium Iodide

B

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<tr>
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C

![Bar chart](chart1.png)

D

![Bar chart](chart2.png)
Figure 5

Graphs showing the levels of CCL2, CCL3, and CCL4 in different time periods from symptom onset (days) in different groups: <7, >7, and Control. Each graph includes data points and error bars indicating the mean and standard deviation. Asterisks indicate statistically significant differences between groups.
### Figure 7

**A**

% cell migration

![Bar graph showing % cell migration across different conditions](image)

**B**

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<td>CXCL6</td>
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</table>

**C**

% cell migration

![Bar graph showing % cell migration across different conditions](image)

---

**Legend**

- CCL2
- CCL3
- CCL4
- CCL8
- <0.1
- >10

---

Relative expression (Log2)