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ORIGINAL ARTICLE

The high-risk corneal regraft model: a justification for tissue matching in humans

Andrea Vitova,[1] Lucia Kuffová,[1] Izabela P. Klaska,[1] Vladimir Holan,[2] Richard J. Cornall[3] and John V. Forrester[1]

1 Section of Immunology and Infection, Division of Applied Medicine, University of Aberdeen, Aberdeen, UK
2 Institute of Molecular Genetics, Laboratory of Transplantation Immunology, Prague, Czech Republic
3 Nuffield Department of Medicine, University of Oxford, Oxford, UK

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Correspondence
Professor John V. Forrester, Section of Immunology and Infection (Ocular Immunology), Division of Applied Medicine, School of Medicine and Dentistry, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen AB25 2ZD, UK.
Tel.: 44 1224 437505;
fax: 44 1224 437506;
e-mail: j.forrester@abdn.ac.uk

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Summary

Models of high-risk corneal graft rejection involve neovascularization induced via innate immune responses, e.g., suture-mediated trauma. We describe a model of high-risk corneal graft rejection using corneal graft donor-recipient pairing based on a single-antigen disparity. Donor corneas from transgenic mice on B10.BR (H-2^k) background, in which hen-egg lysozyme (HEL) as a membrane-bound antigen (mHEL) was expressed under the major histocompatibility complex (MHC) Class I promoter (KLK-mHEL, H-2^k), were transplanted into wild type B10.BR recipient mice. Unmanipulated wild type recipient mice rejected KLK-mHEL grafts (39%) slowly over 50–60 days. Graft rejection incidence was maximized (100%) and tempo accelerated (27 days) by priming with HEL-pulsed syngeneic dendritic cells and less so by increasing T-cell precursor frequency. Rejection also reached maximum levels (100%) and tempo (3–8 days) when mice which had rejected a first graft ('rejectors') were regrafted, and was associated with induction of HEL-specific memory T cells. In contrast, 'acceptors' rejected a second graft at rates and tempo similar to naïve mice. These data reveal the importance of (i) donor MHC antigens as alloantigens for indirect recognition, (ii) alloantigen-specific memory in high-risk graft rejection involving regrafts, and (iii) suggest a role for tissue matching in human corneal graft to avoid sensitization to donor MHC antigens.

Introduction

The cornea's reputation for a high rate of allograft acceptance across full histocompatibility barriers is based mainly on data from corneal allografts performed in low-risk settings [1]. Such allografts are rejected at a lower rate and frequency than vascularized corneal grafts under similar conditions [2–4]. Similarly, experience from clinical practice shows that the historically privileged status of corneal grafts, based on their acceptance across major and minor histocompatibility barriers, has come under scrutiny in recent years with the realization that many recipients present a 'high-risk' for rejection [5,6], whereas even 'low-risk' graft recipients, such as keratoconus patients, have a significant long-term failure rate [7]. In addition, controversy continues concerning the value of tissue matching in clinical corneal graft [8–12]. Induction of the host T-cell corneal alloresponse is regulated by various tolerance mechanisms, which underpin ocular immune privilege, such as local immunosuppressive factors [13], systemically induced antigen (Ag)-specific suppressor CD8 T-cell induction [14], local intragraft $CD4^+$ T-regulatory cells (Tregs) [15], and immunological ignorance (antigenic sequestration) [16–19]. Recently, local factors, such as thrombospondin

expression by the tissues [20,21], have assumed greater importance. Conversely, intragraft Tregs have been ascribed a major, if not a critical role, as graft survival in their absence is not possible [15]. This has also been observed in vascularized solid organ grafts and in part relies upon the presence of blood and lymphatic vessels [22]. Furthermore, adoptive transfer of Tregs from mice, which have accepted an allograft are more effective in protecting naïve mice from graft rejection than Tregs from mice which have previously rejected an allograft [23].

The evidence for the suppressive effect of intragraft Tregs in vascularized solid organ grafts is indeed compelling [24,25], but precisely how they function remains unclear. As the donor cornea is avascular and does not contain T cells [26], the presence of Tregs in accepted grafts requires trafficking of such cells into the stroma and indeed small numbers of such cells have been identified in accepted corneal grafts [15]. However, these may be insufficient to have a significant suppressive effect locally and it is possible that much of the effect of Tregs occurs at the site of priming as suggested in the adoptive transfer studies mentioned above [23]. As naïve T cells do not normally patrol the tissues, it can be assumed that allospecific T cells require to be activated prior to entry to the cornea. Accordingly if T-cell activation is suppressed in the secondary lymphoid tissues, then this process is halted before it can be initiated.

These ideas contrast with the older concept of immunological ignorance in which donor Ag is ignored by the host immune system, presumably by being sequestered behind tissue barriers. In one sense, regulation by Tregs and immunological ignorance are complementary mechanisms of immune regulation acting at different poles of the effector T-cell response. Interpretations of previous experiments have been hampered by the relative lack of Ag specificity in tracking T-cell responses. Corneal allografts are rejected by indirect allorecognition, in which host T cells respond predominantly to minor Ags [1,27–31]. Responses to specific minor Ags have been investigated for instance in studies of skin graft rejection [32–34], whereas in the cornea, most work has been performed on H-Y incompatibility [35,36], but not on transgenically expressed novel Ags in the cornea. A previous study using the DO11.10 mouse model system, examined the immune response to ovalbumin injected into corneal allografts, which is more analogous to an intradermal injection of Ag without adjuvant [37]. Similarly, the immunological mechanisms for 'high-risk' graft failure remain unclear, in part because studies in mice are rarely based on Ag-specific models. Most experimental studies of high-risk corneal graft rejection involve activation of the innate immune system through injury and induction of corneal vascularization [38], although regraft models have been described in both mice and rats [39–41]. However, the lack of Ag specificity in such models presents a hindrance to investigating the specific role of T cells.

Taken together, it is clear that although corneal transplantation is today a common procedure in clinical practice, there are still significant numbers of 'high- and low-risk' patients who sooner or later undergo graft rejection and the mechanisms behind this remain obscure. The purpose of the present study is to examine the immune response to a single Ag in a high-risk regraft model, using a transgenic (tg) model in which there is a single Ag disparity between donor and host.

Materials and methods

Animals

Inbred 8–12-week-old wt B10.BR (H-2^k), KLK-mHEL (H-2^k), 3A9 T-cell receptor-transgenic (TCR-tg) (H-2^k), CBA (H-2^k), and BALB/c (H-2^d) mice were provided by the Medical Research Facility at the University of Aberdeen. KLK mice were generated on a C57BL/6 background and were backcrossed to B10.BR mice for >10 generations [42]. 3A9 TCR-tg mice express a rearranged I-Ak restricted TCR specific for the hen-egg lysozyme (HEL) epitope 46–61 [43] and have also been maintained on a pure B10.BR background. Wt B10.BR mice were littermates of the KLK and 3A9 TCR-tg mice. All procedures were performed according to the regulations of the Animal License Act (UK) and approved by ethical committee of Aberdeen University.

Reverse transcriptase polymerase chain reaction for HEL expression in naïve KLK-mHEL cornea

Positive HEL mRNA expression in KLK-mHEL corneal tissue was confirmed using the reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from naïve KLK-mHEL corneal tissue using the RNeasy Micro Kit (Qiagen Ltd, Crawley, UK) as per manufacturer instruction. The following primers were used for amplification: HEL sense 5′-CAA CAC CCA GGC TAC AAA CC-3′, antisense 5′-GTT TCC ATC GCT GAC GAT CT-3′ (196 bp); β-actin sense 5′-CCT TCG TTG CCG GTC CAC-3′, and antisense 5′-ACA CCC TGG TGC CTA GGG-3′ (166 bp). PCR products were loaded on 1.8% Agarose gel in Tris–borate–ethylenediaminetetraacetic acid buffer and products were visualized by ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) and ultraviolet light.

Western blot analysis of HEL protein

Corneal tissue was cut into pieces, placed in lysis buffer containing 5% Protease Inhibitor Cocktail (Sigma-Aldrich) and left overnight on a rotating drum at 4 °C. Single cell suspension of cells from spleen and bone marrow (femur,

tibia) was lysed with lysis buffer containing complete protease and phosphatase inhibitors cocktail (Roche, West Sussex, UK). Samples were after lysis centrifuged at 16 420 ***g*** for 20 min at 4 °C. Total protein estimation was carried out using the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL, USA). LDS Sample buffer and Reducing agent (NuPAGE; Invitrogen, Carlsbad, CA, USA) were added to 10 μg of sample lysate or to 5 ng of lysozyme (Sigma-Aldrich). Samples were then heated in a heating block at 70 °C for 10 min. Following protein separation using 4–12% BisTris Gel electrophoresis with MES Running buffer and Antioxidant (all NuPAGE; Invitrogen), separated proteins were transferred to Nitrocellulose Membrane (Invitrogen) and blocked with triethanolamine-buffered saline containing 0.1% Tween-20 (Sigma-Aldrich) and 5% skim milk at room temperature for 1 h. The membrane was incubated overnight with IgG fraction of antilysozyme rabbit polyclonal antibody (Rockland Immunochemicals, Philadelphia, PA, USA) at 4 °C followed by incubation with secondary swine antirabbit-biotinylated antibody (Dako, Glostrup, Denmark) and Streptavidin/horseradish peroxidase (Dako) both for 1 h at room temperature. Blots were developed using chemiluminescent substrate LumiGLO (Cell Signaling Technology, Boston, MA, USA). Membranes were stripped using mild stripping buffer (Millipore, Billerica, MA, USA), blocked as above and reprobed with mouse GAPDH antibody (Abcam, Cambridge, USA) followed by incubation with secondary rabbit antimouse HRP antibody (Dako). In some KLK-mHEL animals, corneal epithelium was scratched and CpG (Fisher Scientific UK Ltd, Loughborough, UK) were applied to the abraded corneal surface; 24 h postapplication cornea was removed and Western blot (WB) or staining for further confocal microscopy was performed.

Confocal microscopy

Whole corneas were removed after fixing *in situ* with 4% paraformaldehyde and stained as previously described [30]. Primary MHC class I (AbD Serotec, Kidlington, UK) and HEL (Rockland Immunochemicals, Philadelphia, PA, USA) antibodies followed by appropriate biotinylated secondary antibodies (Dako) and fluorescent colors – Streptavidine-Texas Red (Amersham) and Streptavidine-FITC (Vector, Vector Laboratories, Peterborough, UK). For nuclear staining, DAPI (Invitrogen) was used; corneal whole mounts were examined using a confocal microscopy (LSM700; Carl Zeiss Meditec Ltd, Cambridge, UK).

Transplantation and retransplantation

Orthotopic corneal transplantation was performed as described previously [44,45]. In corneal graft retransplantation experiments, a second graft was sutured in place of the first graft using the same technique [39].

Presensitization with HEL Ag

Wt B10.BR mice were subcutaneously (s.c.) immunized with HEL peptide (Sigma-Aldrich) either diluted in PBS or emulsified 1:1 in complete Freund's adjuvant H37Ra (CFA; Difco, Sparks, MD, USA) (×3@10 μg/mouse/injection on alternate days followed with 7 days gap before corneal transplantation).

Adoptive transfer of T lymphocytes

One day before KLK-mHEL corneal graft transplantation wt B10.BR recipients received an intravenous injection via the tail vein of a single cell suspension, prepared from pooled lymph nodes, of HEL-specific T cells from 3A9 TCR-tg mice (1×10^6 $CD4^+1G12^+$ T cells). The 1G12 anticlonotype antibody was prepared from hybridoma cell line supernatant (a gift from Richard J. Cornall, University of Oxford, UK [46]) and commercially purified (AbD Serotec, Paisley, Scotland). In flow cytometry studies, the 1G12 antibody was detected by fluorochrome-conjugated rat-antimouse IgG1 antibody (BD Biosciences, Franklin Lakes, NJ, USA).

Generation and characterization of bone marrow-derived DC

Bone marrow-derived DC (BMDC) (B10.BR) were cultured (5×10^5/ml) in 10 ml complete RPMI-1640 medium with rGM-CSF (20 ng/ml; R&D Systems, Abingdon, UK) for 6 days. Cells were then replated in fresh medium containing rGM-CSF (10 ng/ml) and pulsed with HEL peptide (20 μg/ml; Grade I, Sigma-Aldrich) ± LPS (1 μg/ml; Sigma-Aldrich) on day 7. Twenty-four hour later, both adherent and nonadherent cells from all three groups (no Ag, HEL, and LPS + HEL) were collected, washed free from residual HEL peptide, and resuspended in PBS. The cells were then (s.c.) injected to the right side of the nape into naïve wt B10.BR mice (×3 doses, 2×10^6 cells per dose, 2 days apart) followed with 7 days gap before corneal transplantation.

Samples of BMDC, the mix of nonadherent and adherent cells, were taken for FACS analysis of surface markers using a BD LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Fluorochrom-conjugated antibodies (BD Biosciences) against CD11b, CD11c, CD40, CD86, and MHC class II were used. Supernatants from cultured DC were collected on day 8 and evaluated for cytokine levels (IL-6, IL-10, IL-12p70, and TNF-α) using

the multiplex Cytometric Bead Array (CBA) assay (BD Biosciences).

In vitro proliferation assay using CFSE

Lymph node (LN) and spleen (SPL) cells from naive wt B10.BR mice and mice after KLK-mHEL corneal retransplantation were stained with 2.5 μM CFSE (Molecular Probes, Carlsbad, CA, USA) according to manufacturer's instructions, washed, and cultured in 96-well round-bottom plates at 3×10^5 cells/200 μl/well with or without HEL peptide (30 μg/ml; Sigma-Aldrich). After 84 h, cells were collected for flow cytometric analysis of proliferating T cells by dilution of CFSE label. A total of 250 000 events per sample were collected using a BD LSRII (BD Biosciences). Data were analyzed using the FlowJo software (Tree Star Inc., Ashland, OR, USA). The final percentage of proliferating cells was calculated as percentage of $CD3^+CD4^+$ proliferating cells after HEL peptide stimulation minus percentage of $CD3^+CD4^+$ proliferating cells from the same tissue without *in vitro* stimulation.

Statistics

For statistical analysis, we used Log-rank test for comparing survival curves and the two-tailed unpaired *t*-test for comparing *in vitro* proliferative responses (GraphPad Prism Software Inc., La Jolla, CA, USA). A *P*-value <0.05 was considered significant.

Results

A single minor-H Ag disparity is sufficient to trigger corneal graft rejection

In this study, we used donor corneal grafts from tg mice on a B10.BR background expressing mHEL under the control of the MHC class I promoter (KLK-mHEL mice). As MHC Class I expression is not strong in normal corneas [47], we wished initially to determine whether HEL Ag was expressed in KLK-mHEL corneal tissue. Positive HEL mRNA expression in KLK corneal tissue was confirmed using RT-PCR (Fig. 1a). However, HEL protein expression was not detected in normal KLK-mHEL cornea by WB, and was only faintly positive in some samples after induction of short-term inflammation in corneas, which had been abraded and exposed to CpG [48] (Fig. 1c). In contrast, splenocytes and bone marrow cells from KLK-mHEL mice consistently expressed high levels of HEL, which was absent from wt mice (Fig. 1b). Confocal microscopy of KLK-mHEL corneas revealed double positive HEL (green)/MHC class I (red) cells only in KLK-mHEL mice after scratching corneal surface and applying CpG; whereas nonscratched KLK-mHEL mice and wt B10.BR mice (both nonscratched and scratched) were negative for HEL (Fig. 1d). It was concluded that normal KLK-mHEL cornea does not express significant levels of HEL Ag consistent with its low constitutive expression of MHC Ag (Fig. 1d), that detection of HEL Ag in mHEL corneas using PCR (Fig. 1a) is likely related to its low content of MHC-expressing myeloid cells [49].

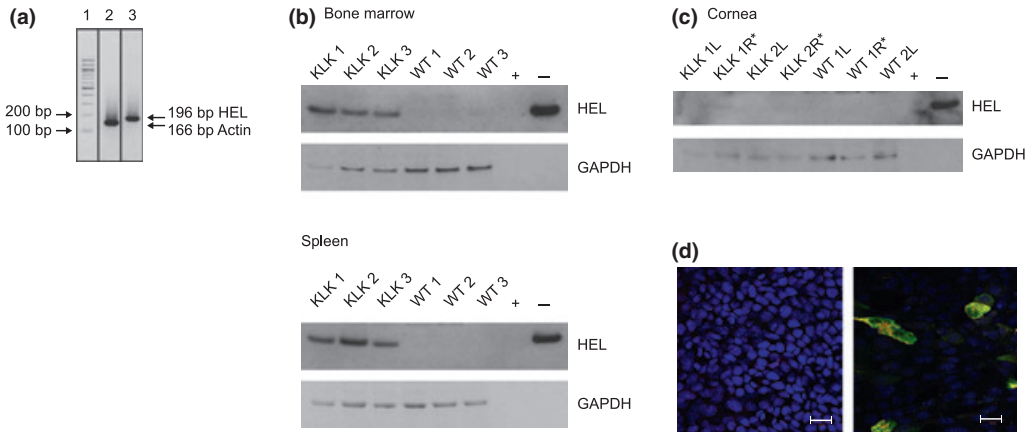

Figure 1 HEL is expressed in KLK-mHEL mice. (a) HEL mRNA expression in naïve KLK-mHEL cornea as detected using RT-PCR. Molecular weight ladder (1), mRNA expression of β-actin (2), and HEL (3) in one KLK-mHEL naïve cornea. (b) HEL protein is present in the bone marrow and spleen from KLK-mHEL mice, but not wt B10.BR mice. Bone marrow and spleen protein extracts from three individual KLK-mHEL mice and three wt B10.BR control mice were analyzed using Western blotting. As a negative control protein lysis buffer was used (–), whereas 5 ng of HEL peptide was used as a positive control (+). (c) Western blotting from KLK-mHEL cornea – HEL expression not detectable in naïve animals and only in some animals with corneal abrasion and CpG application (*). (d) Confocal microscopy, HEL staining (green) only present on scratched corneal surface in KLK-mHEL mouse (right), whereas wt B10.BR mice (left) do not express HEL on any corneal cells normally (data not shown) or when injured (abraded). Costaining with DAPI (blue) and MHC class I (red). Scale bar – 20 μm.

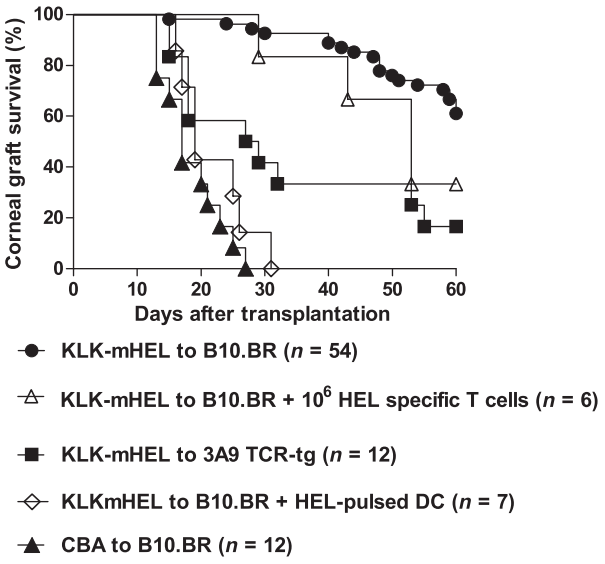

Figure 2 Increased initial T-cell precursor frequency and repeated presensitization of corneal graft recipients with syngeneic HEL-pulsed DC accelerates rejection of HEL Ag incompatible corneal grafts. The curves show survival of single minor-H Ag disparate KLK-mHEL corneal grafts in wt B10.BR mice (full ring); 3A9 TCR-tg mice (full square); wt B10.BR mice adoptively transferred 1 day before grafting with HEL-specific T cells from 3A9 TCR-tg mice (1×10^6 cells, empty triangle); wt B10.BR mice that received three doses (2×10^6 cells per dose) of HEL-peptide pulsed syngeneic DC injected s.c. at 2-day intervals into the right side of the nape followed with 7 days gap before corneal transplantation (empty rhombus); and survival of CBA (H-2^k) corneal grafts expressing multiple minor-H Ags transplanted to wt B10.BR mice (full triangle). Transgenic KLK-mHEL (H-2^k) corneal grafts are created on a B10.BR (H-2^k) background and express membrane-bound HEL Ag.

Transplantation of KLK-mHEL donor cornea to wt B10.BR recipients led to graft rejection in 39% of mice (Fig. 2) albeit slowly over a 60-day period (median is >60 days). The rate of graft rejection was greatly accelerated by priming wt B10.BR recipients with repeated s.c. inoculation ($\times 3$ doses, 2×10^6 cells per dose, 2 days apart) of HEL-pulsed syngeneic DC [MHC Class II^{lo} and $CD86^{lo}$ compared to LPS-activated, TNF-α secreting DC (data not shown)] and the incidence of rejection increased to 100% [mean survival time and standard deviation (MST $\pm$ SD) 21.9 $\pm$ 5.6 days, Fig. 2]. Wt B10.BR controls inoculated with HEL-unpulsed DC rejected donor KLK-mHEL corneal grafts at a similar tempo and frequency to naïve wt B10.BR recipients (data not shown). Interestingly, priming with soluble HEL peptide did not alter graft rejection rate or incidence even after multiple (up to three) inoculations (data not shown), whereas priming with HEL peptides emulsified in CFA had an accelerating effect on graft rejection, although less so than with HEL-pulsed syngeneic DC (median is 37 days; 80% rejected grafts by day 60, $P < 0.01$ vs. naïve wt B10.BR recipients; data not shown). The 100% of fully minor-H Ag disparate grafts (CBA to naïve wt B10.BR recipients) were rejected by day 27 [MST $\pm$ SD 18.4 $\pm$ 4.8 days ($P < 0.0001$ vs. single HEL Ag disparity), Fig. 2].

Increasing the precursor frequency of host Ag-specific T cells either by adoptive transfer of HEL-specific T cells from 3A9 TCR-tg mice (1×10^6 cells) (median is 53 days; 67% rejected; $P > 0.05$ vs. naïve wt B10.BR recipients; Fig. 2), or by transplantation of KLK-mHEL donor corneas to 3A9 TCR-tg recipients (median is 28 days; 83% rejected; $P < 0.0001$ vs. naïve wt B10.BR recipients; Fig. 2), was also less effective in maximizing the corneal graft rejection rate or incidence.

Previous corneal graft rejection in a single disparity combination is a strong stimulus for accelerated second graft rejection and is associated with significant induction of effector/memory T cells

The above data indicated that a single Ag disparity was sufficient, although weakly so, to induce corneal graft rejection, but that the strength of this response could be increased greatly either by priming *via* the innate immune system (CFA or HEL-pulsed syngeneic DC) or by increasing the Ag-specific T-cell precursor frequency. However, whether graft rejection induced by a single antigenic disparity is a sufficient stimulus to prime for accelerated rejection of a second graft, as occurs frequently in clinical practice, remains unknown.

Here, we show in a single disparity corneal graft model that presensitization with a previous corneal graft containing the transgenically expressed HEL Ag induces a rapid rejection of a second HEL-expressing corneal graft, depending on whether the first graft was accepted or rejected. As shown above, 39% of wt B10.BR mice rejected KLK-mHEL corneal grafts with median >60 days (Figs 2 and 3a). Some of these mice received a same-eye second fresh KLK-mHEL corneal graft. First graft 'acceptors' (i.e., mice whose first graft had not been rejected) received their second graft no earlier than 100 days after the first graft and rejected their second graft with an incidence of 43% and median >60 days, i.e., at an incidence and tempo not different from the first graft recipients ($P > 0.05$; $n = 7$; Fig. 3a). In contrast, mice which had previously rejected a first KLK-mHEL graft (first graft becoming opaque during first 15–80 days, herein termed 'rejectors') rapidly and strongly rejected their second graft (incidence 100%; MST $\pm$ SD 5.9 $\pm$ 1.5 days; $n = 7$) (Fig. 3a).

Ag-specific memory responses in 'acceptors' and 'rejectors' were compared 100 days after the second corneal transplantation by collecting LN and SPL cells and assessing T-lymphocyte proliferative responses to HEL peptide *in vitro*. Ag-specific T-cell proliferative responses could

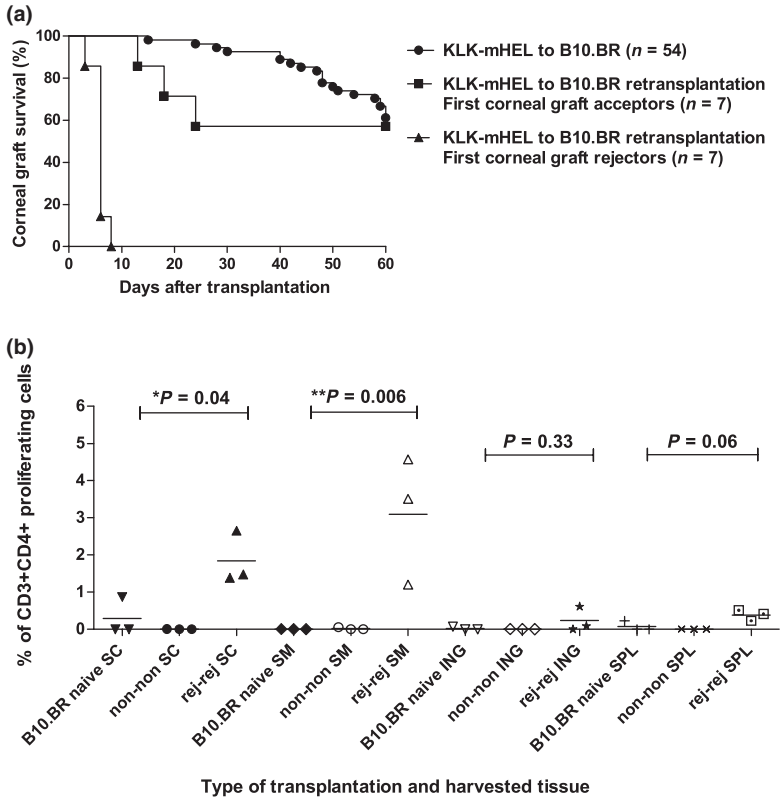

Figure 3 Strong and accelerated rejection of a second KLK-mHEL corneal graft occurs only in recipients that rejected their first KLK-mHEL corneal graft and correlates with the presence of an Ag-specific T-cell memory response. (a) 100 days after receiving a KLK-mHEL corneal graft, wt B10.BR mice were divided into two groups, first corneal graft 'acceptors' (full square) and first corneal graft 'rejectors' (full triangle). Both sets of mice received the second ipsilateral KLK-mHEL corneal graft and graft survival was evaluated as shown in the survival curves. First graft rejectors experienced markedly accelerated rejection of the second graft, whereas first graft acceptors behaved similar to naïve controls receiving only graft, i.e., approximately 40% accepted the second graft. Group of wt B10.BR mice that received only first KLK-mHEL corneal graft (full circle). (b) Superficial cervical (SC), submandibular (SM) and inguinal (ING) LN, and spleen (SPL) from wt B10.BR recipients that either accepted (two time nonrejectors: designated non-non) or rejected their first and their second KLK-mHEL corneal graft (two time rejectors: designated rej-rej) were collected 100 days after the second corneal transplantation. Single-cell suspensions from collected LN and SPL cells were then tested for proliferation responses to HEL peptide *in vitro* using CFSE labeling and expressed as percentage of $CD3^+CD4^+$ cells, i.e., the final percentage of proliferating cells was calculated as percentage of $CD3^+CD4^+$ proliferating cells after HEL peptide stimulation minus percentage of $CD3^+CD4^+$ proliferating cells from the same tissue without *in vitro* stimulation. For statistical analysis was used unpaired *t*-test (two-tail *P*-value; GraphPad Prism Software Inc.) and the data were before analysis arcsine transformed. *P*-value <0.05 was considered significant.

only be detected in the site-specific submandibular (SM) eye-draining LN [39] of 'rejector' mice, i.e., in mice who had rejected their first graft and had an accelerated second corneal graft rejection (rej-rej group, Fig. 3b).

Discussion

Here, we describe a high-risk Ag-specific model of corneal graft rejection in a tg mouse expressing the foreign Ag HEL in the donor cornea under the promoter for MHC Class I (donor mice: KLK-mHEL). Interestingly, although HEL could be detected using PCR in the donor cornea (Fig.1a), it could not be detected reliably at the protein level, indicating that levels of HEL in KLK-mHEL were very low. Membrane-bound HEL in the tg mouse used in this experiment, is expressed using a promoter, which preferentially permits expression in strongly MHC-expressing myeloid cells from the bone marrow [42], and as there are only around 3,000 such cells in the mouse cornea [26], the level of HEL available for Ag recognition in the cornea is low particularly, as overall expression of MHC Class I expression in normal cornea is not high [47].

Despite low corneal levels of HEL protein, donor KLK-mHEL grafts were rejected, albeit slowly and at an incidence of only approximately 40% for primary grafts. This is likely to correlate with the level of MHC Ag expressed by the 'passenger leukocytes' in the donor cornea which, in mice which accepted their grafts ('acceptors'), was probably too low to generate an Ag-specific T-cell response. In contrast, mice which rejected their

primary grafts ('rejectors', defined here as rejection on opacity grading by day 100 postgraft) must have had a sufficient content of donor MHC Class I + HEL + myeloid cells to provide Ag for generation of an indirect host APC-induced T-cell response. Interestingly, the tempo of rejection of the first graft was greatly accelerated to that of fully minor-H Ag disparate grafts (27 days) and the incidence increased to levels of 'high-risk' graft recipients (100%) [45] by priming the mice with HEL-peptide pulsed syngeneic DC (Fig. 2). Priming with HEL in CFA or by increasing the T-cell precursor frequency was somewhat less effective, but still highly significant compared to unmanipulated mice, whereas priming with soluble HEL alone was ineffective.

The most effective procedure for conversion of mice from 'low-risk' to 'high-risk' was, however, to regraft 'rejector' mice with a second KLK-mHEL graft. This induced a very rapid (3–8 days) acceleration, and 100% incidence, of graft rejection. Interestingly, 'rejector' mice showed strongly proliferative HEL-specific T-cell responses after the second graft; in addition, this was restricted to the site-specific SM eye-draining LN [39] suggesting that not only were memory T cells induced by the first graft during the process of rejection, but also that they were located specifically to the LN site draining the eye, i.e., they were not generally distributed.

In contrast, to 'rejector' mice, 'acceptor' mice appeared to respond to a second graft in the same manner as a first graft, i.e., with a slow tempo and an incidence of 43%, which was not significantly different from the kinetics of graft rejection in unmanipulated naïve mice. This result can be interpreted in several ways. At first glance, it would appear that the 'acceptor' mice were 'unaware' or had 'ignored' the first graft, and had failed to develop a T-cell effector memory response to HEL Ag. Immunological ignorance appears to play a role when only the corneal endothelium is the allogeneic target, but in full thickness corneal grafts, immune responses to corneal alloantigens present in the epithelium and stroma can readily be demonstrated [16] and might offer an explanation for the success of procedures, such as Descemet's membrane endothelial keratoplasty (DMEK), where there is a lower incidence of rejection and the possibility of alloantigen restricted to endothelial cells being ignored from immune system might be greater than with a penetrating keratoplasty.

However, it is unlikely that Ags in the donor cornea are not 'seen' by the immune system, as the procedure of corneal graft alone causes breakdown of the blood ocular barrier [50,51] and even syngeneic grafts, if only due to the presence of sutures, cause some degree of corneal vascularization [52]. In addition, it is known that soluble and cell-associated Ags traffic within minutes or hours to the eye-draining LN [30]. A more likely possibility to explain the 'acceptor' recipient response to a second graft is that the first graft has been accepted because of immunoregulatory mechanisms. Recent studies indeed have shown that 'acceptor' mice induce high levels of Tregs, which when adoptively transferred to naïve mice can promote graft acceptance in the adoptive recipients and the response correlates with the level of the transcription factor Foxp3 in the adoptive Tregs [23,53,54]. In contrast, Tregs from 'rejector' mice do not have this effect. In addition, accepted grafts seem to be populated with Tregs, although it remains unclear whether they are functional within the graft itself. Data from accepted skin grafts would suggest that they are functional locally [22,55]. As naïve T cells do not normally enter the tissues, but usually require activation to do so, it would seem to be more efficient to prevent activation and, thereby homing of effector T cells, in the secondary lymphoid tissue rather than at the site of attack. Perhaps, both mechanisms are active.

What determines whether a recipient of a first donor graft becomes a 'rejector' or 'acceptor'? A possibility is the level of Ag-specific priming, which occurs during the initial procedure and whether soluble Ag versus cell-associated Ag predominates in trafficking from the tissue. Soluble Ag is known to be more likely to induce tolerance while cell-associated particulate Ag, particularly that endocytosed and presented by activated Ag presenting cells, is more likely to induce an immune response and thus rejection [56,57]. This has been confirmed here using HEL-pulsed syngeneic DC, which were found to be the most powerful method for priming for rejection. The present model thus provides a means to investigate these mechanisms.

Whatever the precise immunological mechanism of rejection of 'high-risk' versus 'low-risk' grafts, the results of this study also provide some insight into the question of tissue matching for corneal grafts. While the question of matching for the initial graft remains open, if there is a perceived risk that the first graft may fail (e.g., in high risk, previously infected corneas), then typing the initial donor and second and subsequent donors would seem highly appropriate, as it would be important to avoid regrafting with a second donor whose haplotype cross-matched with the first. The current study indicates that the initial rejected graft will have induced allospecific memory T cells in the recipient, which will greatly increase risk of failure of the second graft, if these memory cells are exposed to the same alloantigen. This is probably the most important reason for tissue typing human corneal grafts.

Authorship

JVF, AV, and LK: participated in research design. JVF and AV: participated in the writing of the manuscript. IPK and LK: performed additional research. AV and LK: partici-

pated in the performance of the research. JVF and AV: participated in data analysis. RJC, VH, and LK: participated in critical review of the manuscript.

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