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Cross Presentation of Antigen on MHC Class II via the Draining Lymph Node after Corneal Transplantation in Mice

Lucia Kuffová,* Magdaléna Netuková,* Linda Duncan,* Andrew Porter,† Brigitta Stockinger,‡ and John V. Forrester²*

We investigated Ag trafficking from the cornea and T effector cell activation in secondary lymphoid tissue after corneal transplantation. In preliminary experiments, the central cornea was shown to contain a population of CD45⁺, CD11b⁺, CD11c⁻ cells, with a few MHC class II⁺ cells, and F4/80⁺ cells. However, MHC class II⁺ passenger leukocytes in donor cornea after allografting did not traffic to the draining lymph node. Instead, Ag (plasmid) delivered to the eye via the donor cornea during allograft was detected in host CD11c⁺ and F4/80⁺ APC in the draining lymph nodes and spleen. The earliest detection of APC-associated Ag was at 6 h in the draining lymph node and 24 h in the spleen. After 48 h Ag was not detected in the draining lymph node but was still present in the spleen. Ag applied to the donor corneal epithelium before allografting induced Ag-specific T cell activation and expansion in the draining lymph node with a peak response at 4–6 days, indicating that cross-presentation of Ag had occurred. We conclude therefore, that Ag is transported from the donor cornea within host APC and that this event occurs within hours after grafting. Ag is cross-presented to host CD4⁺ T cells on MHC class II and leads to the activation of Ag-specific effector T cells and clonal expansion in the draining lymph node. *The Journal of Immunology, 2008, 180: 1353–1361.

Corneal graft rejection predominantly occurs via the indirect route of alloantigen presentation (1–3) because passenger leukocytes are thought to be absent from healthy donor cornea (4). In addition, only CD4⁺ T cells contribute to the rejection process despite the presence of allospecific CD8⁺ T cells in mice who reject fully mismatched corneal grafts (1, 5). Recent studies in a murine corneal graft model have suggested that the anterior corneal stroma contains a population of CD14⁺CD11c⁻ MHC class II⁺ leukocytes that are induced to express MHC class II after trafficking to the host draining lymph node, but the precise nature of these cells is not clear and their role in graft rejection is unexplored (6). Furthermore, data from others have contested the presence of significant numbers of resident CD11c⁻ dendritic cells in the cornea and instead report that the population of CD45⁺ resident leukocytes in the central cornea (i.e., that part of the cornea that constitutes the donor graft) is exclusively one of macrophages and not of dendritic cells (7, 8). This is similar to the MHC class II⁻, CD45R0⁻/⁺ resident leukocyte cell population in the retina that lacks the phenotype of dendritic cells (9, 10), although recently a restricted population of MHC class II⁺, 33DI⁺ cells has been described at the retinal periphery (11).

Although the submandibular draining lymph node (DLN) is clearly involved in T cell priming for eye-derived Ags (12, 13), direct evidence for trafficking of specific Ag-loaded APC from the cornea to the DLN or spleen has not as yet been shown. In contrast, soluble Ag released into the ocular chambers finds its way to several local cervical and distant mesenteric lymph nodes and the spleen (14) by both the lymphatic and vascular routes (15). In addition, it has been shown that the induction of CD8⁺ T suppressor cells involved in immune deviation after the inoculation of Ag into the eye occurs in the spleen but not in the DLN (16, 17).

Studies of Ag trafficking from the eye to secondary lymphoid tissues are usually performed after the inoculation of soluble Ag intraocularly (12, 14, 18). We wished to determine how cell-associated Ag (such as alloantigen) is transported to the draining lymphoid tissues and in particular to seek evidence for the cross-presentation of donor corneal Ag. Cross-presentation of Ag is usually considered in terms of the presentation of exogenous Ags by a nonclassical, alternative Ag processing and priming route via MHC class I (19), but it has also been described in the context of Ag presentation on MHC class II, for instance after the uptake of apoptotic cells (20). We thus applied Ag to the donor cornea before grafting in the form of a DNA plasmid comprising a construct encoding a portion of the mouse C5' protein that becomes incorporated and transcribed in cells after uptake but is not secreted because the transcribed protein lacks a secretory component (21). Transplantation of the plasmid-containing cornea, therefore, would require donor cell-associated Ag to be cross-presented to host T cells after the phagocytosis of donor cells by host APC if T cell activation is to occur. We find that Ag is transported from the eye under all three experimental conditions in CD11c⁺ APC and can be found in the secondary lymphoid tissues in both CD11c⁺ dendritic cells and F4/80⁺ macrophages of host origin. In addition, we

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Abbreviations used in this paper: DLN, draining lymph node; eGFP, enhanced GFP; Tg, transgenic.

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show that the prior application of Ag to the donor cornea induces early activation and proliferation of Ag-specific T cells in the DLN. These data support a mechanism for the cross-presentation of donor Ag on MHC class II by host APC as an initiating event during the process of corneal graft rejection.

Materials and Methods

Mice

Animals were provided by the National Institute for Medical Research (London, U.K.) and by Harlan Olac. All animals were housed according to the guidelines described in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Vision and Ophthalmic Research and according to the Animal License Act (U.K.). A18.C5-TCR transgenic (Tg):Rag-1-/- (A18 Rag-1-/-), A1-C5-TCR Tg:Rag-1-/- bred onto Thy.1 background (A1 Rag-1-/-), and CD4.A18: C5-TCR Tg:Rag-1-/- (CD4.A18 Rag-1-/-) were maintained under specific pathogen-free conditions at the Medical Research Facility, University of Aberdeen, Aberdeen, U.K. A18 Rag-1-/-, A1 Rag-1-/-, and CD4.A18 Rag-1-/- mice recognize epitope 106–121 of the serum protein C5 in the context of H2-Eb by using a receptor composed of V8.3 and Vn.11.1a as described previously (22). C5/BL/J10 (H2b) and C5/BL/J6 (H2b) mice and the transgenic strain C5/BL/J6-Tg(act-EGFP)OsbY01 expressing enhanced GFP (eGFP) on C57BL/6 background (H2b), all aged 6–8 wk, were used as corneal donors. A18 Rag-1-/- (H2b), Al Rag-1-/- (H2b), and BALB/c (H2b) mice of the same age and sex were used as corneal graft recipients. The controls, nongrafted A18 Rag-1-/-, Al Rag-1-/-, and CD4.A18 Rag-1-/- (used as donor T cells for transfer experiment) mice, were also sex and age matched.

Corneal graft procedure

The mouse keratoplasty technique was adopted from previously published procedures (23) and modified by the present authors. Briefly, a 2-mm trephine donor corneal button was transplanted to a 1.5-mm host bed and sutured in place with a continuous 11-0 suture (Ethicon) that was retained for the duration of the experiment. The corneas were examined three times per week under the operating microscope for signs of rejection, namely increased corneal opacity and thickness (24). The standard procedure involved strain pairs as follows: C57BL/6 or C57BL/10 (H2b) donors and A18 Rag-1-/-, A1 Rag-1-/-, and CD4.A18 Rag-1-/- mice as recipients. These strain pairs were noncompatible across both MHC and non-MHC loci.

Construction of DNA vaccine and plasmid purification

The C5′ and eGFP DNA plasmids were cloned under the human CMV promoter (21). Ligated plasmids were transfected into Escherichia coli bacterial DH5α, and the plasmids were purified as described by the manufacturer (Qiagen).

Vaccination procedure

To track Ag, the DNA vaccines (10 μg/mouse in a 5-μl volume) were applied as droplets to the donor cornea (B10 group) after mild epithelial abrasion. The plasmid was applied for 15 min, after which the cornea was thoroughly washed with saline solution. Antibiotic ointment (Polymax; Dominic Pharma) was applied to the surface of the cornea. After 24 h the previously abraded cornea was grafted to the recipient (BALB/c or A18 Rag-1-/-) and samples were taken at various times thereafter to detect the vaccine (see below). Control experiments were also performed by: 1) applying vaccine directly into the anterior chamber of mice at the end of grafting (AC group) instead of prior application to the donor cornea; and 2) applying vaccine to the lightly abraded cornea of normal, nongrafted mice. At various time points (15 min, 2 h, 6 h, 16 h, 24 h, 48 h, 4 days, 6 days, 9 days, 12 days, 24 days, 36 days, and 60 days) after vaccination and/or after corneal transplantation, the submarginal draining lymph nodes and the spleen were removed for further analysis (see Results).

CFSE labeling

Spleen and lymph nodes of CD4 A18 Rag-1-/- mice after isolation were used as the source of donor CD3+ T cells in transfer experiments. Cell division was assessed by monitoring dilution CFSE labeling (Molecular Probes) using flow cytometry. Cells were resuspended in PBS at a concentration of 10^6 cells/ml and incubated with CFSE at a final concentration of 5 μM for 10 min at 37°C, followed by two washes in RPMI 1640 medium containing 10% FCS. Labeled cells (1 × 10^6 CD3+ T cells/an- mal) were i.v. injected into A1 Rag-1-/- recipients before corneal transplantation procedure.

Tissue retrieval and preparation

Mice were killed with an overdose of CO2, and the eyes were enucleated. For cell tracking studies, DLN and spleen tissues were retrieved and immersed in OCT medium (Miles) and snap frozen in liquid N2-cooled isopentane. They were stored at −80°C until used. Multiple sections were taken and staining in duplicate was performed for each Ab tested.

Preparation of corneal flat mounts

Flat mounts of intact corneas (to demonstrate epithelial leukocyte populations) and deep epithelialized corneas (to demonstrate stromal leukocyte populations because the penetration of Abs through the epithelium into the stroma in intact corneas is variable) were performed. Corneas were prepared both by acetone fixation and light paraformaldehyde fixation as described previously (6, 7). In our hands acetone fixation, even when accompanied with extensive blocking procedures including Fc and strain-specific serum blocking, produced widespread nonspecific staining of corneal stromal cellular elements with secondary Ab combinations. In contrast, light paraformaldehyde fixation as detailed below allowed reproducible specific staining for each of the Abs studied.

Detection of epithelial leukocytes in intact corneas

Mice were euthanized and corneas were immediately fixed in situ with 4% paraformaldehyde in PBS for 20–30 min. The corneas were then excised and fixed for a further 1 h with the same fixative. The corneas were washed five times in PBS and preblocked with strain-specific serum. Fc block (rat anti-mouse CD16/CD32) was used for detecting CD11c Ag with hamster anti-mouse CD11c and also when biotinylated anti-mouse Abs were used (see below). After blocking, the corneas were incubated overnight at 4°C with 100 μl of primary Ab diluted in PBS. After vigorous washing, the corneas were incubated with fluorescently labeled streptavidin for 1 h at room temperature when the primary Ab was biotinylated. When the corneas were incubated with purified nonconjugated primary Ab, their further incubation was with a biotinylated secondary Ab for 2 h at room temperature. Between incubations, the corneas were washed five times with PBS and finally incubated with fluorescently labeled streptavidin for 1 h. They were then mounted in Vectashield in 18 × 18-mm wells prepared with nail polish on glass slides and covered with coverslips.

Detection of corneal stromal leukocytes in deep epithelialized corneas

Mice were euthanized and the corneas excised. The epithelium was removed after incubating the corneas in PBS containing 20 mM EDTA for 20 min at 37°C. The corneas were then fixed for 30 min at 4°C in 1% paraformaldehyde in PBS. After fixation, the samples were washed in PBS and blocked for 20 min at 37°C with strain-specific serum and with Fc-block diluted in PBS-BGEN (PBS containing 3% BSA, 0.25% gelatin, 5 mM EDTA, and 0.025% Nonidet-P40 (nonionic detergent)) as described above. After blocking, the corneas were incubated overnight at 4°C with 100 μl of primary Ab diluted in PBS-BGEN. The tissue was then washed five times in PBS as described above. Corneas that were incubated with biotinylated primary Ab were further incubated with 100 μl of fluorescently labeled streptavidin diluted in PBS-BGEN for 1 h at room temperature. Corneas incubated with the purified primary Ab were then incubated with the biotinylated secondary Ab diluted in PBS-BGEN for 2 h at room temperature, washed five times, and incubated with fluorescently labeled streptavidin diluted in PBS-BGEN for 1 h. This was followed by five washes in PBS and fixation in 1% paraformaldehyde for 30 min at 4°C. Corneas were then washed and mounted in Vectashield.

Antibodies

The primary Abs used for flow cytometry and immunohistochemistry were obtained as follows: CD3-allophycocyanin (pan T cells) (clone 17A2); CD4-FITC (helper T cells) (clone L3T4); CD8-PE (cytotoxic T cells) (clone 53.58); CD11c-FITC (DC) (clone HL3); CD11c-PE (DC) (clone HL3); CD44-PE (clone IM7); Ly5.2-PerCP (clone 104); CD62L-PE (L-selectin) (clone 2B4); I-Ad-PE (clone AMS-32.1); and I-Ab-FITC (clone AF6-120.1). CD40-PE (B cells, bone-marrow DC, follicular DC, and thymic epithelium) (clone 53-5.8)); CD11c-PE (DC) (clone HL3); CD11c-FITC (DC) (clone HL3); CD44-PE (clone IM7); Ly5.2-PerCP (clone 104); CD62L-PE (L-selectin) (clone MEL-17); CD69-PE (very early activation Ag) (clone H1.2F3); CD40-PE (B cells, bone-marrow DC, follicular DC, and thymic epithelium) (clone 3/23); CD86-PE (macrophages, DC, low levels T and B cells) (clone 2C3); CD11b-PE (clone M1/70); Fc block (CD16/CD32) (clone 2.4G2); I-A^d-PE (clone AMS-32.1); and I-A^k-FITC (clone AF6–120.1) all from BD Pharmingen. The mAbs F4/80 (clone C1-A3.1) and CD45 (clone YW6.2.3) were from Serotec.
Cell sorting

Positive selection of lymph node dendritic cells was conducted by magnetic cell sorting on a MACS RS+ separation column (Miltenyi Biotec) according to the manufacturer’s instructions. Lymphoid tissues were digested with a mixture of 0.1% DNase I (Sigma-Aldrich) and 1.0 mg/ml collagenase (CLS4; Worthington Biochemical) at 37°C for 1 h. For positive selection of DC, cells were stained with MACS CD11c microbeads. Positively selected CD11c DCs were collected from the separation column for further analysis.

Immunohistology

Immunohistochemistry was performed on CD11c+ sorted dendritic cell populations prepared as cytospins and on frozen sections of spleen, transplanted eyes, and submandibular DLN. The cell cytospins were stained with the primary mAb to CD11c (Serotec) followed by secondary biotinylated rabbit anti-rat mAb (DakoCytomation) and streptavidin Fast Red (Amersham Biosciences). The sections were stained with CD11c (Serotec) and F4/80 mAb (Serotec) using the additional secondary and tertiary labels as described above. Sections and cytospins were viewed under a confocal microscope.

For confocal whole mounts, staining procedures were performed with the following Abs: hamster anti-mouse CD11c (clone HL3) (BD Pharmingen); rat anti-mouse CD16/CD32 (clone 2.4G2) (BD Pharmingen); rat anti-mouse CD45 (clone 30-F11) (BD Pharmingen); rat anti-mouse F4/80 (clone CL-A:3-1) (Serotec); rat anti-mouse Ly-6C (clone Gr-1) and Ly-6C (clone RB6-8C5) (BD Pharmingen); biotinylated rat anti-mouse CD11b (clone M1/70) (BD Pharmingen); biotinylated mouse anti-collagenase (clone AF6-120.1) (BD Pharmingen); and biotinylated mouse anti-mouse I-Ax (clone AMS-32.1) (BD Pharmingen). The secondary Abs used were biotinylated goat anti-hamster IgG (clone G700-204 G94-56) (BD Pharmingen), biotinylated rabbit anti-rat immunoglobulins (DakoCytomation), and streptavidin conjugated with rhodamine (TRTC) and with FITC purchased from Jackson ImmunoResearch Laboratories.

Confocal microscopy

Cell cytospins and sections were viewed with a Bio-Rad MRC 1024 confocal microscope. Cells stained with GFP and/or Texas Red were scanned using a krypton/argon laser (all lines) with emission maximum at 520 nm and 590 nm, respectively, using wavelength specific filters. An oil-immersion objective (×60) was used in conjunction with an iris setting of 2–3.5 mm for image collection. Serial horizontal sections were collected and analyzed with Bio-Rad MRC-1024 LaserSharp image analysis program, version 2.1a.

Corneal whole mounts were viewed with a Zeiss confocal microscope and images were captured and analyzed using inbuilt software. Cell populations in different regions of the cornea on corneal flat mounts were counted directly from digital images. Central, paracentral and peripheral areas of each cornea as reported previously (6, 7) were assessed separately. At least three different corneas were examined for each Ab tested. To count the number of positively labeled cells in the corneal stroma and the epithelium, a series of multiple z-sections were generated and single images were created for each sample.

Flow cytometry

Spleen and DLN were removed from transplanted animals at the above indicated times after corneal transplantation. Each sample from an individual mouse was separately prepared and analyzed i.e., no pooling of lymph node cells was done. Samples from at least four individual mice were analyzed for each data time point unless otherwise stated.

Rat and hamster mAbs specific for mouse cell surface markers and mono-chrome-isotype controls were purchased from BD Pharmingen and Serotec. Staining was performed as previously described (10) using FACS buffer (1% BSA, PBS, 10 mM Na3) for washes. In brief, two-color and three-color labeling of single cell suspensions were performed by incubating the cells with rat anti-mouse mAbs directly conjugated with fluorochrome for 30 min followed by two washes, data acquisition, and analysis. Negative controls (identical species and isotype) and single fluorochrome controls were performed to allow accurate breakthrough compensation. Two-color and three-color phenotypic analyses of lymph node and spleen were performed using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest acquisition and analysis software (BD Biosciences). A total of 10,000 events from each sample was collected. The lymphocyte gate was set on the CD3+ cell population and further analysis of lymphocyte surface markers was done within this gate. Analysis of fluorescence was performed after further back gating to exclude dead cells and aggregates.

### Table I. Distribution of leukocyte phenotypes in flat mounts of mouse corneal stroma

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Periphery</th>
<th>Paracentral</th>
<th>Central</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>253 (58)</td>
<td>197 (34)</td>
<td>174 (30)</td>
</tr>
<tr>
<td>CD11b</td>
<td>213 (64)</td>
<td>183 (60)</td>
<td>178 (52)</td>
</tr>
<tr>
<td>F4/80</td>
<td>128 (28)</td>
<td>88 (33)</td>
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</tr>
<tr>
<td>CD11c</td>
<td>39 (35)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MHC Class II</td>
<td>92 (35)</td>
<td>66 (21)</td>
<td>55 (26)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent the mean (SD).

The data from the CFSE proliferation experiment were analyzed by the FlowJo program (Becton Dickinson).

Statistics

Results from a minimum of four animals for each time point were expressed as means ± SD. Statistical analyses were performed using a one-way ANOVA test and Tukey multiple comparison tests using the GraphPad InStat program (GraphPad Software). Comparisons were performed between different time points and data from normal lymph node and spleen cells and also between peaks at different times after grafting.

Results

**CD11c+ dendritic cells are absent from the normal central cornea but rapidly infiltrate the epithelium and anterior stroma after abrasion**

Cross-presentation of Ag is mediated by dendritic cells (20). To investigate the cross-presentation of Ag after corneal grafting, it was important to resolve the question of whether dendritic cells are present in the normal donor cornea (6, 7).

Immunostaining of corneal whole mounts indicated that leukocyte cell surface Ag expression varied for different regions of the cornea (designated central, paracentral, and peripheral in keeping with previous descriptions) (6, 7). In all three regions a prominent population of CD45+ cells was observed in the stroma, increasing from 174 (30) cells/mm² in the center to 253 (58) cells/mm² in the periphery (see Table I) (Numbers quoted throughout represent mean (e.g., 253) with SD in parentheses (e.g., (58).) These cells were distributed throughout the stroma but predominated slightly toward the anterior third of the tissue. A similar number of CD11b+ cells were found in each region of the stroma with slightly fewer cells toward the periphery (Fig. 1e and Table I). No CD11c+ cells were observed in the central or paracentral stroma (Fig. 1b), whereas infrequent CD11c+ cells were observed in the periphery and usually in discrete foci (Table I). A small proportion of cells in the stroma of BALB/c mice expressed low to moderate levels of MHC class II Ag (Fig. 1c). These cells were distributed throughout all three regions, increasing slightly toward the periphery of the cornea. Interestingly, no MHC class II+ cells were identified in the central or paracentral regions of C57BL/6 corneas (data not shown). However, F4/80+ presumed macrophages were present in all three regions of the stroma and epithelium, although they were significantly less frequent in the central corneal stroma (Table I). In the corneal epithelium CD45+ and CD11b+ cell surface staining followed a similar pattern in all three regions of the cornea, but no CD11c+ cells were found in any region of the normal corneal epithelium (data not shown).

Although CD11c+ cells were absent from the central and paracentral corneal stroma, they infiltrated this region in response to inflammation. Twenty-four hours after centrally abrading the epithelium, the corneal stroma was fully reepithelialized and was infiltrated by a small number of CD11c+ cells in its anterior layers (Fig. 1d). They numbered ~29 (18) cells/mm². In addition,
CD11c\(^+\) cells were observed in the corneal epithelium in large numbers as early as 1–2 h after abrasion. It thus appears that while the normal central corneal stroma and epithelium do not contain CD11c\(^+\) dendritic cells, mild inflammation induced by epithelial abrasion can induce infiltration of both the healed epithelium and the underlying stroma by Gr-1-CD11c\(^+\) dendritic cells. Furthermore, light abrasion of the corneal epithelium combined with application of an eGFP plasmid to the central corneal surface led to an uptake of the construct by epithelial cells and by CD11c\(^+\) cells in the cornea (data not shown).

We next wished to determine whether corneal allografting was associated with an influx of CD11c\(^+\) cells into the cornea. Transplantation of a normal donor cornea or a cornea whose epithelium had healed after epithelial debridement 24 h previously led, to the infiltration of CD11c\(^+\) dendritic cells into both the host and the donor epithelium within 1 h of grafting and substantially so by 6 h. Where previously abraded corneas were used as donors, the following CD11c cell numbers were found: at 1 h 24.0 ± 13.0 and at 6 h 50.6 ± 16.7 in the donor epithelium; at 1 h 19.3 ± 12.9 and at 6 h 140.5 ± 61.4 in the host epithelium. This compared with the following CD11c numbers found when naive corneas were used as donors: 9.7 ± 8.9 cells (at 1 h) and 13.6 ± 6.6 (at 6 h) in the donor; 14.5 ± 9.0 (at 1 h) and 57.5 ± 55.3 (at 6 h) in the host. Previous studies have shown that in accepted corneal allografts the donor epithelium is replaced by the host epithelium within days after grafting while the donor stroma and endothelium persist as alloantigenic targets (25).

Infiltration of the stroma of the donor cornea after allografting by host CD11c\(^+\) cells occurred significantly later than infiltration of the epithelium. During the initial 3 days after grafting there was a marked increase in the numbers of CD11c\(^+\) cells in the peripheral host cornea, whereas the central donor cornea remained negative for CD11c (Fig. 2 a, b, and d). By 3–5 days a few CD11c\(^+\) cells were detected in the donor corneal stroma (Fig. 2 d), but by 9 days there was a marked influx of CD11c\(^+\) stromal cells (Fig. 2, c and d). At this stage, the corneas showed no signs of clinical rejection as determined by opacification (23, 24), which first became evident by day 14–15 post transplantation (Fig. 2 d).
Donor corneal leukocytes do not traffic to the DLN after corneal grafting

The above data indicate that although the cornea contains a population of CD45+ leukocytes, some of which express low/moderate levels of MHC class II, the central 2.0-mm portion of the cornea, which is used for allografts, is devoid of CD11c+ dendritic cells. Because it was important in the context of alloantigen transport from the cornea to determine whether donor CD11c+MHC class II+ cells could traffic to the DLN, we conducted the following experiments. Donor corneas were debrided of epithelium and allowed to heal. Twenty-four hours later they contained an average of 29 (18) CD11c+ donor dendritic cells/mm2 in the anterior stroma and many more in the epithelium (see above). The donor corneas were then transplanted to recipient mice and, after a further 24 h, DLN were harvested and evaluated for the presence of donor MHC class II+ cells. No evidence of donor MHC class II Ag could be detected in the submandibular DLN (Fig. 3, a and b), but increased expression of host MHC class II Ag was found (Fig. 3c).

Similar experiments were performed using the C57BL/6-Tg(act-EGFP)Osby01 mouse, which expresses eGFP in all nucleated cells. Donor corneas from eGFP mice were debrided as described above and, after 24 h, transplanted to BALB/c hosts. However, no eGFP-labeled cells could be detected in the host DLN (Fig. 3d) despite the fact that eGFP+CD11b+ cells were readily detectable in the donor corneal stroma (Fig. 3d, inset). There was no evidence of donor C57BL/6 (I-Ab) MHC class II Ag in the host BALB/c (I-Ad) DLN when allografts (C57BL/6→BALB/c) were compared with syngeneic grafts (BALB/c→BALB/c) (Fig. 3, e and f). In all mice (whether they received a C57BL/6 or a BALB/c corneal graft) there was a commensurate increase of CD11c+ MHC class II (I-Ad+) population in the DLN in comparison with naive mice (data not shown), presumably reflecting the innate response to the surgical trauma.

CD11c+ mouse dendritic cells up-regulate MHC class II upon migration from a site of inflammation to the DLN (26, 27). However, after corneal transplantation we could find no evidence of donor MHC class II in the DLN. Thus, despite the presence of a resident population of CD45+CD11b+ cells (~200 cells/mm2) in the normal central and paracentral corneal stroma and a population of donor dendritic cells in the previously abraded grafted cornea, there is no evidence that they migrate to the draining lymph node in sufficient numbers after grafting to allow their detection.

Ag deposited in the donor cornea is transported to the DLN in host CD11c+ dendritic cells

To track the movement of Ag from the donor cornea to the secondary lymphoid tissue, we applied an eGFP-expressing plasmid to the donor cornea 24 h before grafting and examined the DLN and spleen at various times after grafting by confocal microscopy and flow cytometry. To compare the trafficking of Ag from the donor cornea with Ag trafficking generally from the anterior segment of the eye, we included two “control” experiments, namely application of the same plasmid to the nongrafted host cornea and intraocular inoculation of the plasmid into the anterior chamber of mice that received untreated donor corneal grafts.

In all three groups, GFP-labeled cells were first detected in the submandibular DLN 6 h after application (Fig. 4a). Ag was detectable in both CD11c+ dendritic cells (Fig. 4e) and F4/80+ macrophages (data not shown). No Ag was detectable in spleen tissues at this stage (Fig. 4b). By 24 h Ag was only detectable in mice that had received grafts. However, by 48 h Ag was only detectable in the DLN after delivery via the donor cornea. No Ag was detectable in the DLN in any group after 7 days.

In the spleen, Ag was first detected in CD11c+ dendritic cells after 24 h, but only in the animals that had received corneal grafts and not after direct application to the intact host cornea. In addition, Ag was detectable in spleen macrophages at this time only after delivery via the donor cornea. By 48 h, Ag was detectable in spleen dendritic cells (Fig. 4c) and macrophages in both sets of grafted mice. At this stage there was also some weak Ag positivity in dendritic cells after direct application to the intact cornea. No Ag was detectable in the spleen after 7 days.

Confirmation that GFP Ag was present intracellularly in CD11c+ dendritic cells was established by isolating dendritic cells using magnetic bead separation technology (see Materials and Methods). The GFP label was first detected in purified CD11c+ cells 6 h after plasmid application to the anterior chamber of the eye (Fig. 4, d, e, and f) but was difficult to find in isolated cells after 24 h. Cytospin counts indicated that 10% of CD11c+ cells isolated by this procedure were GFP+.
Flow cytometry data from the identical experiment as that described above on cytospins revealed significantly increased numbers of eGFPhighCD11c+ cells in draining LN 24 h after corneal grafting in animals that received corneal graft and eGFP DNA plasmid compared with direct application to the eye (Table II).

These results indicate that cell-associated Ag present in the donor cornea is transported to the secondary lymphoid tissues in host APC for presentation to T cells. In addition, Ag uptake and transport from the donor cornea by host APC is more effective and persistent than similar uptake and transport either from the naive host cornea or from the ocular chambers after surgical trauma. In addition, they show that transport of Ag to the DLN occurs before that to the spleen.

Donor corneal Ag induces T cell activation and proliferation in the DLN

We next wished to determine whether the observed differences in the efficiency of Ag transport after delivery via the donor cornea compared with direct application to the eye were translated into differences in CD4+ T cell activation. To study the kinetics of T cell activation, we made use of the A18 Rag-1-/- mice that were turned to normal by 24 days postgraft.

No changes in T cell populations were observed in the postgraft period in spleen cells, which may have been partly due to the very low numbers of T cells detected in the spleen in A18 Rag-1-/- mice (~1–2%, data not shown).

In an attempt to detect Ag-specific proliferation in the DLN after corneal transplantation, we used the following strategy; recipient mice were prepared by transferring CD4+ T cells isolated from CD4 A18 Rag-1-/- mice (Ly5.2+) and stained with CFSE, into naive A1 Rag-1-/- (Ly5.1+) (1×106 CD3+ cells per mouse i.v.) mice before corneal grafting. Donor mice (C57BL/6) underwent plasmid-containing donor corneal tissue was transplanted 24 h before corneal transplantation. In this way C5' plasmid-containing donor corneal tissue was transplanted into the recipient bed of A1 Rag-1-/- (Ly5.1+) mice that

These mice exclusively contain a monoclonal CD4 T cell population specific for peptide 106–121 of the complement component C5' presented in the context of H2-Eb, and therefore any change in T cell numbers and activation markers represent an Ag-specific response to the plasmid. Using CD3 as a general T cell marker and gating on the CD3+CD4+ T cell population (Fig. 5), T cell responses after the application of a C5'-containing plasmid to the eye were studied by flow cytometry. The low number of T cells overall in unmanipulated mice is due to their short half-life in the periphery (28).

T cell numbers remained fairly constant through the first 48 h after grafting a C5'-containing donor cornea to A18 Rag-1-/- recipients. However, by day 4 there was an increase in the numbers of Ag-specific T cells in the DLN and by day 6 this increase was statistically significant (Fig. 5A) (p < 0.01). T cell numbers returned to normal by 24 days postgraft.

T cell expansion was accompanied by an increase in the numbers of T cells expressing the activation marker CD69, which was also statistically significant at day 6 (Fig. 5B) (p < 0.001). In addition, there was an increase in the overall intensity of CD69 expression in the T cells at the corresponding time points after grafting (Fig. 5B).

FIGURE 4. Detection of GFP label in CD11c+ and F4/80+ cells in the submandibular DLN after the application of GFP-containing plasmid to the eye. a, GFP+CD11c+ cells in lymph nodes fluorescing yellow (fluorescent Texas Red and GFP merged image) 6 h after delivery via donor cornea. b, CD11c+GFP+ Texas Red fluorescent cells in spleen 6 h after delivery via the donor cornea. c, CD11c+GFP+ merged image; yellow fluorescent cells in spleen 48 h after Ag delivery via donor cornea. d–f, Cytospins preparations of magnetic bead-purified CD11c+ cells from DLN at 0 h (GFP−) (d), 6 h (GFP+) (e), and 24 h (GFP−) (f) after application of the plasmid to the cornea. Bars on a–c represent 20 μm, and those on d–f represent 10 μm.

FIGURE 5. Changes in the proportion of CD3+CD4+ T cells in the submandibular DLN of A18 Rag-1-/- mice after C5' plasmid delivery via a C57BL/10 donor corneal graft 24 h after application of the plasmid to the donor cornea. A, Time course postgraft of numbers of CD3+CD4+ T cells in DLN; results are expressed as mean ± SD (n = 4 or more at each time point); *, p < 0.05; **, p < 0.001. B, Time course of CD69 expression on CD3+CD4+ population in terms of the percentage of positive T cells in the DLN (diamonds) and the mean fluorescence intensity (bar histogram; *, < 0.05; ***, p < 0.001).

Table II. Presence of eGFPhighCD11c+ cells in draining LN after corneal transplantation

<table>
<thead>
<tr>
<th>Procedure</th>
<th>eGFPhighCD11c+ (Percentage (% of Total))</th>
<th>Mean (±SD)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corneal graft and eGFP plasmid</td>
<td>1.226 (0.74)</td>
<td>P &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Corneal graft only</td>
<td>0.43 (0.06)</td>
<td>P &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Naive mice</td>
<td>0.19 (0.08)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

An eGFP DNA plasmid (10 μl) was injected into the anterior chamber at the completion of corneal grafting. Twenty-four hours later the draining LN were harvested and stained with CD11c mAb. Flow cytometry results present the percentage (%) of total cells in draining LN; p values indicate a comparison between the experimental group and naive mice (n = 3).
A) above (data not shown). the group (B) as above. Similar histogram as that observed in two generations of proliferating cells were seen.

C/H11001

Three generations of the proliferating CD3/Ly5.2 corneal transplantation (C57BL/6 donor with C5

descendants. Control group of mice received CFSE-labeled cells and a s.c.

population on day 5 after corneal transplantation. A separate mice were analyzed by flow cytometry gating on the Ly5.2


cells in the spleen in A18 Rag-1

themselves phagocytosed by resident macrophages and den-

are then phagocytosed by infiltrating APC. These Ag-loaded

mice that re-

cells migrate to the secondary lymphoid tissue (Fig. 4) where

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in vivo. Only recently has a route for Ag transport to the DLN from the eye has been recognized (6, 12, 13, 29),

because it was previously thought that the ocular chambers connected directly to the spleen via the venous circulation (30).

Most recently, the transport of soluble Ag from the anterior chamber has been shown to track to local cervical and distal mesenteric lymph nodes as well as to the spleen (14, 15, 18).

Thus, it appears that soluble Ag draining from the eye is distributed to regional and nonregional lymph nodes in a fluid phase via conventional lymphatics.

However, the above experiments did not address the fate of cell-bound Ag. Recent studies indicate that priming of the immune response to corneal allografts occurs in a single DLN, namely the submandibular lymph node, suggesting that cell-bound Ag is transported selectively to this node (13). The experiments reported here were designed to exclusively track internalized, cell-associated Ag using GFP- and C5

expressing plasmids (21, 22). The data show that Ag applied to the eye in this form can be detected in the DLN and the spleen in host APC (macrophages and dendritic cells). It has also been shown previously (21) and confirmed in this study (data not shown) that Ag applied to the surface tissues in the form of a plasmid is initially taken up by corneal epithelial cells, some of which are then phagocytosed by infiltrating APC. These Ag-loaded cells migrate to the secondary lymphoid tissue (Fig. 4) where they either present Ag to T cells (Fig. 5 and Fig. 6) or are themselves phagocytosed by resident macrophages and dendritic cells. Therefore, it can be deduced from this and previous studies (12, 13) that the transport of cell-associated ocular Ags within APC is restricted to two discrete routes, namely via lymphatics to a site-specific DLN (the submandibular in the mouse) and via the circulation to the spleen. The data also indicate that Ag transported intracellularly in CD11c cells to the DLN is followed by the activation of Ag-specific T cells in DLN. No evidence for T cell activation in the spleen was found in these experiments. However, this is most likely due to the limitations of the model used in this study because there are very few T cells in the spleen in A18 Rag-1

and A1 Rag-1

mice. In tracking experiments, cell-associated Ag reaches the spleen (Fig. 4) and, predictably, arrives later in the spleen than in the DLN. It is likely that the soluble Ag released from ocular tissues is distributed to several regional and nonregional lymph nodes as well as to the spleen (14) and may be poorly taken up by corneal cells (31).

The data from the present study also confirm that the cross-presentation of cell-associated Ag on MHC class II occurs in vivo, in this case after corneal grafting. Cross-presentation of Ag may occur on MHC class I or class II (20, 32) to CD8 and

showed two rounds of proliferation 5 days postgraft. Spleen T cells from both of the above groups of mice failed to show any proliferation (Fig. 6C). Naive A1 Rag-1

animals were negative for CD3/Ly5.2 T lymphocytes (data not shown).

Discussion

The work reported here has shown that, after corneal transplantation, cell-associated donor Ag is transported to the DLN in host APC leading to early activation and expansion of Ag-specific CD4

T cells and that cycles of T cell expansion and contraction may recur over several days in the DLN. The data support the view that cross-presentation of Ag to T cells on MHC class II occurs in vivo.

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CD4 T cells, respectively, and is considered an important mechanism for the induction of tolerance via apoptotic cells and for the rejection of allografts and tumors (33–35). The phenomenon has been described mostly for cross-priming of CD8 T cells via MHC class I and has been shown to be as efficient as conventional priming of CD4 T cells via MHC class II (36). The data for cross-primer on MHC class II is scant, and indeed clear evidence against the cross-primer of soluble free Ags and haptenhs has been obtained (37). However, cell-associated OVA can be efficiently cross-presented on MHC class II, although less so than on MHC class I (38). In addition, necrotic cellular fragments are better cross-presented on MHC class II whereas apoptotic cell material is presented preferentially on MHC class I (39).

We show in this study that the endogenous cell-associated Ag present in donor corneal cells is captured by host APC, which then induce expansion of the Ag-specific CD4+ T cell population in the DLN, thus providing evidence of the cross-presentation of specific Ag in vivo on MHC class II in an allograft model. Whether this occurs by endocytosis of dead or dying donor cells or through the “nibbling” of live donor cells by host dendritic cells is not clear (40). However, because macrophages do not appear to be able to acquire Ag from live cells in this manner (40), the presence of GFP-labeled plasmid in F4/80+ APC (Fig. 1 and 2); these findings concur with those of a previous report indicating that the population of CD45+ cells in normal mouse cornea are CD11c+CD11b+ (7).

In conclusion, this work has shown that cell-associated Ag is transported from donor corneal allografts in host dendritic cells by two separate routes i.e., in lymphatics to the DLN and in blood vessels to the spleen. Host dendritic cells are rapidly recruited after grafting to the corneal epithelium and anterior stroma, where they endocytose Ag. They traffic first to the DLN within a few hours where they cross-present on MHC class II and induce Ag-specific T cell activation and expansion after a few days, to be followed by later cycles of contraction and expansion of the CD4 T cell pool. The traffic of dendritic cells to the spleen occurs later than to the lymph node (24–48 h) and in this study was not associated with significant T cell activation. However, this is most likely due to the very low numbers of T cells present in the spleen of A18 Rag-1−/− and A1 Rag-1−/− mice, and the question of T effector cell activation in the spleen after cell-associated Ag traffic from the eye remains open.

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Disclosures

The authors have no financial conflict of interest.

References


