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Depletion of Passenger Leukocytes from Corneal Grafts: An Effective Means of Promoting Transplant Survival?

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Abstract

Purpose—To develop and compare effective strategies for depleting graft-derived passenger leukocytes which include antigen-presenting cells from corneal buttons, and to assess the effectiveness of this strategy in promoting graft survival using a high-risk (HR) model of corneal transplantation.

Methods—Corneal buttons harvested from C57BL/6 mice were used in three *ex vivo* strategies of passenger leukocyte depletion. Two strategies involved storage in Optisol-GS medium at different temperatures for prolonged periods. A third strategy utilized complement-dependent cytotoxicity (CDC) by treating the buttons with anti-CD45 mAb plus complement. Whole-mount corneal buttons or cells from enzyme digested corneas were analyzed using confocal microscopy or flow cytometry, respectively, for the pan-leukocyte surface marker CD45. HR host beds were created and used to evaluate the efficacy of passenger leukocyte depletion on transplant survival.

Results—Passenger leukocyte numbers in the buttons were significantly reduced by all three treatments. CDC was the most efficient strategy for passenger leukocyte depletion with 39% reduction (P < 0.00005) of CD45$^+$ cells, and negligible damage to the endothelial layer, achievable within 24 h. However, passenger leukocyte depletion failed to improve HR graft longevity.

Conclusions—Anti-CD45 antibody plus complement-mediated targeting of donor tissue is the most efficient way to deplete corneal passenger leukocytes and can considerably reduce the time required for cell depletion. However, depletion of graft passenger leukocytes does not have a significant effect on promoting graft survival even in the HR setting.

INTRODUCTION

Despite the successful outcome of corneal transplantation in non-vascularized, or so-called "low-risk (LR)", recipient beds corneal transplantation performed in vascularized and inflamed "high-risk (HR)" host beds has shown little improvement in survival over the past several decades. Indeed, while systemic corticosteroids and immunosuppressive agents may be partially effective in preventing graft rejection, their use is limited because of a wide range of side effects, including infection, cataract, and glaucoma. Moreover, even with potent immune suppression, rejection rates in HR corneal transplantation can be as high as 50–90%.[1, 2] There is, therefore, a compelling need to improve the survival of HR grafts in a manner that minimizes the impact on host immune competence.

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The most common cause of corneal graft failure remains allograft rejection, during which host alloreactive T cells are activated and once peripheralized to the graft, can result in graft destruction.[3] Allograft rejection is triggered by two distinct, but not mutually exclusive pathways of allorecognition mediated by either donor-derived antigen-presenting cells (APCs) or recipient APCs— the direct and indirect pathways of allosensitization respectively. In the direct pathway, donor APCs present intact major histocompatibility complex (MHC) class II molecules residing on their surface to T cells. In contrast, in the indirect pathway recipient APCs present processed MHC or minor antigens to T cells.[4–8] The direct and indirect pathways play distinct roles in corneal alloimmunity. Using a murine orthotopic corneal transplantation model, Huq et al. demonstrated that T cells activated via the direct pathway are detected and donor-derived APCs are functionally capable of priming host T cells in HR graft recipients. They also found that transplantation using MHC class II knockout donor tissue leads to significantly improved survival of HR allografts.[6] Relatedly, Simon et al. discovered that prolonged storage of donor corneas prevented allograft rejection particularly in HR corneal transplantation but offered no mechanistic information to explain this finding, although they postulated that depletion of donor APCs through prolonged storage could reduce graft immunogenicity.[9]

CD45 is a transmembrane molecule found on the surface of all bone marrow-derived nucleated hematopoietic cells and their precursors, hence, a leukocyte common antigen.[10] It is well known that the cornea contains a heterogeneous population of bone marrow (BM)-derived cells which express the leukocyte common antigen, CD45.[11, 12, 13, 14] Given the capacity of CD45$^+$ cells to potentially prime T cells, and thereby initiate alloimmune responses, it is of interest to investigate the effect of graft passenger leukocyte depletion on corneal transplant survival. To this end, complement-dependent cytotoxicity (CDC) is a mechanism of killing cells in which antibody binds to the receptor of a target cell, then fixes and activates the complement system. The end result is the formation of a membrane attack complex that makes a hole within the cell membrane, causing cell lysis and death. CDC can potentially provide a powerful strategy for depleting graft passenger leukocytes prior to corneal transplantation, and has been shown to be effective to this end in renal transplantation[15]. The current study was conducted to compare the efficiency of *ex vivo* donor passenger leukocyte depletion using CDC and other strategies, as well as to investigate the acceptance of the APC-depleted corneal grafts in HR corneal transplants.

METHODS

Mice

C57BL/6 and BALB/c mice (male, 6–14 weeks) were purchased from Taconic Farms (Germantown, NY). Corneal buttons from C57BL/6 mice were used as sources of donor tissue because they are MHC and multiple minor H disparate to BALB/c mice which were used as recipients in the corneal transplantation experiments. For the *ex vivo* experiments, mice were euthanized by cervical dislocation and the corneal buttons were harvested. All protocols were approved by the Schepens Eye Research Institute Animal Care and Use Committee, and the mice were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunofluorescent Staining of CD45 on Corneal Buttons

To count the number of CD45$^+$ cells in the cornea, the corneal buttons were stained immunohistochemically with anti-CD45. All staining procedures were conducted in 96-well plates at room temperature (RT). Antibodies were purchased from BD Pharmingen unless otherwise specified. All antibodies were diluted 1:100 in 2% bovine serum albumin in phosphate-buffered saline (PBS-BSA). Whole-mount corneal buttons (2 mm diameter) from

C57BL/6 mice, either stored in Optisol-GS® medium or freshly excised from the mice, were fixed with acetone for 15 min, then blocked with anti-Fc receptor (anti-FcR) monocolonal antibody (mAb) CD16/CD32 for 30 min to prevent nonspecific staining. The corneas were then either stained with rat anti-mouse CD45-FITC (clone30-F11) and the isotype-matched control antibody, rat IgG2b-FITC, or with Alexa Fluor® 488 anti-mouse CD45 and the isotype control antibody Alexa Fluor® 488 rat IgG2b; both Alexa Fluor® 488 antibodies were obtained from BioLegend, San Diego, CA. After CD45 staining, the corneal buttons were labeled with a nuclear stain To-Pro-3 (1 μM, Invitrogen-Molecular Probes) for 30 min at RT. Following three washings with PBS for 5 min each, corneas were mounted with a mounting medium (Vector Shield, Burlingame, CA) and analyzed by confocal microscopy.

Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End Labeling (TUNEL) Assay

The TUNEL assay was used along with CD45 staining to detect the apoptotic CD45^{+} cells in the whole-mount corneal buttons stored for prolonged periods. First, whole-mount corneal buttons were fixed with acetone for 15 min at RT. TUNEL staining was then carried out following the instructions provided by the manufacturer (Roche). Afterwards, the same procedures described above for CD45 and nuclear staining were performed. Apoptosis of CD45^{+} cells was assessed by confocal microscopy.

Cell Counting and Imaging with Laser Scanning Confocal Microscopy

Confocal laser scanning microscopy was carried out using a Leica TCS SP spectral confocal laser scanning microscope equipped with an air-cooled argon-krypton ion-laser system (Leica Microsystems Heidelberg, Heidelberg, Germany). Magnification was achieved using a Nikon oil-immersion lens at 60x. Corneal cells were counted in a masked manner. The central area of each 2 mm button was defined as the central 1mm section; the remainder defined as the paracentral cornea (the full diameter of a murine cornea is 3mm). To count the cells, 1–3 and 6–8 consistent and equally spaced fields were assessed from the central and the paracentral areas, respectively. Images were recorded by continuously focusing the optical dissection through the full-thickness of the cornea within the fixed area of a field. Any cells straddling the upper and left adjacent boundaries of each field were excluded. Because the cells are not uniformly distributed in the cornea, the cell counts of the unit area from different locations were averaged and multiplied by the corresponding amount of the central and paracentral area to obtain the total number of cells in each corneal button, then expressed and plotted as average cell densities by dividing the total number of cells by the total area of the corneal button.

Detection of CD45^{+} cells in Optisol-GS® Medium with Laser Scanning Cytometry (LSC)

Corneal buttons were stored in 12-well culture dishes at 37°C for different periods of time. To determine whether CD45^{+} cells emigrated from the corneas into the medium, both adherent cells and non-adherent cells were collected from the medium of culture dishes. Isolated cells were washed once with PBS, and blocked by incubation with anti-FcR mAb (CD16/CD32) for 20 min. The cells were then stained with anti-CD45-FITC. To label the nuclei, cells were then fixed with 0.5% paraformaldehyde at 4°C overnight, washed twice with PBS and stained with Hoechst 33258 (Invitrogen, USA) following the manufacturer's instructions. After washing with PBS, 10 μl of the resuspended pellet resuspension was placed onto a spot in the center of a glass slide. The slides were coverslipped and sealed for LSC (Compucyte Inc., Cambridge, MA) measurements. LSC results were expressed as percentages of positive cells over and above the negative isotype control–stained cells.

Corneal CD45^{+} Cell Depletion by Complement-dependent Cytotoxicity (CDC)

To efficiently deplete CD45^{+} cells from the corneal buttons other than passively extending the storage time, the procedure of CDC was carried out using rat anti-mouse CD45 (clone30-F11)

and Low-Tox®-M rabbit complement (CL3051, Cedarlane, US) following the manufacturer's protocol, with minor modifications. The control mAb was the isotype-matched rat IgG2b. Corneal buttons were incubated at 4°C overnight with anti-CD45 or isotype control mAb in Optisol-GS® medium at a dilution of 1:100. After 3 gentle washings with HBSS+ for 2 min each on ice, the corneal buttons were incubated at 37°C for 1–3 h with different concentrations of complement. The corneal buttons were then gently washed again on ice with HBSS+ 3 times and proceeded to either confocal microscopic enumeration of CD45+ cells after staining the whole-mount corneal buttons, or flow cytometric analysis on digested corneal buttons as described below.

To confirm the depletion of CD45+ cells from the tissue (as determined by the confocal microscopic enumeration), digestion of corneal buttons was carried out for flow cytometric analysis. After washing with PBS on ice, the CDC-treated corneal buttons were incubated for 30 minutes at 37°C in HBSS+ medium supplemented with 2 mg/ml collagenase D and 0.5 mg/ml DNase (both from Roche). The corneal buttons were then pressed gently and passed through a nylon mesh screen to remove debris and to generate single-cell suspensions. Cells were washed in PBS twice, and blocked by anti-FcR for 20 min on ice, and then incubated with anti-CD45-FITC alone or in combination with a dead cell marker, LIVE/DEAD® Fixable Red Dead Cell Stain Kit (LIVE/DEAD®-Red, Invitrogen-Molecular Probes) following the manufacturer's instructions. Cell suspensions were washed in PBS-BSA and then resuspended in PBS for flow cytometric analysis. Flow cytometry was performed using a Coulter Epics-XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA), and Summit Software v4.3 (Dako USA) was used for data analysis.

Corneal Endothelium Morphologic Study with Zonula Occludens (ZO)-1 Staining

To assess the status of the endothelial layer as a result of the depletion strategies, ZO-1 staining was performed to delineate the tight junctions between endothelial cells,[16, 17] thereby allowing their enumeration and morphologic characterization. Rabbit anti-ZO-1 mAb (40–2300, Zymed Laboratories, Inc., South San Francisco, CA) was used as the primary antibody, and diluted at a concentration of 1 μg/mL in PBS-BSA. Normal Rabbit IgG was used for the isotype control. The duration of the primary mAb staining was 2 h, followed by the secondary antibody, goat anti-rabbit IgG-rhodamine (Santa Cruz Biotechnology, CA) staining for 1 h both at RT. After ZO-1 staining, the corneal buttons were labeled with the nuclear stain To-Pro-3 for 30 min at RT. Following three washings with PBS for 5 min each, corneas were mounted with Vector Shield mounting medium and coverslipped for confocal microscopic analysis.

High-Risk Corneal Transplantation

The well established procedures for corneal HR transplantation have been described previously.[6, 18] Briefly, three interrupted 8-shaped sutures (11-0 nylon, 50-um diameter needle, Sharpoint, Vanguard, TX) were placed in the central cornea of BALB/c mice to induce inflammation and neovascularization (HR graft beds) 14 days before orthotopic corneal transplantation. Corneal grafts of 2 mm diameter were excised from C57BL/6 mice with a trephine (Storz Instrument Co., St. Louis, MO) and microscissors. Three groups of donor buttons were treated with anti-CD45 plus complement, isotype control mAb plus complement, or Optisol-GS® medium only just before the transplantation. The recipient bed was prepared by excising a 1.5-mm tissue in the central cornea of BALB/c host mice. The prepared donor corneal grafts were then transplanted onto the host bed with eight interrupted 11-0 nylon sutures. The sutures were removed 7 days post-transplantation. Corneal grafts were assessed for graft survival by slit lamp biomicroscopy 2 times a week for 8 weeks according to a standardized corneal opacity scoring system[19] with score 0 being a clear graft and 5 representing maximum stromal opacity. Grafts with consistent opacity scores ≥ 3 at 2 weeks, or ≥ 2 at or after 3 weeks were defined to have been rejected. At week-8 post-transplantation

the grafted corneas of different groups were harvested and analyzed for infiltrating immune cells. Corneal digestion for preparing single cell suspension and flow cytometry (using anti-CD45-FITC and anti-CD3-PE mAbs, eBioscience Inc.) was performed as described above.

Statistical Analyses

Results were expressed as mean ± SEM. When the data were fitted with a regression line, the R-squared (R^2) was calculated as a parameter for the quality of the fit using Microsoft Excel. GraphPad Prism (ver. 3.0; GraphPad, San Diego, CA) was used for all the statistical analyses descried below. When comparing more than 2 groups for cell densities in the corneal buttons, one-way analysis of variance (ANOVA) tests were performed, followed by Dunnett's Multiple Comparison Test in which each experimental group was compared with the control group. Statistical comparisons of CD45^{+} cell counts between the complement plus anti-CD45 mAb and the complement plus isotype control mAb groups were performed using the unpaired Student's *t* test. A *P* value < 0.05 was considered as statistically significant. Graft survival results were plotted as Kaplan–Meier curves.

RESULTS

CD45^{+} Cell Numbers Decreased over Time with Prolonged Storage

Depletion of BM-derived cells from the cornea was successfully achieved when the corneas were stored in the medium at both 4°C and 37°C over time. The FITC (green)-stained cells in the corneal buttons were CD45^{+} (Fig. 1A and 1B). The staining was verified by including a negatively stained isotype control antibody in each batch of slides. Representative images in Figure 1 showed that the number of CD45^{+} cells decreased significantly when the corneal buttons were stored in Optisol-GS® at 4°C for 14 days (Fig. 1B) as compared to baseline numbers in fresh corneas (Fig. 1A). Cell enumeration data are plotted in Figure 1C and fitted with regression lines. The R^2 values indicated that approximately 99% of the data can be fitted with a 2-degree polynomial function for the 4°C curve, and about 90% of the data can be fitted with an exponential function for the 37°C curve. The number of CD45^{+} cells decreased faster at 37°C than at 4°C.

Apoptotic CD45^{+} Cells Increased with Storage Time

To determine whether the CD45^{+} cells undergo apoptotic death in corneas stored for prolonged periods, groups of corneal buttons stored in the medium for up to 28 days were stained for the TUNEL assay along with Alexa Fluor® 488 anti-CD45 staining. CD45^{+} TUNEL^{+} cells were detected in corneas that were stored longer than 2 weeks (Fig. 2A). Figure 2B displays the densities of CD45^{+} cells in the corneal buttons, showing a significant decrease over time (line, *P* < 0.05); along with an increasing proportion of apoptotic CD45^{+} cells (bars and the percentages). Apoptotic CD45^{+} cell densities in the 2-, 3-, and 4-week groups were significantly higher than those of the fresh corneas (*P* < 0.05 in the 2-week group, and *P* < 0.01 in the 3-, and 4-week groups).

Concomitant Increase of CD45^{+} Cell Numbers in the Medium at 37°C

Cells with variable morphology were readily observed in the Optisol-GS® medium when buttons were stored at 37°C. However, no evidence of cells was found in the media at 4°C during the storage period with minimal sloughing of the epithelium. Since tissue APCs are capable of emigrating out of tissues,[20, 21] the cells in the medium were assayed for CD45 expression. LSC (a slide-based cytometry system) was chosen over flow cytometry to define the cells because LSC is more suitable for detecting relatively small populations of cells. Concurrent with a decrease in the number of CD45^{+} cells in the corneal buttons (Fig. 1B solid curve), there was an increase of cells in the medium during the first week (Fig. 3A). LSC

analysis on the cells in the medium indicated that the percentage of CD45$^+$ cells started to increase on day 2 and peaked around day 7, and then started to decrease afterward (the experiments were repeated several times and the representative data are plotted in Fig. 3B, n = 40 corneal buttons in each time point).

Efficient Depletion of CD45$^+$ Cells Achieved with Complement-dependent Cytotoxicity (CDC)

The primary protocol of CDC method was developed and tested using bone marrow-derived dendritic cells as prototypic CD45$^+$ cells. The cells were treated with anti-CD45 antibody and complement, and the degree of cell killing was assessed (data not shown). The optimized conditions for complement concentration and incubation time that could result in maximal depletion of resident CD45$^+$ cells, with minimal toxicity to the endothelial cells, were tested and applied to the donor buttons to deplete resident CD45$^+$ leukocytes. Different combinations of complement concentrations (5–60%) and incubation time (0.5–3 h) were further tested on donor buttons, including evaluation of endothelial cell health; representative data on CDC-treated corneal buttons are plotted in Fig. 4A. Statistically, when compared with the untreated control groups, the anti-CD45 mAb plus complement treated corneal buttons had significantly fewer CD45$^+$ cells in the 20% and 60% Low-Tox® groups, with the respective percent reduction of CD45$^+$ cells being 39% (N=5) and 46% (N=3), ($P < 0.00005$) (Fig. 4A). The efficacy of this treatment was further supported by flow cytometric analysis on digested corneal buttons (Fig. 4B). The two-color flow cytometric analysis for CD45 expression (FITC) and cell death (LIVE/DEAD®-Red) revealed that nearly half (48.8%) of the CD45$^+$ cells were dying/dead after CDC treatment, not including the ones that were already dead (Fig. 4B).

The corneal endothelium serves a critical role in maintaining corneal clarity.[22, 23] To ensure the safety of CDC treatment to the endothelium, the corneal buttons were stained for ZO-1 so as to delineate their numbers and morphology; indeed, CDC treatment using Lo-Tox® complement showed minimal damage to the endothelial cells (Fig. 4C). Since treatment for 1 h incubation at 37°C with 20% complement after anti-CD45 mAb incubation overnight at 4° C caused negligible endothelial changes, yet substantial degrees of CD45$^+$ cell depletion, these parameters were determined as optimal for in vivo transplantation.

An additional experiment was performed to gain insight into the lifespan, and degree of CD45 expression of BM-derived dendritic cells stored in Optisol-GS® for up to 10 days. Our flow cytometric analysis of cell viability and CD45 expression revealed that approximately 70% of the cells are dead/dying on day 2, while significant diminishment in CD45 expression is not reached until 2 days later (Fig. 4D), suggesting that cell viability is lost before complete loss of surface CD45 expression.

Fate of CDC-treated Donor Grafts on High-Risk Corneal Transplantation

Donor buttons were either (i) treated with anti-CD45 plus complement, (ii) treated with isotype Ab plus complement, or (iii) kept in Optisol storage medium alone, prior to transplantation in high-risk host beds. All three groups had a swift rejection starting day 17 after transplantation, with the survival rates being 22%, 33%, and 30% for the "anti-CD45 plus complement", "isotype Ab plus complement", and "Optisol only" groups, respectively (Fig. 5A). The anti-CD45 plus complement group did not differ significantly from its two control groups–isotype Ab plus complement, and Optisol only (Fig. 5A). In addition, the transplanted corneas of different groups were harvested at week 8 post-transplantation to quantify and analyze the phenotypes of infiltrating immunocytes (Fig. 5B). The anti-CD45 plus complement treated group showed slightly higher infiltration of CD45$^+$ cells compared to the isotype plus complement and Optisol-only groups. However, the degree of CD3$^+$ cell infiltration was comparable among the three different groups.

DISCUSSION

The concept that depletion of class II-expressing "passenger" leucocytes could promote long-term survival of allografts by reducing immunogenicity was established more than 20 years ago[24–26] and has played a central role in solid organ (heart, kidney, etc.) transplantation. For this reason, and because we (and subsequently many others) showed that the cornea also bears bone marrow-derived cells,[11, 12] we hypothesized that their depletion will promote graft survival. Distinct *ex vivo* strategies of CD45^{+} cell depletion from the corneal buttons were successively developed in the current study, and the most efficient method was assessed for HR corneal transplantation. The fact that the manipulation did not improve the survival suggests that perhaps these bone marrow-derived cells may also be relevant in promotion of graft tolerance.

Our CD45^{+} cell density data in the normal corneal buttons (day 0) are comparable to those reported by Brissette-Storkus et al., despite the different mouse strains.[27] Our data for CD45^{+} cell numbers in the cornea over time in the 4°C medium show slightly lower values (Fig. 1C dotted line) than those in Figure 2B at corresponding time points. This is likely due to the different staining reagents; Alexa 488 anti-CD45 seems to be more sensitive than anti-CD45-FITC. Both sets of data show similar trends, however; that is, CD45^{+} cell density decreases over time in the medium at 4°C. Indeed, the increased apoptotic CD45^{+} cell density concomitant with decreased CD45^{+} cell density, detected by two different staining reagents, provides evidence that the reduction in CD45^{+} cells in the whole mount corneal buttons over prolonged storage time is not artifact.

The decrease in the number of CD45^{+} cells assayed in the medium after 7 days of storage (Fig. 3B) may be accounted for by the short lifespan of the cells in the medium. The work by Chinnery et al. has shown a turnover rate for BM-derived corneal cells of ~24% by 2 weeks in an *in vivo* model.[28] Our study, however, investigated cell viability/lifespan in an *in vitro* system, which may explain the shorter lifespan observed. Because of the delay in complete loss of CD45 expression following cell death (Fig. 4D), we postulate that the actual number of viable CD45^{+} cells in the corneal buttons after the depletion manipulations is lower than the staining data alone may suggest.

Because CD45 is a transmembrane molecule found on the surface of all nucleated hematopoietic cells,[10] it offers a means of targeting all leukocyte populations, including passenger APCs in the graft tissue, with the aim of reducing graft alloimmunity. All professional (bone marrow-derived) APCs are CD45^{+}, and hence targeting CD45^{+} would allow depletion of APC populations. Anti-CD45 mAbs have been successfully applied to deplete renal allografts of passenger leukocytes in allogeneic transplantation.[29] In contrast, the CDC method employed in this study provides a more precise way of targeting intragraft leukocytes because the complement system can be activated by specific complement-fixing-antibodies directed at specific cell surface (e.g. CD45) epitopes. The anti-CD45 antibody used in the current study, rat IgG2b, clone 30-F11, has also been previously used to readily lyse CD45^{+} cells in the presence of complement.[30] A clinical trial of *ex vivo* perfusion of two complement fixing anti-CD45 mAbs into kidneys (for the purpose of depleting donor-derived CD45^{+} cells which include APCs) prior to transplantation achieved significant reduction in the incidence of rejection, and improvement of long-term renal function.[15] The current study provides the first data regarding the feasibility of *ex vivo* CD45^{+} cell depletion in corneal graft tissue.

A reduction in MHC class I and minor H antigen expression in the preserved corneal grafts has been found to improve the acceptance in corneal allografts in a normal-risk corneal transplantation model.[31] However, it is not known whether the approach would have the same effect in HR transplantation where graft-derived leukocytes are thought to be particularly

relevant for alloimmunization.[6] Furthermore, preservation of corneal buttons requires longer waiting time and can be detrimental to endothelial cells. Comparing the various methods for $CD45^+$ cell depletion in the current study, the corneas that are stored at 37°C undergo a faster depletion than those stored at 4°C for prolonged periods. However, anti-CD45 antibody plus complement-mediated targeting of donor buttons is an even more efficient way to deplete passenger leukocytes *ex vivo* in that a majority of $CD45^+$ cells can be depleted within a day. This reduces the length of time required for prolonged storage which can be deleterious for the viability of both the epithelial and endothelial layers, the latter especially being essential for corneal transplant clarity.[22, 23, 32]

In the study reported by Huq et al.,[6] corneal transplantation using MHC class II knockout donor tissue led to significantly improved survival of HR allografts in mice. That experimental setting completely eliminated all donor MHC class II expression, thereby not only preventing direct sensitization, but also indirect sensitization to donor MHC class II antigens—since host APCs would also be unable to process and present donor class II to T cells. In the present study reliant on passenger leukocyte depletion, however, the direct pathway only would be suppressed, with a reduced capacity of donor APCs to present donor MHC molecules to host T cells; in contrast, host APCs would still be able to process donor class II molecules expressed by non-leukocyte populations. Thus, the fact that we did not observe an improved transplantation survival in our *in vivo* transplantation study may be accounted for by at least two factors leading to graft rejection—(i) the lack of effect of this donor passenger leukocyte depletion strategy on the indirect pathway mediated by host APCs, and/or (ii) suppression of a possible tolerogenic effect of graft (donor)-derived cells on host immunity. Indeed, there is accumulating strong evidence that immature dendritic cells of either donor or host origin play a fundamental role in transplant tolerance by perhaps activating regulatory T cells (Tregs).[33–35] In fact, a growing number of studies are focused at the therapeutic use of immature dendritic cells on induction of Tregs in transplantation, raising the specter that graft passenger leukocyte depletion may also have deleterious consequences in terms of Treg induction. Further experiments are required to investigate this provocative hypothesis.

Acknowledgments

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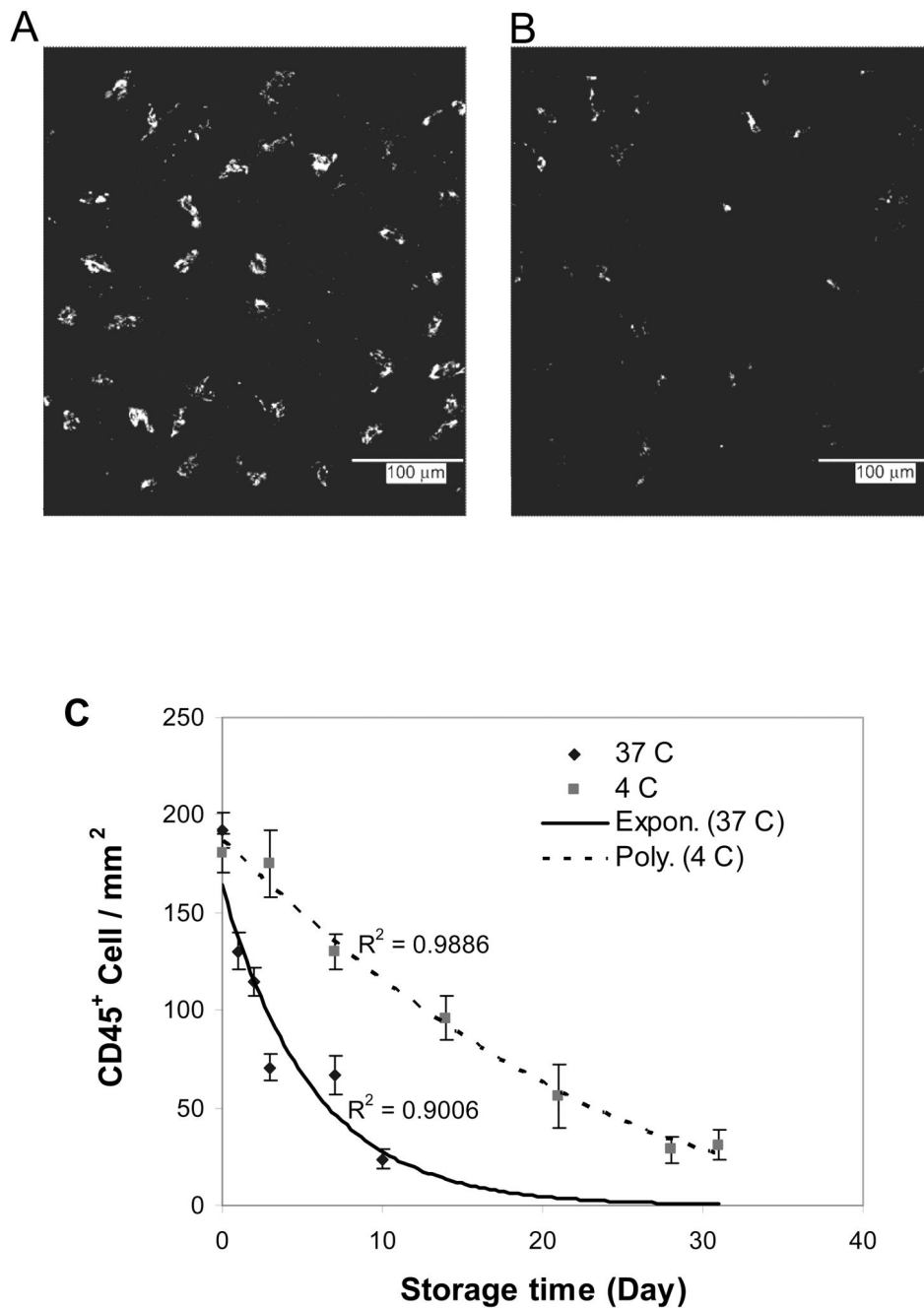
Figure 1.
Enumeration of CD45^{+} cells in corneal buttons stored in Optisol-GS® medium. Representative Confocal micrograph of whole-mount corneal buttons, fresh **(A)** or stored for 14 days **(B)** in Optisol-GS® at 4°C. The unit bar in the pictures represents 100 μm. **(C)** Corneal CD45^{+} cells in the buttons decreased over time when the buttons were stored in medium at either 4°C (n = 5, 3, 8, 9, 5, 4, 5 corneal buttons per time group, respectively. $P < 0.005$ at day 7 and afterwards) or 37°C (n = 9, 7, 7, 7, 10, 9 corneal buttons per time group, respectively. $P < 0.0005$ in all storage time groups). Trendlines were fitted to the data and the quality of the fit was calculated as R^2.
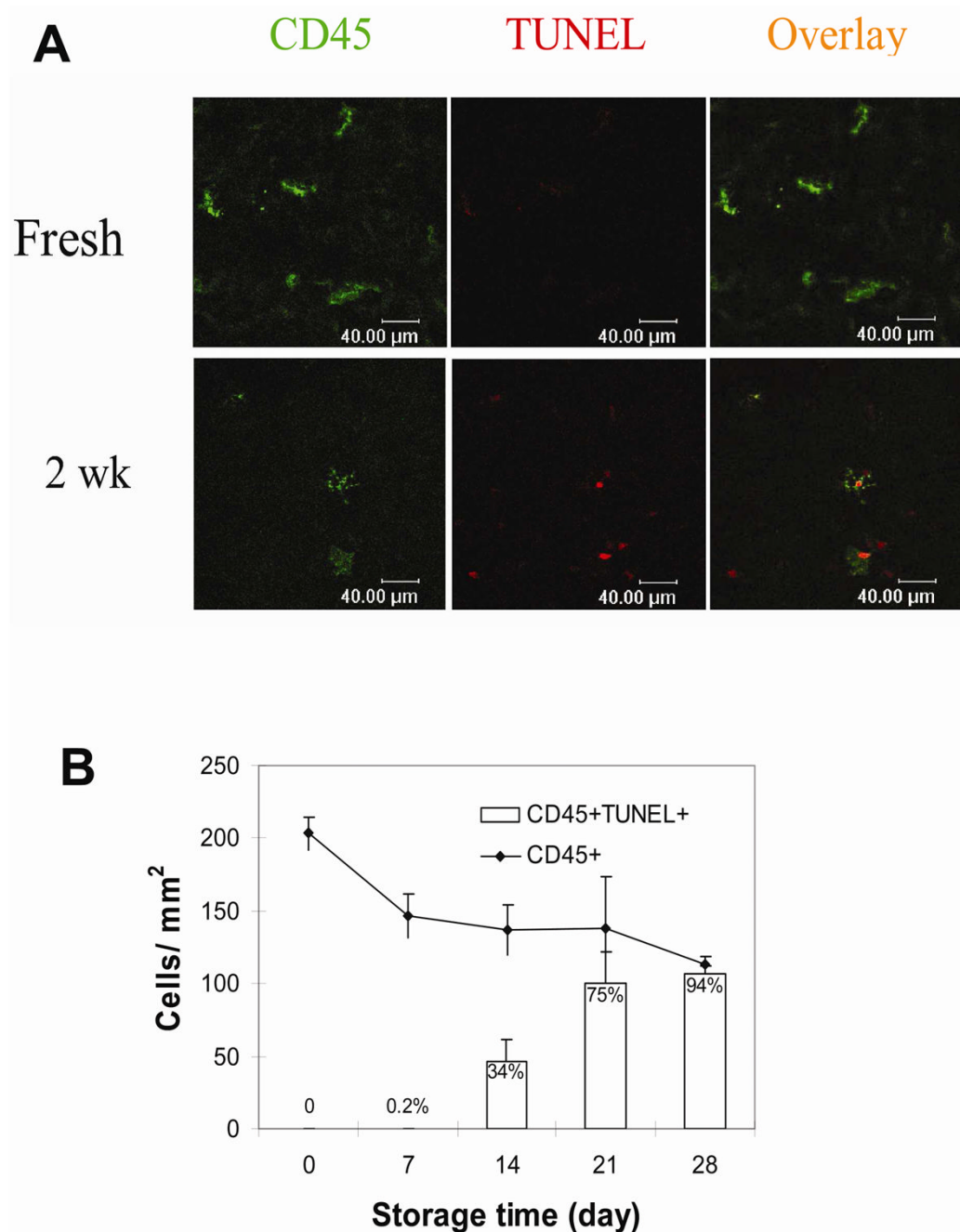
Figure 2.
Detection of apoptotic (TUNEL^{+}) CD45 cells in corneal buttons. (n = 5, 6, 4, 4, 4 corneal buttons per time group, respectively). **(A)** Confocal micrographs of whole-mount corneal buttons stored in Optisol-GS$^{®}$ medium at 4°C for 2 weeks compared with the fresh cornea (green = CD45-Alexa 488, red = TUNEL). **(B)** Proportion of apoptotic cells (bars) as percentage of total CD45^{+} cells (line) in the buttons stored in medium at 4°C over time. The percentage shown on the bars indicates the proportion of apoptotic cells. Apoptotic CD45^{+} cell densities in the 2-, 3-, and 4-week groups were significantly higher than those of the fresh corneas ($P < 0.05$ in the 14-day group, and $P < 0.01$ in the 21-day, and 28-day groups).
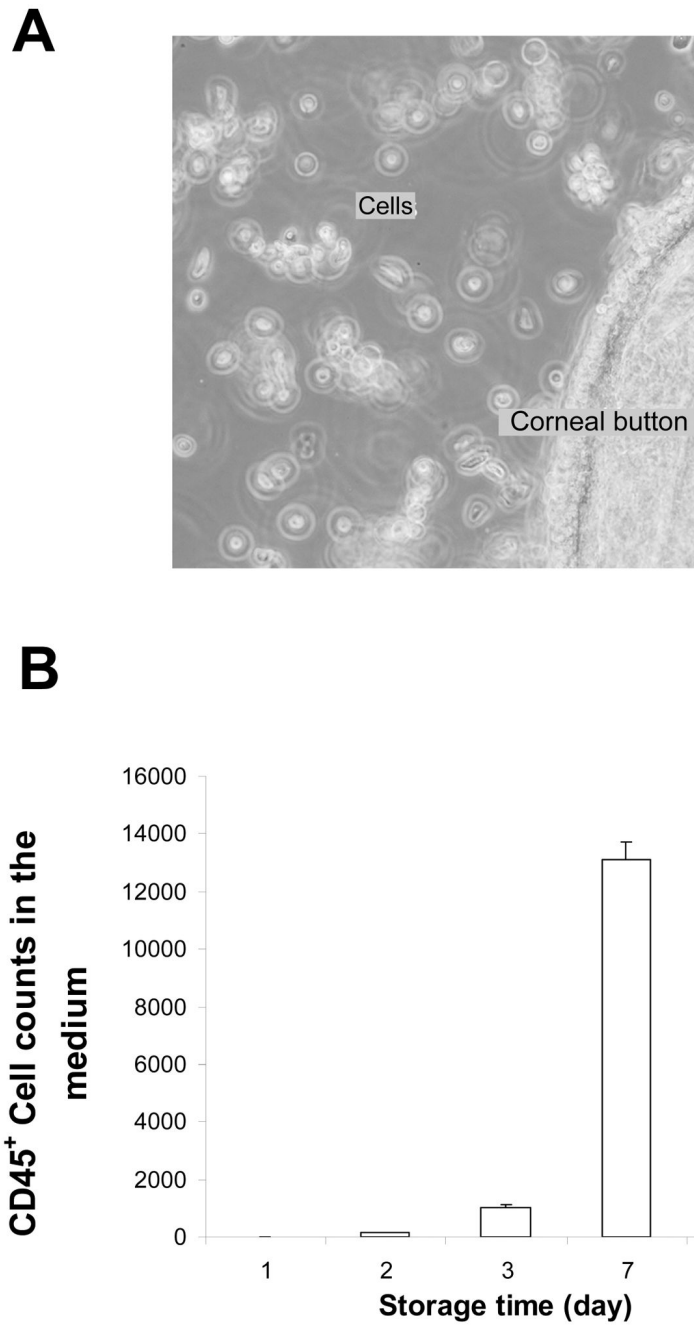
A

B

Figure 3.
Detection of CD45$^+$ cells in Optisol-GS$^®$ medium at 37°C. **(A)** By 3 days of storage a morphologically heterogeneous population of cells could be detected in the medium. **(B)** Total CD45$^+$ cells in the storage medium detected with laser scanning cytometry and calculated per corneal button.
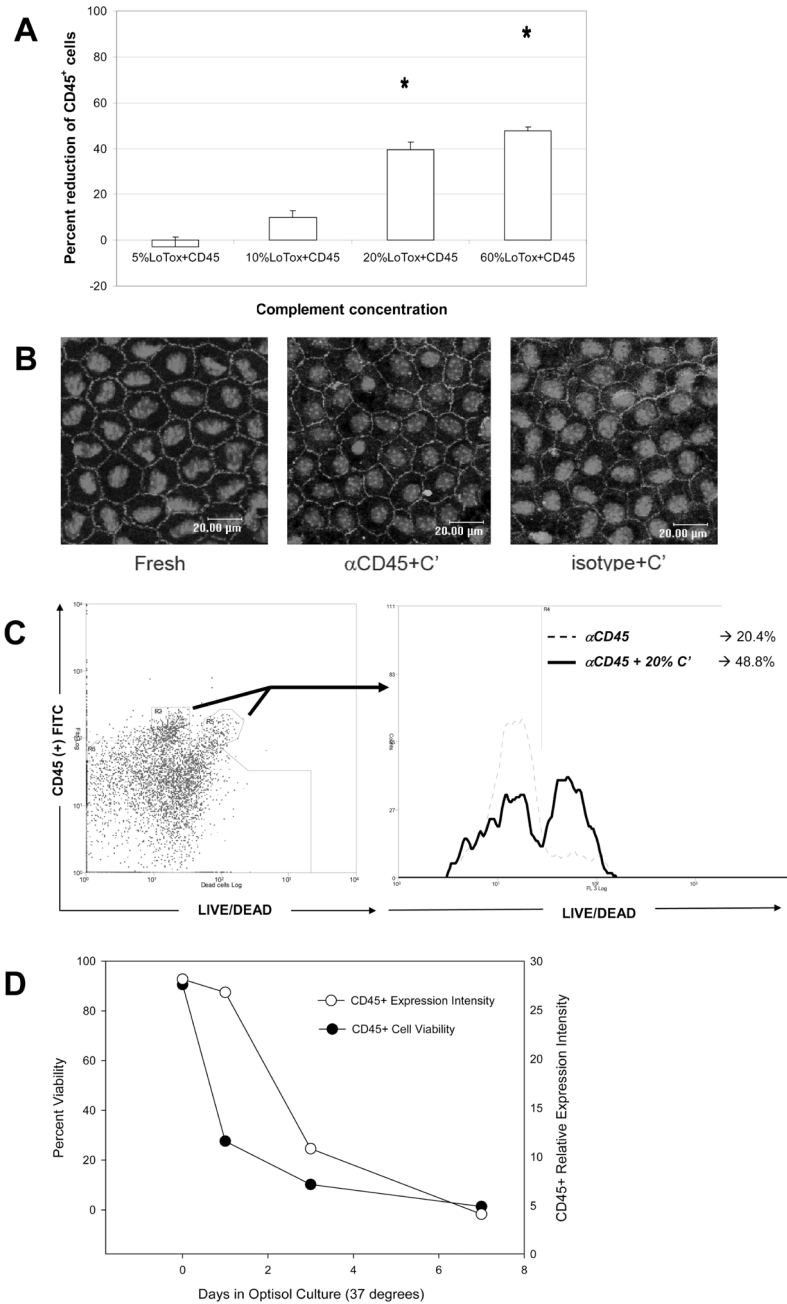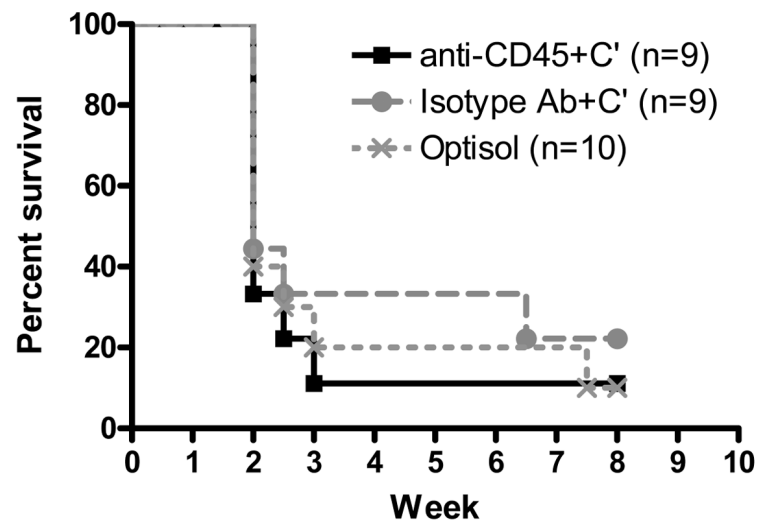
Figure 4.
Evaluation of *ex vivo* treatment of complement-dependant cytotoxicity (CDC) on corneal buttons. (**A**) Percent reduction of CD45^{+} cells in the corneal buttons with CDC treatment when compared to the untreated control group (n =7 [control], and 5, 10, 5, 3 corneal buttons per complement concentration groups respectively, *$P < 0.00005$). (**B**) Two-color flow cytometric analysis for CD45 expression (FITC) and cell death (LIVE/DEAD®-Red) on digested corneal buttons treated with CDC. CD45^{+} gates are indicated in cell density plots (arrow). Frequencies of cell death in CD45^{+} gated populations were analyzed in the adjacent histogram. (**C**) Confocal micrographs of the endothelial layer of the CDC-treated corneas stained for ZO-1 in grayscale. Normal endothelial mosaic and cell density are shown. (**D**) Cell viability vs. CD45 expression

of BM-derived dendritic cells stored in Optisol-GS for up to 7 days, showing cell viability was lost before complete loss of surface CD45 expression.
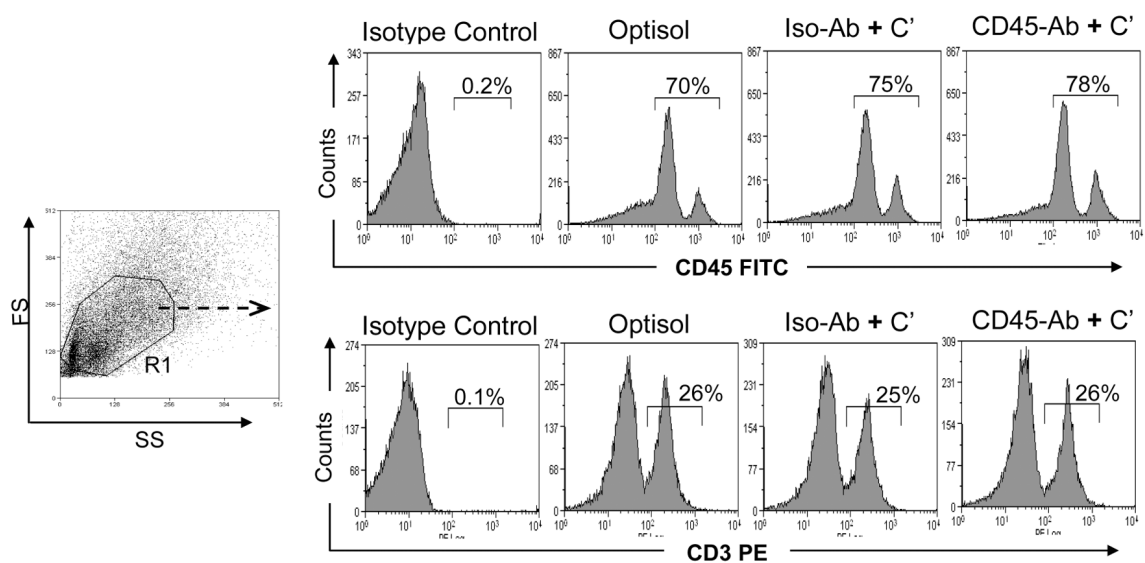
Figure 5.
In vivo studies of CDC-treated donor buttons on HR corneal transplantation. **(A)** Kaplan-Meier survival curves of CDC-treated corneal grafts and the control groups in HR corneal transplantation; no significant difference was found ($P > 0.05$). **(B)** Flow cytometric analysis of infiltrating total CD45$^+$ cells and CD3$^+$ T cells in transplanted corneas at week 8 post-transplantation.