Early subretinal allograft rejection is characterized by innate immune activity.

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Abstract

Successful subretinal transplantation is limited by considerable early graft loss, despite pharmacological suppression of adaptive immunity. We postulated that early innate immune activity is a dominant factor in determining graft survival and chose a non-immunosuppressed mouse model of retinal pigment epithelial (RPE) cell transplantation to explore this.

Expression of almost all measured cytokines by DH01 RPE cells increased significantly following graft preparation and the neutrophil chemoattractant, KC/GRO/CINC, was most significantly increased. Subretinal allografts of DH01 cells (C57BL/10 origin) into healthy, non-immunosuppressed C57BL/6 murine eyes were harvested and fixed at 1, 3, 7 and 28 days post-operatively and subsequently cryosectioned and stained. Graft cells were detected using SV40 large T antigen (SV40T) immunolabeling and apoptosis/necrosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Sections were also immunolabeled for macrophage (CD11b & F4/80), neutrophil (Gr1 Ly-6G), and T-lymphocyte (CD3-ε) infiltration. Images captured with an Olympus FV1000 confocal microscope were analyzed using Imaris software.

The proportion of the subretinal bolus comprising graft cells (SV40T+) was significantly (p<0.001) reduced between post-operative day (POD) 3 (90% ± 4%) and POD 7 (20% ± 7%). CD11b+, F4/80+ and Gr1 Ly-6G+ cells increased significantly (p<0.05) from POD 1 and predominated over SV40T+ cells by POD 7. Co-labeling confocal microscopic analysis demonstrated graft engulfment by neutrophils and macrophages at POD 7 and reconstruction of z-stacked confocal images confirmed SV40T inside Gr1 Ly-6G+ cells. Expression of CD3-ε was low and did not differ significantly between time-points. By POD 28, no graft cells were detectable and few inflammatory cells remained.

These studies reveal for the first time a critical role for innate immune mechanisms early in subretinal graft rejection. The future success of subretinal transplantation will require more emphasis on techniques to limit innate immune-mediated graft loss, rather than focusing exclusively on suppression of the adaptive immune response.
Keywords: Retinal transplantation, Innate immunity, Cell transplantation, Retinal pigment epithelium, Neutrophil, Macrophage

INTRODUCTION

Retinal degenerative disease, including neovascular and non-neovascular age-related macular degeneration, are the commonest cause of blindness in the developed world. Anti-vascular endothelial growth factor (anti-VEGF) agents prevent loss of vision and improve visual acuity in patients with neovascular degenerations. However, no effective treatment exists for the more common non-neovascular degenerations. Transplantation of cells to the subretinal space (SRS) to replace degenerate or dysfunctional cells has been proposed as a treatment strategy, and achieves anatomical and visual rescue in animal models of retinal degenerative disease and in affected humans.

Rapid graft loss and decline of anatomical and functional benefit following transplantation remains a major challenge. This occurs despite suppression of the T-lymphocyte mediated adaptive immune response with drugs such as cyclosporine and azathioprine. These immunosuppressive regimes still have graft survival rates of just 11% at 4 weeks and 0.2% at 28 weeks. Indeed, rabbit allograft failure is not associated with lymphocyte infiltration and is not altered by cyclosporine-immunosuppression.

Poor graft survival in the SRS is paradoxical, as this site is characterized by a suppressed antigen-specific adaptive immune response. The RPE expresses CD95 ligand that induces T-cell apoptosis, secretes TGF-b, which is important in the induction of tolerance, and can also phagocytize T-lymphocytes. We hypothesized that cells transplanted to the immune-deviant SRS are destroyed by an alternate immune mechanism rather than via classic T-lymphocyte mediated immunological rejection.
Innate immune responses are characterized by upregulation of pro-inflammatory cytokines\textsuperscript{37} and a coordinated local and systemic inflammatory response\textsuperscript{38}. A critical role for innate immunity in acute allograft rejection is now emerging\textsuperscript{39-41}, and has been shown to be crucial in skin\textsuperscript{42} and pancreatic islet cell\textsuperscript{43} allograft rejection. However, the role of innate immunity in subretinal transplantation has not been studied. We hypothesized that subretinal graft failure is a consequence of innate immune mechanisms and used a mouse RPE allograft model to investigate this. The purpose of this study was to examine the mechanisms underlying early allograft cell loss in the SRS rather than to achieve a structural or functional benefit following transplantation. Accordingly, we used healthy non-immunosuppressed hosts to investigate our hypothesis.

**MATERIALS AND METHODS**

**Ethics statement**

Ethical approval for this research was obtained from University College Dublin’s Animal Research Ethics Committee (AREC-P-07-09-Keegan). All procedures involving the use of mice were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use and Care of Animals in Ophthalmic and Vision Research and all efforts were made to minimize suffering.

**Cell line derivation**

The DH01 RPE line was prepared from RPE cultured from a healthy C57BL/10.RIII-H-2r mouse as previously described\textsuperscript{44} and immortalized using supernatant from the SVU 19.5 cell line\textsuperscript{45} secreting retrovirus encoding a temperature-sensitive non-SV40 origin-binding U19 mutant of the SV40T antigen and the neomycin resistance gene\textsuperscript{46}. 
Cell culture

DH01 cells were routinely cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich Ireland, Wicklow, Ireland) supplemented with 1% fetal calf serum (Sigma-Aldrich, Ireland) 200 mM L-glutamine (Sigma-Aldrich, Ireland) and 5 mg/ml penicillin/streptomycin (Sigma-Aldrich, Ireland). As these cells harbor SV40T they were grown at 33° C in 5% CO₂.

Transplant conditions (TC) and baseline conditions (BC)

TC cells were prepared as a highly concentrated cell suspension (50,000 cells/μl), deprived of serum (suspended in serum-free medium), and kept on ice for the maximum period that typically exists between harvesting and transplanting the graft (4 hours). To determine the effect of graft cell suspension preparation on cytokine production, we compared cytokine expression by DH01 cells immediately following resuspension in full media (BC) and after being prepared for subretinal transplantation (TC).

Preparation of samples for cytokine quantification

Cytokine production by DH01 was quantified under BC and following the stress of the graft cell suspension preparation procedure (TC). Cells were cultured in full medium, trypsinized (Trypsin from Sigma-Aldrich, Ireland) and seeded in triplicate to 24-well culture plates with 2x10⁵ cells/well to yield confluent cultures. BC cells were seeded in 0.5 ml of serum-free medium. TC cells were subjected to the process involved in preparation of a graft cell suspension as described above, and after 4 hours on ice were seeded in 0.5 ml serum-free medium. Following culture for 24 hours, the medium was removed and stored at -80°C for subsequent analysis.

Multiplex cytokine assay

A 9-plex multi-spot T-helper 1 (TH1) / T-helper 2 (TH2) cytokine assay kit (Meso Scale Discovery-MSD, K15013B-1) was used according to the manufacturer’s instructions to quantify the following cytokines: IFN-γ, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12 total and KC/GRO/CINC (CXCL1). An 8-point calibration curve from 0-10,000 pg/ml was constructed using a plot of signal
intensity from a series of known concentrations of the multiplex standard provided by the kit manufacturer. Cytokine levels were calculated using the MSD Sector Imager 2400 and Discovery Software 2.0 (MSD, Rockville, MD, USA). Concentrations were determined in triplicate and expressed in pg/ml.

**IL-6 enzyme-linked immunosorbent assay (ELISA)**

IL-6 was not included on the MSD TH1/TH2 multiplex kit. Therefore, expression of this cytokine was measured separately using an IL-6 ELISA kit (Invitrogen, KMC0061). A 6-point calibration curve from 0-500 pg/ml was generated from standard mouse IL-6 reconstituted in standard diluent buffer. ELISA was carried out as per the manufacturer’s instructions. Absorbance at 450nm was measured using a spectrophotometer (SpectraMax M2, Molecular Devices). Sample IL-6 concentrations were calculated against the graph of the standard curve. Concentrations were determined in triplicate and expressed in pg/ml.

**Subretinal RPE cell transplantation**

Male C57BL/6 mice were obtained from Harlan UK (Bicester, UK). All surgery was performed under ketamine (Ketalar, 75 mg/kg, C&M Vetlink, Limerick, Ireland) and medetomidine (Domitor, 0.5 mg/kg, C&M Vetlink) anesthesia, and the mice were recovered with atipamezole (Antisedan, 1 mg/kg, C&M Vetlink). Euthanasia was with sodium pentobarbitone (Euthatal 140 mg/kg, C&M Vetlink) followed by cervical dislocation. All drugs were delivered by intra-peritoneal injection, with the exception of atipamezole, which was delivered by subcutaneous injection.

DH01 (C57BL/10 origin) cell suspensions (50,000 cells/µl DMEM) were prepared from pre-confluent cultures as described under TC above. We have previously demonstrated that preparing DH01 cell suspensions in this manner results in 95% graft cell viability at the time of transplantation 44. Subretinal transplants were delivered transsclerally through glass micro-injection pipettes (BioMedical Instruments, Germany) to the dorso-temporal subretinal space of healthy non-immunosuppressed C57BL/6 mice. Eyes received 2 µl of the graft cell suspension (n=16). Graft position and size was verified by fundoscopy under the operating microscope. To distinguish a host inflammatory response to the
surgical procedure, as distinct from a response directed specifically against the allograft, sham surgery controls that received 2 µl vehicle only (serum-free medium) were also performed (n=16). The animals were euthanized and eyes harvested on POD 1, 3, 7 and 28 (n=4/group/time-point). In order to establish the baseline expression of markers of interest, unoperated eyes were also harvested from naïve mice that received neither graft nor sham surgery to either eye (n=4). The eyes were fixed in 4% paraformaldehyde, cryoprotected in sucrose, embedded in OCT under liquid nitrogen and stored at -80°C. Sections (7µm) were cut on a Leica cryostat and stained as described below.

Graft detection (SV40T), TUNEL-labeling and identification of the host immune response to subretinal RPE transplants

To examine temporal graft survival, graft cells were identified using a specific primary antibody to SV40T (Santa Cruz, SC-20800, 1:100) and goat-anti-rabbit Texas Red-labeled secondary antibody (Jackson, 111-075-003, 1:100). DNA strand breaks were detected by TUNEL as previously described 47.

To examine the host immune response to subretinal DH01 allografts, cryosections were immunolabeled for the SV40T antigen (Santa Cruz, SC-20800, 1:100) using a donkey-anti rabbit FITC-labeled secondary antibody (Jackson 711-095-152, 1:100). In addition, sections were immunolabeled to detect macrophages (CD11b and F4/80), neutrophils (Gr1 Ly-6G) or T-lymphocytes (CD3-ε). Rat anti-mouse CD11b (AbD Serotec MCA711, 1:100), rat anti-mouse F4/80 (AbD Serotec MCA 497EL, 1:25) and rat anti-mouse Gr1 Ly-6G (R&D Systems, MAB1037, 1:100) primary antibodies were secondarily immunolabeled using donkey anti-rat TRITC-labeled secondary antibody (Jackson, 712-025-150, 1:50). Goat anti-mouse CD3-ε (Santa Cruz, sc-1127, 1:100) was secondarily immunolabeled using donkey anti-goat TRITC-labeled secondary antibody (Jackson, 705-025-003, 1:100).

For all immunohistochemistry performed, serum from the host species of the relevant secondary antibody (1:50) was used to block non-specific binding and 4,6-diamidino-2-phenylindole (DAPI 10 ng/ml, Sigma-Aldrich, D9542)
counterstaining was used to enable visualization of nuclei. Phosphate-buffered saline (Sigma-Aldrich, Ireland) was used to dilute all reagents and for three 5-minute washes between steps. After the final wash, sections were mounted using Vectashield® HardSet™ (Vector laboratories).

**Confocal Microscopy**

Immunolabeling was visualized using an Olympus FV1000 confocal microscope. Differential interference contrast microscopy (DIC) images were taken at the time of fluorescent confocal microscopy to more accurately identify the SRS. Z-stack images were taken through areas of interest to enable 3-dimensional (3D) image reconstruction using image analysis software as described below. Captured images were viewed using Olympus Fluoview Ver. 1.4a software.

**Image Analysis**

Four transplanted eyes and 4 sham-treated eyes were examined for each post-operative time-point. Four unoperated eyes were also examined. In order to maintain consistency in analyses of transplanted eyes, cryosections through the center of the subretinal cell bolus where the greatest numbers of cells were present were used for all eyes. For sham-treated eyes, cryosections in the region of the injection site were used. All sections were immunolabeled for SV40T to identify transplanted cells and counterstained with DAPI to label all nuclei. Sections were also immunolabeled to detect one of the following: DNA nicks (TUNEL), macrophages (CD11b and F4/80), neutrophils (Gr1 Ly-6G) or T-cells (CD3-ε).

Single optical sections from 4 transplanted eyes and 4 sham-treated eyes per time-point, in addition to 4 unoperated control eyes were analyzed using Imaris image analysis software (Bitplane AG, Switzerland). For sections fluorescently labeled to detect both SV40T and TUNEL, the following counts of cells in the SRS were performed: total number of cells (DAPI+); number of graft cells (DAPI+/SV40T+); and number of graft cells with DNA nicks (DAPI+/SV40T+/TUNEL+). As the total number of cells in the SRS varied between images, the proportion of the subretinal cell bolus comprising grafted cells was
calculated as SV40T+ cells per 100 cells (DAPI+) in the SRS. The proportion of graft cells (SV40T+) that had DNA nicks (SV40T+/TUNEL+) was also calculated.

As immune cell infiltration of the SRS was identified by cell specific surface markers (CD11b, F4/80, Gr1 Ly-6G and CD3-ε), it was not possible to accurately estimate cell number. Therefore, sections immunolabeled for these markers in conjunction with the graft cell marker (SV40T) were analyzed by calculating the area of immunolabeling using Imaris. To correct for differing sizes of subretinal boluses, the numbers of nuclei (DAPI) in the SRS were also counted and the areas of immunolabeling expressed as µm²/100 nuclei in the SRS. This enabled determination of the temporal change in expression of the graft cell marker (SV40T) relative to markers of infiltrating immune cells.

The proportion of the area of SV40T+ immunolabeling that co-labeled with immune cell markers (CD11b, F4/80, Gr1 Ly-6G and CD3-ε) was also calculated using Imaris. This allowed determination of altered temporal co-labeling of the graft cell marker with markers of infiltrating cells.

Z-stack confocal images of selected regions of SV40T co-labeling with immune cell markers were processed using Imaris and reconstructed in 3D form using isosurface rendering to determine the nature of the relationship between co-labeling markers at a cellular level.

**Statistical analysis**

Data were analyzed using GraphPad Prism 5 statistical software (GraphPad, San Diego, CA, USA). Data are expressed as mean ± standard deviation (SD) and p<0.05 was considered statistically significant.

*Comparisons of cytokine levels*

Student’s t-tests assuming unequal variances were used to determine the significance of differences between sample means. Results are presented as mean ± SD.
Comparisons of cell numbers in the SRS at different post-operative time-points

At POD 1, 3, 7 and 28, counts of cells in the SRS were performed to determine the total number of cells; the proportion of cells expressing SV40T; and the proportion of SV40T+ cells that were also TUNEL+. One-way analysis of variance (ANOVA) followed by all pairwise post-hoc t-tests with Bonferroni Correction were used to determine the significance of differences between sample means.

Analysis of areas of immunolabeling

The areas of immunolabeling and the total numbers of nuclei in the SRS were calculated for each immunolabeling combination at POD 1, 3, 7 and 28. Thus, areas of immunolabeling/100 nuclei were determined. To assess for changes in marker expression between time-points, areas of immunolabeling/100 nuclei were treated as normally distributed and analyzed with ANOVA and Tukey’s post-hoc test.

Analysis of immunofluorescent co-labeling

The proportions of SV40T immunolabeling that co-labeled with immunolabeled infiltrating cells were calculated using Imaris. The co-labeling as proportions are likely not normally distributed and were analyzed with nonparametric methods, Kruskal-Wallis test and Dunn’s post-hoc comparisons.

RESULTS

Cytokine expression increases significantly following graft preparation

Cytokine expression by the mouse RPE cell line, DH01, was quantified under BC and TC prior to transplantation (Table 1). With the exception of IL-4, expression of all cytokines increased significantly (p<0.05) following graft preparation (TC). However KC/GRO/CINC was expressed at considerably greater levels under both BC and TC, compared to all other cytokines measured. KC/GRO/CINC also had the greatest increase (6-fold increase; p=0.022) in expression following TC. A 3-fold increase (all ps<0.001) in expressed IL-1β, IL-5, IL-6 and IL-12 occurred following TC. There was also significantly increased expression of IL-10 (2.1-fold
increase; p=0.024) and IL-2 (2.7-fold increase, p=0.002) following graft preparation (TC) compared to baseline levels (BC).

Retinas from unoperated and sham-treated eyes of C57BL/6 mice contain F4/80+ cells and few TUNEL+ cells, but do not comprise CD11b+, Gr1 Ly-6G+ or CD3-ε+ cells.

To establish the baseline expression of markers of interest in the C57BL/6 mouse retina, cryosections from unoperated eyes (n=4) and sham-treated eyes at POD 1, 3, 7 and 28 (n=4/time-point) were labeled for macrophage (CD11b and F4/80), neutrophil (Gr1 Ly-6G) and T-lymphocyte (CD3-ε) markers. TUNEL-labeling was also performed to examine for baseline apoptosis and necrosis in the retina. All sections were additionally labeled for SV40T to establish levels of background staining for the graft cell marker. All nuclei were counterstained with DAPI (Fig. 1).

The unoperated retina was characterized by the presence of F4/80+ cells, particularly in the ganglion cell layer (Fig. 1k). Low background levels of TUNEL+ cells were also present in the retina (Fig. 1a). SV40T, CD11b, Gr1 Ly-6G or CD3-ε labeling was not observed and no cells were present in the SRS.

Sham-treated eyes did not demonstrate SV40T immunolabeling but had low levels of TUNEL+ cells in the inner and outer nuclear layers of the retina (Fig. 1b-e). At all post-operative time points, sham-operated eyes did not have cells in the SRS and the retina did not stain for CD11b, Gr1 Ly-6G or CD3-ε. However, F4/80+ cells were seen in retinas of sham-operated eyes, particularly in the ganglion cell layer in a pattern analogous to that observed in unoperated eyes (Fig. 1l-o).

The first week post-transplantation is characterized by rapid loss of DH01 RPE allograft cells, but low levels of graft apoptosis and necrosis

We sought to ascertain whether apoptosis or necrosis were predominant mechanisms of graft failure in the early post-operative period. DH01 (C57BL/10 origin) cell suspension allografts (in serum-free medium) were transplanted to the SRS of C57BL/6 mice. Subretinal DH01 allografts were examined at POD 1, 3, 7 and 28 (n=4 eyes/time-point). Cryosections were immunohistochemically
labeled for SV40T (Texas red) to identify transplanted cells and TUNEL-labeled (FITC, green) to examine for DNA strand nicks. All nuclei were identified using DAPI (blue) counterstaining.

Subretinal DH01 allograft cells were distinguishable from host cells by SV40T immunolabeling (Fig. 2A). At POD 1 and POD 3 most cells in the subretinal bolus were identified as graft cells by the expression of the SV40T in the nucleus (Fig. 2A: a, b). By POD 7, there was a dramatic reduction in the proportion of cells in the subretinal bolus expressing SV40T (Fig. 2A: c). TUNEL-labeling demonstrated that few cells in the subretinal bolus had DNA strand nicks at each time-point. No graft cells were identified at POD 28 (Fig. 2A: d). Few cells in the nuclear layers of the retina were TUNEL+ following subretinal cell transplantation (Fig. 2A: a-d). This observation relates to the retinal detachment induced to place the graft and was also evident in sham-treated eyes (Fig. 1: b-e).

Cell counts were performed using Imaris image analysis software. The proportion of all cells (DAPI+) identifiable as graft cells (DAPI+/SV40T+) in the SRS was quantified. The proportion of graft cells (DAPI+/SV40T+) with DNA strand nicks (DAPI+/SV40T+/TUNEL+) was also quantified. There was no significant change in the total number of cells in the SRS between POD 1 (435 ± 143), POD 3 (487 ± 164) and POD 7 (607 ± 45), but total cell number decreased significantly (p<0.05) between POD 7 (607 ± 45) and POD 28 (49 ± 49) (Fig. 2B). There was no significant difference in the proportion of the subretinal bolus comprised of graft cells between POD 1 (93% ± 1%) and POD 3 (90% ± 4%). However, there was a significant (p<0.001) reduction in the proportion of the subretinal bolus comprised of graft cells between POD 3 (90% ± 4%) and POD 7 (20% ± 7%) (Fig. 2C). No graft cells survived to POD 28. Levels of graft cells with DNA strand nicks remained low at every time-point (POD 1: 2% ± 0.3%; POD 3: 0.4% ± 0.3%; POD 7: 5% ± 2%) and did not differ significantly between time-points (Fig. 2C).
The period of graft loss is characterized by infiltrating cells expressing markers of innate immunity, but not T-lymphocytes

Figure 2 illustrates a significant (p<0.001) decrease in the proportion of cells in the subretinal bolus expressing SV40T between POD 3 and POD 7, with total graft cell loss by POD 28. Subretinal DH01 allografts were also examined at POD 1, 3, 7 and 28 (n = 4 eyes per time-point) for evidence of infiltrating macrophages (CD11b+ and F4/80+) and neutrophils (Gr1 Ly-6G+). T-lymphocytes (CD3-ε+) are traditionally regarded as mediating allograft rejection in conventional (non-immune deviant) sites and these cells were also sought.

In eyes that received subretinal transplants, the period of graft loss between POD 1 and POD 7 was characterized by infiltration of the SRS by cells expressing markers of innate immunity (Fig. 3A). CD11b and F4/80 are cell-surface markers used to identify macrophages and Gr1 Ly-6G is a cell surface marker used to identify neutrophils. Low levels of infiltrating CD11b+, F4/80+, and Gr1 Ly-6G+ cells were seen at POD 1 and POD 3, with a marked increase evident at POD 7. No graft cells were identified at POD 28. Only 1 of 4 eyes at POD 28 had cells remaining in the SRS and these were F4/80+ (Fig. 3A: h). Few T-lymphocytes (CD3-ε+) were seen at each time point (Fig. 3A: m-p). At POD 28 the neural retina overlying the graft site had folds in the outer nuclear layer, but otherwise appeared undamaged (Fig. 3A: d, h, l, p).

The area of immunolabeling as a function of the number of nuclei present in the SRS was calculated and expressed as μm²/100 nuclei. A significant (p<0.05) increase in expression of the innate immune cell markers CD11b, F4/80 and Gr1 Ly-6G coinciding with a significant (p<0.05) decrease in immunolabeling of the graft cell marker (SV40T) was identified between POD 1 and POD 7 (Fig. 3B). Expression of the T-cell marker CD3-ε remained very low across all time-points and was not expressed at significantly different levels between time-points (Fig. 3B).
Macrophages and neutrophils co-label with, and engulf, graft cells during the first post-operative week

Areas of co-labeling of the graft cell marker (SV40T) with the innate immune cell markers (CD11b, F4/80 and Gr1 Ly-6G) were apparent on confocal microscopy images. The proportion of SV40T immunolabeling that co-labeled with infiltrating immune cell markers on POD 1, 3, 7 and 28 was calculated. CD11b, F4/80 and Gr1 Ly-6G all co-labeled with SV40T at significantly (p<0.05) increased levels by POD 7 (Fig. 4F). However, co-labeling of SV40T with the T-lymphocyte marker (CD3-ε) remained low at all time-points and did not change significantly across time-points (Fig. 4F).

To better discriminate areas of co-labeling, high power z-stack confocal images were taken through these areas. SV40T immunolabeling was apparent inside the cell membranes of CD11b+ (Fig. 4A) and F4/80+ (Fig. 4B) cells. DIC images also identified pigment granules inside the cell membranes of these cells. Similarly, Gr1 Ly-6G+/SV40T+ co-labeling was apparent inside cell membranes of Gr1 Ly-6G+ cells (Fig. 4C-D). Reconstruction of z-stacked confocal images taken through such areas of co-labeling confirms SV40T inside Gr1 Ly-6G+ cells (Fig. 4E). The images presented in Figure 4 are consistent with phagocytosis of graft cells by macrophages and neutrophils.

DISCUSSION

We examined cytokine expression by DH01 RPE cells under BC and following preparation of a concentrated graft cell suspension (TC), to determine whether the graft preparation technique might influence the host immune response following transplantation. Graft preparation (TC) increased significantly the expression of almost all cytokines examined (Table 1). Notably, KC/GRO/CINC was expressed at by far the greatest levels and also demonstrated the greatest fold increase (6-fold) in expression following graft preparation. Mouse GRO and the chemoattractant KC are structural and functional homologues of the human chemokine IL-848. IL-8 is a potent neutrophil chemoattractant and stimulates phagocytosis, superoxide radical production and cytoplasmic degranulation49,50. Thus, production by graft cells of high levels of KC/GRO/CINC in the SRS in the
period following transplantation would increase the risk of monocyte migration and inflammation at the graft site. KC/GRO/CINC, or IL-8, has not been identified previously as a potentially significant mediator of the immune response against subretinal grafts.

TC caused a 3-fold increase in expression of the inflammatory cytokines IL-1β, IL-5, IL-6 and IL-12. While increased expression of IL-1 and IL-6 at the graft site has been reported following human RPE xenografts to the SRS of non-dystrophic Royal College of Surgeons (RCS) rats\(^{51}\), the effects of these cytokines in subretinal cell transplantation are unknown. IL-1 has many pro-inflammatory effects including mediating a neutrophilic inflammatory response to dying cells in vivo\(^{52}\). RPE allografts to rabbits cause elevated IL-6 in the vitreous in the first week post-operatively\(^{53}\). IL-6 is a key mediator of allograft rejection and increased expression is highly correlated with human cardiac allograft rejection\(^{54}\). Graft-produced IL-6 promotes T-cell activation and cardiac allograft rejection in the mouse\(^{55}\).

DH01 expression of IL-10 increased significantly (2.1-fold increase; \(p=0.024\)) following graft preparation (TC) compared to baseline levels (BC). Production of IL-10 by the RPE plays a key role in modulating the posterior ocular immune microenvironment and suppressing delayed-type hypersensitivity (DTH) by diverting the immune response from a TH1- to a TH2-type immune response\(^{56,57}\). Increased IL-10 expression by DH01 RPE cells at the time of transplantation would therefore inhibit a T-lymphocytic host response against the subretinal graft.

IL-2 supports T-lymphocyte proliferation\(^{58-62}\) and survival\(^{63}\), as well as the differentiation of naive T-lymphocytes into effector and memory T-cells\(^{64-66}\). However, IL-2 also has an immunosuppressive function by promoting regulatory T cell (Treg) production and homeostasis\(^{67}\) and plays a fundamental role in immune regulation and tolerance in vivo\(^{68-73}\). While DH01 production of IL-2 increased significantly (2.7-fold increase, \(p=0.002\)) following graft preparation, absolute levels of IL-2 production under both BC and TC were relatively low.
In the context of consistently poor graft survival in the SRS, we sought to examine graft survival in the early post-operative period. We have previously demonstrated that our technique for preparing DH01 graft cell suspensions results in 95% cell viability at the time of transplantation. Expression of SV40T by the DH01 cell line enabled examination of the fate of transplanted cells during the first post-operative month. The proportion of the subretinal bolus composed of graft cells reduced from 90% on POD 3 to just 20% on POD 7, and no graft cells survived to POD 28 (Fig. 2).

Our finding of rapid graft loss in the SRS is consistent with other studies. Human RPE cells xenografted to the SRS of cyclosporine-immunosuppressed RCS rats are quickly lost with just 27% survival at POD 7 and 11% at POD 28. Similarly, just 3% of subretinal porcine fetal RPE cells xenografted to rabbits survived to one month in immune-competent animals and only 10.5% survived in rabbits receiving daily triple systemic immunosuppressive therapy with prednisone, cyclosporine and azathioprine.

In contrast to solid tissue or organ transplants, cell transplants are commonly derived from cell cultures and delivered as a cell suspension (e.g. stem cells, Schwann cells, pancreatic islet cells and RPE cells). Transplantation paradigms involving cell suspension grafts have poor rates of graft survival. Human fetal dopaminergic neuronal xenografts to cyclosporine-immunosuppressed rats had only 5-6% survival, and poor graft survival is also a feature of fetal nigral allografts in humans.

TUNEL-labeling identifies apoptotic cells by detecting DNA fragmentation. However, necrosis is also accompanied by DNA breaks and thus TUNEL-labeling also identifies necrotic cells. It is unlikely that the rapid graft cell loss observed in this study was due to apoptosis or necrosis as rates of TUNEL-labeling of graft cells remained low at all time points (Fig. 2A). Graft cell apoptosis/necrosis was just 2% at POD 1, <1% at POD 3 and 5% at POD 7 (Fig. 2C). We hypothesized that the rapid graft cell loss observed during the first post-operative week was a consequence of a host immune response against the subretinal allograft.
On POD 1 and POD 3, 90% of cells in the subretinal space were identifiable as graft cells (SV40T\(^+\)). However, by POD 7 this figure reduced to just 20% (Fig. 2C). Thus, the remaining 80% of cells at this time must be host-derived. We characterized the cellular composition of the subretinal bolus during the first post-operative month and found that macrophages (CD11b and F4/80) infiltrated subretinal grafts on POD 1 and POD 3 and predominated over transplanted cells by POD 7 (Fig. 3A: a-h and Fig. 3B).

There was also a significant increase (p<0.05) in immunohistochemical co-labeling of the graft cell marker (SV40T) with macrophages (CD11b, F4/80) at POD 7 (Fig. 4F). High-power images of areas of co-labeling confirmed the presence of the graft cell marker inside macrophages (Fig. 4A and B). Thus, we have confirmed that macrophages not only associate with subretinal allografts, but also engulf transplanted cells.

The interaction of graft cells with F4/80\(^+\) macrophages in the subretinal space is particularly interesting because the mouse macrophage F4/80 receptor is known to play a critical role in the generation of antigen-specific efferent Treg cells that suppress antigen-specific DTH responses\(^{83}\). However, while F4/80\(^+\) macrophages may inhibit a T-lymphocytic adaptive immune response, our observations reveal direct engulfment of transplanted cells by F4/80\(^+\) macrophages. Macrophages have been reported to associate with subretinal grafts in many host species including the mouse\(^{84}\), rat\(^{14,26,27}\), rabbit\(^{29-31,85-87}\), pig\(^{88}\) and primate\(^{8}\). While these studies reported macrophages associating with subretinal grafts, they did not correlate macrophage infiltration with graft cell loss. The study presented is the first investigation to make this connection.

The observation of positive immunolabeling for F4/80, but not for CD11b, in the ganglion cell layer was unexpected, and may be a consequence of the particularly high levels of expression of F4/80 by amoeboid ganglion cell layer microglia compared to ramified parenchymal microglia\(^{89}\). Although the immunofluorescent techniques employed in this study were insufficient to detect the CD11b antigens expressed by ganglion cell layer microglia, they clearly identified CD11b cells in the SRS.
Zecher et al. described an innate immune response to allografts mediated by macrophages that is independent of natural killer cells and T-lymphocytes. Graft survival in the early post-operative period is promoted by macrophage inhibition in cellular transplants of pancreatic islet cells and spleen cells. Macrophage depletion via subconjunctival administration of clodronate liposomes also promotes long-term survival of high-risk corneal grafts. We observed a significant macrophage response in the subretinal graft coinciding with the period of graft cell loss during the first post-operative week. It is likely that perioperative macrophage depletion or inhibition may also prove useful in promoting subretinal graft survival.

Having found that DH01 cells prepared for transplantation expressed large amounts of the neutrophil chemoattractant KC/GRO/CINC, we also investigated whether neutrophils infiltrated the subretinal graft. We used an antibody against Gr1 Ly-6G which detects the myeloid differentiation antigen, Gr1, and specifically identifies neutrophils as distinct from monocyte-macrophages via Ly-6G. We found a significant increase in Gr1 Ly-6G+ cells infiltrating the subretinal cell bolus during the first post-operative week (Fig. 3A: i-k and Fig. 3B). Moreover, by POD 7 there was also a significant increase in immunofluorescent co-labeling of transplanted cells with this neutrophil cell marker (Fig. 4F). 3D image reconstruction of areas of co-labeling demonstrated the presence of the graft cell marker internal to the neutrophil cell membrane confirming engulfment of graft cells by infiltrating neutrophils (Fig. 4C-E). Neutrophils have not previously been identified in subretinal grafts.

Neutrophils have recently been implicated as mediators of cardiac graft failure. They are the first leukocyte to infiltrate cardiac allografts, arriving within one hour following transplantation. Neutrophil infiltration also correlates with cardiac allograft rejection severity, and cardiac allograft survival is promoted by neutrophil depletion and inhibition of neutrophil infiltration. Statins inhibit neutrophil transendothelial migration and when used in patients with cardiac allografts they lower the incidence of cardiac allograft vasculopathy and reduce the severity of allograft rejection. Thus, the use of perioperative...
statins may also prove beneficial in suppressing neutrophil infiltration and promoting subretinal graft survival.

T-lymphocyte (CD3-ε) infiltration was not a feature of this study (Fig. 3A: m-p and Fig. 3B). T-lymphocytes have also been notable by their absence in subretinal transplants to the rat26,27, rabbit29-31,85-87 and pig34. This is consistent with the deviant immune environment of the SRS32-34 characterized by a suppressed T-lymphocytic adaptive immune response35,36,104,105. Interestingly, after finding no significant differences between cyclosporine-immunosuppressed and control animals in RPE cell suspension allografts in the rabbit, Crafoord et al. concluded that graft failure was caused by either immunological mechanisms not inhibited by cyclosporine or by non-immunologic events31. The study presented here describes the cellular infiltrate in the SRS at specific time-points following subretinal allograft transplantation. It is possible that a T-lymphocyte response was occurring at the regional lymph nodes, however this possibility was not investigated. Nevertheless, we found that the majority of graft cell loss had occurred by POD 7. This is much earlier than acute allograft rejection mediated by T-lymphocytes, which occurs at 10-13 days following transplantation106.

Lopez et al. suggested that RPE cells transplanted to the SRS of the RCS rat could phagocytize shed outer segments7. This conclusion was made because histology showed pigmented cells incorporated into the host RPE layer, in the SRS and also in between photoreceptor outer segments. These cells were assumed to be transplanted cells. However, the electron microscopy images revealed 10 times as many phagosomes in the putative transplanted RPE cells compared to normal rat RPE cells. The staining and imaging techniques were insufficient to determine if the phagosomes seen were truly in transplanted RPE cells or another cell type. In the present study, the DIC images clearly demonstrate pigment granules in macrophages (Fig. 4A and B) and neutrophils (Fig. 4C). We propose that infiltrating macrophages and neutrophils, rather than transplanted RPE cells may be responsible for the hyperphagocytosis observed by Lopez et al.
Retinal detachment activates retinal microglia\textsuperscript{107}. We observed a considerable infiltrate of macrophages that appeared to arrive from the retinal side of the subretinal graft (Fig. 3A: a, b, f). It is possible that the macrophage infiltration represented activated retinal microglia as CD11b\textsuperscript{108} and F4/80\textsuperscript{109} also identify retinal microglia. The macrophage or microglial response we observed was likely exaggerated due to the high-density cell suspension graft delivered. However, it is conceivable that a low-density cell suspension graft or even a simple subretinal injection of saline may result in lower levels of microglia activation and subretinal infiltration that proves beneficial to the host. Activated retinal microglia may assume a phagocytic phenotype and migrate to the subretinal space, thereby adopting the role of outer segment phagocytosis and enabling photoreceptor rescue. This explanation would theoretically explain the following phenomena of subretinal transplantation observed particularly, though not exclusively, in the RCS rat: (1) transplantation of many different cell types to the SRS results in photoreceptor rescue\textsuperscript{14,16,76,110}, (2) sham surgery has anatomical\textsuperscript{76,111} and functional benefits\textsuperscript{18-21} and even subretinal saline alone promotes photoreceptor survival\textsuperscript{112}; (3) the area of rescue may extend beyond the area of the graft\textsuperscript{17}; (4) macrophages or macrophage-like cells have repeatedly been seen at the graft site\textsuperscript{6,14,26,27,29,31,84-88}; (5) temporal photoreceptor rescue may outlast graft survival\textsuperscript{18}.

Our observation of infiltrating cells expressing the neutrophil marker Gr1 Ly-6G is novel. Neutrophils are distinguished from monocyte-macrophages via their expression of Ly-6G\textsuperscript{95}. Neutrophils in the eye are CD11b\textsuperscript{-}/Gr1 Ly-6G\textsuperscript{+113}. However, neutrophils are negative for F4/80\textsuperscript{114}. It follows therefore that the CD11b\textsuperscript{+} cellular infiltrate observed during the first week following transplantation in the present study may comprise a combination of CD11b\textsuperscript{+} / F4/80\textsuperscript{-} / Gr1 Ly-6G\textsuperscript{-} microglia and CD11b\textsuperscript{+} / F4/80\textsuperscript{-} / Gr1 Ly-6G\textsuperscript{+} neutrophils. However, the immunohistochemical methods in our study could not sub-classify the cellular infiltrate with certainty. Further studies such as multi-labeled flow cytometric analysis of cells extracted from the SRS, or following whole-eye dissociation, would be helpful in more precisely phenotyping the infiltrate. Nevertheless, the data presented here reveal for the first time a critical role for
the innate immune system early in subretinal graft rejection, while T-lymphocytes did not feature at all during the main period of graft loss (POD 3-7).

The rapid loss of graft cells observed in this study was accompanied by comparatively very low levels of graft cell death as examined by TUNEL-labeling (Fig. 2A and C). TUNEL-labeling identifies apoptotic and necrotic cells by detecting DNA strand breaks\(^{80-82}\). However, it is possible that the graft cell loss observed in the present study occurred via a process other than apoptosis or necrosis, such as by autophagy\(^{115,116}\). Autophagic cell death is characterized by increased autophagosomes/autolysosomes and extensive cytoplasmic vacuolization, but with relatively minor changes to the nucleus and chromatin\(^{117}\). Therefore, this mode of cell death could remain undetected by TUNEL-labeling. Autophagic cell death is triggered by stresses including nutrient deprivation\(^{118-121}\) and hypoxia\(^{119}\). In order to limit potential immunological triggers against subretinal cell suspension transplants, such grafts are normally delivered suspended in serum-free medium\(^{18,20,76}\). For this reason, we also suspended DH01 cells in serum-free medium immediately prior to transplantation. It may be that the stresses of serum-deprivation and the other processes involved in preparing a highly concentrated graft cell suspension (TC) trigger autophagic graft cell death. Cells that undergo autophagic cell death may be cleared by phagocytosis\(^{122-124}\) in a process that limits inflammatory and immunological responses\(^{125}\). Consistent with these features of autophagic cell death, there was clear evidence of graft engulfment by macrophages and neutrophils in this study (Fig. 4) and the retina overlying the graft site remained remarkably preserved with no substantial evidence of inflammatory scarring at POD 28 (Fig. 3A: d, h, l, p).

Toll-like receptors (TLRs) are phylogenetically ancient mediators of innate immunity that detect microbes via pathogen-associated molecular patterns (PAMPs)\(^{38,126}\). Stressed and dying cells express endogenous TLR ligands termed damage-associated molecular patterns (DAMPs)\(^{127}\). Activation of the innate immune system via TLRs plays an important role in allograft rejection\(^{39,40}\). Blocking TLR4-mediated graft rejection prolongs pancreatic islet cell transplant survival\(^{43}\). TLR4 is constitutively expressed by RPE cells\(^{128,129}\), photoreceptors\(^{130}\)
and by resident antigen-presenting cells in the normal human uveal tract\textsuperscript{131}. However, there have been no studies to date regarding the role of TLRs in subretinal cell transplantation. Cell-suspension grafts are exposed to many potential stressors including enzymatic cleavage, centrifugation, resuspension in serum-free medium and delivery through non-biological injectors. It is likely that these processes not only incite increased inflammatory cytokine and chemokine production by graft cells as demonstrated in this study, but may also provoke increased expression of DAMPs. Following transplantation the to the SRS, excessive DAMP expression by transplanted cells could propagate an innate immune response by binding host TLRs, thereby triggering host cytokine and chemokine expression capable of recruiting microglia/macrophages from the overlying retina as well as neutrophils from the circulation. DAMPs may also trigger complement-activation\textsuperscript{132,133} and the release of anaphylatoxins capable of mediating leukocyte chemotaxis\textsuperscript{134,135}. Future strategies to suppress the host immune response against cells transplanted to the subretinal space should focus more on limiting the effect of infiltrating macrophages and neutrophils by specifically targeting these mediators of the innate immune response. Potential agents include clodronate\textsuperscript{91,93}, statins\textsuperscript{102,103}, resveratrol\textsuperscript{136,137} and minocycline\textsuperscript{138,139}. Controlling innate immune and inflammatory responses following transplantation will be particularly important in the context of treating conditions such as age-related macular degeneration, which is characterized by increased macrophage infiltration at the affected site\textsuperscript{140-142}.

The rapid innate immune response observed in our study is likely a consequence of multiple inciting factors including increased expression of inflammatory cytokines and DAMPs by cells prepared for transplantation. Such factors are independent of genetic mismatch and would not be restricted to allogeneic cell transplants. Consequently, the innate immune response observed in our study is also likely to occur in response to other cell transplants including syngeneic, autologous and stem cell transplants. The future success of subretinal transplantation will require more emphasis to be placed on optimizing techniques to prepare and deliver grafts of high quality that limit rapid innate
immune-mediated clearance of transplanted cells. Furthermore, pharmacological strategies in the field of subretinal transplantation must go beyond merely suppressing adaptive immunity in the host, and focus on methods of inhibiting the early innate immune response.

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References


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Farrokhi-Siar L, Rezai KA, Semnani RT, Patel SC, Ernest JT, Peterson EJ, Koretzky GA, van Seventer GA. Human fetal retinal pigment epithelial cells induce


89. Dick AD. Influence of microglia on retinal progenitor cell turnover and cell replacement. Eye (Lond) 2009;23(10):1939-45.


### Table 1. DH01 cytokine expression under BC and TC.

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<th>Cytokine</th>
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<sup>a</sup> Values represent pg/ml and are expressed as mean ± SD.
Figure Legends

Figure 1. Retinas from unoperated and sham-treated eyes of C57BL/6 mice contain F4/80$^+$ cells and few TUNEL$^+$ cells, but do not comprise SV40T$^+$, CD11b$^+$, Gr1 Ly-6G$^+$ or CD3-$\epsilon^+$ cells.
Representative confocal microscopy images of sections from unoperated and sham-treated eyes immunolabeled for SV40T (Texas red: a-e; FITC, green: f-y) to identify background expression of the graft cell marker and either TUNEL-labeled (FITC, green) to identify DNA strand nicks (a-e), or immunolabeled for the immune cell surface markers CD11b (f-j), F4/80 (k-o), Gr1 Ly-6G (p-t) or CD3-$\epsilon$ (u-y) (all TRITC, red). All nuclei were counterstained with DAPI (blue). Unoperated and sham-treated retinas demonstrate F4/80$^+$ cells in the ganglion cell layer, and low background levels of TUNEL$^+$ cells in the inner and outer nuclear layers. SV40T$^+$, CD11b$^+$, Gr1 Ly-6G$^+$ or CD3-$\epsilon^+$ cells were not observed. Scale bar 50µm.

Figure 2. DH01 cells allografted to the SRS of healthy non-immunosuppressed C57BL/6 mice are lost by POD 28.

A. Representative confocal microscopy images of sections immunolabeled for SV40T (Texas red) to identify transplanted cells and TUNEL-labeled (FITC, green) to identify DNA strand nicks. All nuclei were counterstained with DAPI (blue). At POD 1 (a) and POD 3 (b) most cells in the subretinal bolus are graft cells, but at POD 7 (c) few cells express SV40T (red). No graft cells are identified at POD 28 (d). TUNEL-labeling in the subretinal cell bolus remains low at each time-point. High-power inset at POD 7 shows an example of an apoptotic/necrotic graft cell (DAPI$^+$/SV40T$^+$/TUNEL$^+$). Scale bar 50µm. Scale bar in high-power inset (c) 10µm. B. There was no significant change in the total number of cells in the SRS between POD 1 (435 ± 143), POD 3 (487 ± 164) and POD 7 (607 ± 45), but the total cell number decreased significantly (p<0.05)
between POD 7 (607 ± 45) and POD 28 (49 ± 49). C. The proportion of the subretinal bolus (red line) comprised of graft cells did not change significantly between POD 1 (93% ± 1%) and POD 3 (90% ± 4%). However, there was a significant (p<0.001) reduction in the proportion of the subretinal bolus comprised of graft cells between POD 3 (90% ± 4%) and POD 7 (20% ± 7%). No graft cells survived to POD 28. Levels of graft cell apoptosis/necrosis (DAPI+/SV40T+/TUNEL−) (green line) were low at each time-point (POD 1: 2% ± 0.3%; POD 3: 0.4% ± 0.3%; POD 7: 5% ± 2%) and did not differ significantly between time-points.

Figure 3. The period of DH01 allograft loss in the SRS of healthy non-immunosuppressed C57BL/6 mice is characterized by infiltrating cells expressing markers of innate immunity, but not T-lymphocytes.

A. Representative confocal microscopy images of sections immunolabeled for SV40T (FITC, green) to identify transplanted cells and either CD11b (a-d), F4/80 (e-h), Gr1 Ly-6G (i-l) or CD3-ε (m-p) (all TRITC, red). All nuclei were counterstained with DAPI (blue). At POD 1 the subretinal graft site is primarily composed of SV40T+ graft cells but by POD 7 the proportion of cells in the subretinal bolus expressing this graft cell marker has considerably decreased. CD11b+ (a-c), F4/80+ (e-g) and Gr1 Ly-6G+ (i-k) cells infiltrate the graft in increasing numbers between POD 1 and POD 7. No graft cells are seen at POD 28 (d, h, l, p). One of 4 eyes at POD 28 still has cells in the SRS and these are F4/80+ (h). Graft cell loss is not associated with CD3-ε+ T-cell infiltration (m-p). Scale bar 50µm. Scale bars in high-power insets 20µm. B. The period of graft cell loss coincides with increasing numbers of infiltrating CD11b+, F4/80+ and Gr1 Ly-6G+ cells that predominate over SV40T+ graft cells by POD 7. However, CD3-ε immunolabeling remains low across all time-points.

Figure 4. Co-labeling of DH01 allografts with infiltrating CD11b+, F4/80+ and Gr1 Ly-6G+ cells peaks at POD 7 with evidence of graft engulfment by these innate immune cells.
Large areas of SV40T were observed to co-label with CD11b+, F4/80+ and Gr1 Ly-6G+ on POD 7. Co-labeling of SV40T with the T-lymphocyte marker (CD3-ε) remained low at all time-points. Representative images of the immunofluorescent co-labeling evident on POD 7 are shown. A. The cell in the center of this image has positive cell membrane immunolabeling for the macrophage marker CD11b (TRITC, red) on POD 7. SV40T (FITC, green) immunolabeling can be seen within this CD11b+ cell suggesting engulfment of graft by the macrophage. The DIC image also identifies pigment granules within CD11b+ cells. Scale bar 20μm. B. Multiple cells in this image have cell membrane immunolabeling for the macrophage marker F4/80 (TRITC, red) on POD 7. SV40T (FITC, green) immunolabeling can be seen within these F4/80+ cells. This suggests engulfment of graft by macrophages. The DIC image also identifies pigment granules within F4/80+ cells. Scale bar 20μm. C. Many cells in this POD 7 subretinal graft have immunofluorescent co-labeling (orange) for Gr1 Ly-6G (TRITC, red) and SV40T (FITC, green). Scale bar 10μm. D. Sequential single optical sections from the z-stack confocal image through the highlighted Gr1 Ly-6G+ neutrophil on POD 7 is consistent with phagocytosis of SV40T from the neutrophil cell membrane. E. The cell highlighted in C was also reconstructed in 3D form using Imaris image analysis software. The red channel (Gr1 Ly-6G) was removed from the top portion of the cell to enable visualization inside the cell membrane. The reconstructed image confirms the presence of graft (SV40T, green) inside the neutrophil cell membrane. F. The proportions of SV40T that co-labeled with CD11b, F4/80, Gr1 Ly-6G and CD3-ε were analyzed. Co-labeling of SV40T with all infiltrating immune cell markers was minimal on POD 1 and POD 3. However, there was a statistically significant increase in the proportion of graft co-labeling with F4/80, CD11b and Gr1 Ly-6G between POD 1 and POD 7 (p<0.05). Co-labeling of SV40T with the T-lymphocyte marker (CD3-ε) remained low at all time-points and there was no statistically significant difference in SV40T/CD3-ε co-labeling between time-points.
Figure 1
Figure 2
Figure 3
Figure 4