LABORATORY SCIENCE

Retinal pigment epithelial cells phagocytosis of T lymphocytes: possible implication in the immune privilege of the eye

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Aim: To investigate the capability of retinal pigment epithelium (RPE) cells to phagocytose T lymphocytes and to further analyse the immunobiological consequences of this phagocytosis.
Methods: Human RPE cells pretreated or not by cytochalasin, a phagocytosis inhibitor, were co-cultured with T lymphocytes for different time points. Phagocytosis was investigated by optic microscopy, electron microscopy, and flow cytometry. T cell proliferation was measured by ^{3}H thymidine incorporation. RPE interleukin 1β mRNA expression was quantified by real time PCR.
Results: RPE cells phagocytose apoptotic and non-apoptotic T lymphocytes, in a time dependent manner. This is an active process mediated through actin polymerisation, blocked by cytochalasin E treatment. Inhibition of RPE cell phagocytosis capabilities within RPE-T cell co-cultures led to an increase of lectin induced T cell proliferation and an upregulation of interleukin 1β mRNA expression in RPE cells.
Conclusions: It is postulated that T lymphocyte phagocytosis by RPE cells might, by decreasing the total number of T lymphocytes, removing apoptotic lymphocytes, and downregulating the expression of IL-1β, participate in vivo in the induction and maintenance of the immune privilege of the eye, preventing the development of intraocular inflammation.

The eye is an immune privileged site, protected from the immune system by different mechanisms.[1] At the level of the posterior segment of the eye, retinal pigment epithelial (RPE) cells have a crucial role in the induction and maintenance of this immune privilege.[2] RPE cells constitute the external blood-retinal barrier, an interface between the retina and the choroidal circulation. Accordingly, one of RPE cell functions is to impede immune effector cells, mainly lymphocytes, to accumulate in the choroid, penetrate the retina, and damage this fragile tissue. Failure in this barrier role leads to a breakdown of the immune privilege with intraocular inflammation development, as happens in posterior uveitis.

Much work has been done to better understand the mechanisms underlying the RPE protection role, especially how these cells interact with lymphocytes. It is now evident that active processes are likely to be involved. Liverdsidge *et al* first showed that RPE cells could inhibit lymphocyte proliferation.[3] From then on, three main pathways have been proposed to explain this RPE immunosuppression.[4–11] Firstly, RPE cells might inhibit T cell proliferation through the secretion of inhibitory factors like prostaglandins and immunosuppressive cytokines. Among the latter, transforming growth factor β, or IL-1 receptor antagonist have been proposed.[4 5] Secondly, the apoptosis phenomenon might also contribute to the RPE induced immunosuppression. Apoptosis is a programmed cell death commonly used by the immune system to eliminate cells, without causing inflammatory reaction, as it is the case during necrosis. Moreover, apoptotic bodies phagocytosis has been shown to downregulate pro-inflammatory cytokines secretion in macrophages.[6] Interestingly, several studies have demonstrated that apoptosis occurs within T cells following co-incubation with RPE cells, suggesting that this could be an important mechanism in RPE immunosuppression.[7–9] Finally, we and others have shown that, by lacking expression of a complete set of co-stimulatory molecules, RPE cells only partially activate lymphocytes and act as deviant antigen presenting cells, driving the

lymphocyte to a state of hyporesponsiveness rather than activation.[10 11]

Considering that human RPE (hRPE) are known as phagocytic cells, since they largely phagocytose photoreceptor outer segments, we postulated that phagocytosis may also contribute to the RPE immunosuppression directly by decreasing T cell number and clearing apoptotic lymphocytes, and indirectly by downregulating pro-inflammatory cytokine secretion. We thus first investigated the capability of RPE cells to phagocytose T lymphocytes and then studied the immunobiological consequences of such phagocytosis.

MATERIALS AND METHODS

Cells

Human RPE (hRPE) cells were isolated from human healthy donor eyes as previously described.[12] Briefly, cells were trypsinised (0.25%) from Bruch's membrane into serum free Dulbecco's Modified Eagle Medium (Gibco BRL, Life Technology, Merelbeke, Belgium). Isolated RPE cells were then grown on large culture flasks at 500 000 cells/flask in complete DMEM medium consisting in DMEM supplemented with 10% fetal calf serum (FCS, Gibco), penicillin 100 U/ml and streptomycin 100 mg/ml (Gibco). HRPE cells were used between passages 3 and 5. The purity of the hRPE cultures was checked by morphological criteria and by immunohistochemistry for positive staining with antibodies against cytokeratins 7, 8a/b, AE-1, AE-3, and CAM-5.2. ARPE-19, a spontaneously arising hRPE cell line was purchased at the American Type Culture Collection (VA, USA) and cultured in a 1:1 mixture of DMEM and Ham's F12 with 2.5 mM L-glutamine, 10% fetal bovine serum, penicillin 100 U/ml, and streptomycin 100 mg/ml. Human peripheral blood mononuclear cells (PBMC) of healthy donors were isolated from buffy coats by density gradient centrifugation on Lymphoprep solution (Nycomed,
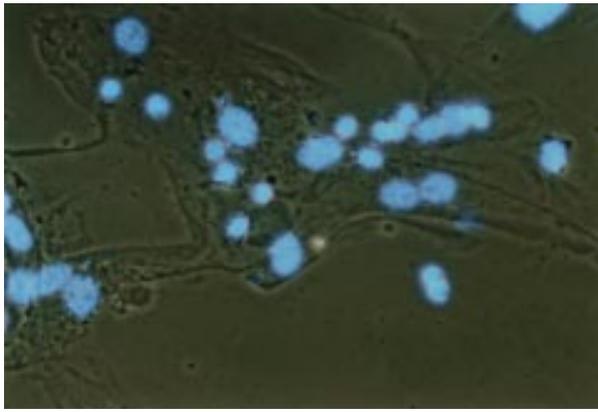
Figure 1 Photomicrograph of Hoechst staining of 24 hour co-culture hRPE cells/T lymphocytes. RPE cells (200 000 cells/well) were allow to adhere overnight at 37°C in 24 well flat bottomed tissue culture plates in complete medium, before allogeneic purified T lymphocytes (500 000 T cells/well) were added. After 24 hours of co-culture, Hoechst 33342, a bisbenzimidazole DNA binding dye was added to the culture at 10 µg/ml, for 30 minutes at 37°C. The supernatant was discarded, and cells trypsinised to disrupt cell-matrix and cell-cell binding, dislodging lymphocytes just attached and not ingested by RPE cells. Thereafter, the collected cells were washed, cytospun, and visualised under ultraviolet light. On the photomicrograph RPE cells (large dark blue nucleus and large cytoplasm) have phagocytosed T cells (small light blue nucleus and no cytoplasm). Magnification ×400.

Oslo, Norway). T lymphocytes were then purified by Lymphokwik T (One Lamba Inc, Los Angeles, CA, USA) according to the manufacturer's procedure. Their purity was greater than 98%.

Cell cultures
HRPE cells, from cultures established from two different donors, or ARPE-19 cells (200 000 cells/well) were allow to adhere overnight at 37°C in 24 wells flat bottomed tissue culture plates (Gibco) in complete DMEM medium, before allogeneic purified T lymphocytes (500 000 T cells/well) were added. Co-cultures were performed for various periods, in a total volume of 1 ml of RPMI 1640 complete medium, consisting of RPMI 1640 supplemented with 10% FCS, 1% glutamine, 1% non-essential amino acid, penicillin 100 U/ml and 100 µg/ml streptomycin, and 5×10^{-5} molar of 2-mercaptoethanol. In some settings, phagocytosis was blocked by pre-incubating adherent RPE cells with cytochalasin E (20 µM) (Sigma, St Louis, MO, USA) for 30 minutes at 37°C.

Hoechst staining
After 24 hours of co-culture, Hoechst 33342, a bisbenzimidazole DNA binding dye (Boehringer Mannheim GmbH) was added to the culture at 10 µg/ml, for 30 minutes at 37°C. Culture supernatant was then discarded and the adherent RPE cells washed to eliminate floating cells. Cells were then trypsinised to disrupt cell-matrix and cell-cell binding. This trypsinisation step is classically used in similar settings to dislodge lymphocytes that were attached and not ingested by phagocytes.[13] Cells were then washed, collected, cytospun, and visualised on an inverted microscope under ultraviolet light.

Electron microscopy
Similarly, after 24 hours co-culture with allogeneic purified T lymphocytes, adherent hRPE cells were carefully washed, trypsinised, collected, and prepared for electron microscopy analysis as described elsewhere.[14] Serial and ultrathin sections were examined in a Philips 30' electron microscope after staining with uranyl acetate and lead citrate.
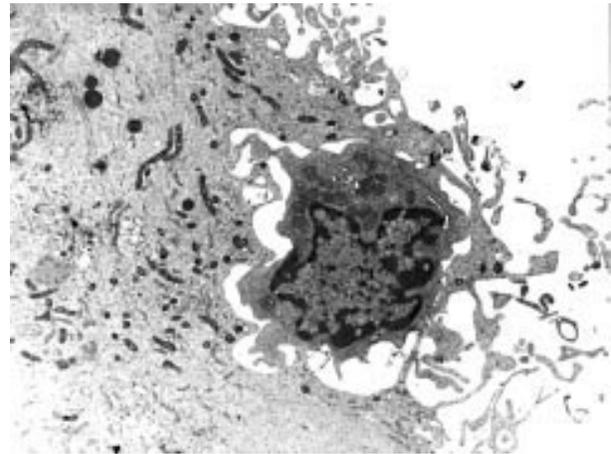
Figure 2 Electron micrograph of phagocytic hRPE cells after 24 hours of co-culture with T lymphocytes. After 24 hours of co-culture with T cells, adherent RPE cells were carefully washed, collected, and prepared for transmission electron microscopy. Electron micrograph of a lymphocyte, without any apoptotic morphological changes being engulfed by a RPE cell. Magnification ×4500.

Analysis of phagocytosis by flow cytometry
T lymphocytes were first labelled with the PKH26-GL Fluorescent Cell Linker Kit (Sigma, St Louis, MO, USA) following manufacturer's instructions; 500 000 red labelled T cells were then added to plated RPE cells as described above. In some experiments, apoptotic T lymphocytes were used; apoptosis was induced by 9 Gy irradiation and checked by Annexin staining. After 2, 6, or 24 hours of co-culture, non-adherent cells were harvested and pooled with trypsinised adherent cells. Experiments with apoptotic cells were not continued till 6 and 24 hours, as by that time, apoptosis could have been induced by RPE cells in the non-apoptotic T cells group. Recovered cells were then washed and resuspended in FACS buffer before being analysed using a BD FACScan flow cytometer and a CELLQuest software (Becton-Dickinson, Mountain View, CA, USA). RPE cells having phagocytosed T cells were identified and quantified as red labelled cells within a Forward scatter (FSC)/side scatter (SSC) dot plot region (G1) targeting RPE cells only.

T lymphocyte proliferation assay
Allogeneic purified T lymphocytes (500 000 cells/ml) were co-cultured, in absence or presence of PHA (at 2 µg/ml), with irradiated (30 Gy) hRPE cells or ARPE-19 cells (500 000 cells/ml) in 96 well flat bottomed tissue culture plates, in a total volume of 200 µl complete RPMI medium. In some settings, adherent RPE cells were pre-incubated in plates with cytochalasin E (20 µM) for 30 minutes at 37°C before addition of T cells. T cell proliferation was measured on day 8, by thymidine incorporation, after a 18 hour pulse with ³H thymidine (1 µCi/well).

Real time RT-PCR for IL-1β and β-actin mRNA
Allogeneic purified T lymphocytes (500 000 cells/ml) were co-cultured, as described above, with ARPE-19 cells (500 000 cells/ml) in 24 well flat bottomed tissue culture plates, in a total volume of 1 ml for 5 hours at 37°C. Total cells were then collected and resuspended in 300 µl lysis buffer from the "MagNA Pure LC mRNA Isolation Kit I" (Roche Diagnostics), which was used to extract mRNA on the MagNA Pure instruments (Roche Diagnostics), following manufacturer's instructions (final elution volume 80 µl). Reverse transcription and real time PCR were performed in one step, following the standard procedure described in the Lightcycler-RNA Master Hybridisation Probes Kit" (Roche Diagnostics), starting from 5 µl of the mRNA preparation. Primers and probes sequence, and PCR conditions have been previously described.[15]

Table 1 Analysis of RPE cells granularity after co-culture with T lymphocytes T lymphocytes (T Ly) were first red labelled using the PKH26 dye and then co-cultured (500 000 T cells) with hRPE cells (200 000 cells). After 24 hours, all cells were prepared for flow cytometry. The FACScan analysis was done within a defined FSC/SSC subregion (G1) where all RPE cells were located, whereas none of the T cells was found. FSC/SSC dot plot analysis of RPE cells reveals increased granularity (SSC value) of the RPE cell population, confirming the engulfment of particles by RPE cells. Results are expressed as values (SSC values) from three independent experiments

	RPE	RPE/T Ly (0 hour)	RPE/T Ly (24 hours)
Exp1	239	240	637*
Exp2	274	217	409*
Exp3	290	245	600*

*Statistically significant with p<0.05 versus time 0 hour.

Statistical analysis

Statistical analysis were performed using a two tail *t* test with 95% confidence interval.

RESULTS

RPE cells phagocytose T cells: microscopy detection

RPE phagocytosis of T cells was first observed by direct microscopy analysis of RPE/T cells co-cultures incubated with the Hoechst DNA binding dye which labels in blue the nucleus of all cells. Figure 1 shows two representative pictures where individual RPE cells (large nucleus in dark blue) have ingested one or more T lymphocytes (small nucleus in light blue). Since cells were recovered by trypsinisation that disrupts cell-matrix but also cell-cell binding, and then washed, it is unlikely that those lymphocytes are only attached to RPE cells.[13] We then looked for ultrastructural details by electron microscopy analysis. Figure 2 strongly suggests that RPE cells have phagocytosed lymphocytes. We found engulfed lymphocytes without any morphological apoptotic changes (Fig 2 left) as well as others with apoptotic features (Fig 2 middle and right).

RPE cells phagocytose T cells: quantitative analysis by flow cytometry

Confluent hRPE cells were co-cultured with red labelled T lymphocytes for 2, 6, or 24 hours before being trypsinised and analysed by flow cytometry. We have defined, within the forward scatter/side scatter (FSC/SSC) dot plot, a subregion G1 corresponding to the large majority of RPE cells, where nearly none Fl-2 positive (red) T cells were localised (<2%, data not shown). Controls consist in RPE cells alone, being Fl-2 negative and T cells alone, being FL-2 positive (data not shown). By performing FSC/SSC dot plot analysis within the G1 subregion we first observed increased granularity of the RPE cell population after 24 hours of co-culture with T cells, confirming that particle ingestion occurred (Table 1. We then quantified T cell phagocytosis by hRPE cells by analysing each individual sample for the presence of FL-2 staining within the G1 subregion. Results are presented as phagocytosis rates evaluated in three independent experiments (Table 2. Quantification of phagocytosis showed that after 2, 6, or 24 hours co-culture respectively, a mean of 13 (SD 5.8%), 25% (9.3%), or 46% (15.5%) of RPE cells have ingested at least one T cell. Table 2 also showed that hRPE cells phagocytosed apoptotic and non-apoptotic cells with the same affinity. Similar results were obtained with ARPE-19 cells (data not shown).

RPE cells phagocytosis of T lymphocytes is an active process.

In order to test if phagocytosis was mediated through actin polymerisation, we performed blocking experiments by pre-incubating adherent hRPE cells or ARPE cells with cytochalasin E 20 µM for 30 minutes at 37°C. As shown in Figure 3 treatment of RPE cells with cytochalasin E inhibited 2 hours of phagocytosis by 70%.

### RPE cells phagocytose T cells: immunobiological relevance

Role of phagocytosis in RPE cell induced T lymphocyte inhibition of proliferation

We have previously described that when T cells were activated with PHA at the same time as being co-cultured with allogeneic irradiated hRPE cells their proliferation rate was strongly decreased compared to incubation with PHA alone.[10] Results

Table 2 Quantitative analysis of RPE cell phagocytosis of T cells; 500 000 red labelled T cells (T Ly) were co-cultured for 2, 6, and 24 hours with overnight plated unlabelled hRPE cells (200 000 cells). T cell apoptosis (T Ly apo) was induced by irradiation at 9 Gy and checked by Annexin-FITC staining. Cells were prepared for flow cytometry and analysed for the presence of red positive cells within the G1 subregion. Values represent percentages of RPE cells having ingested T lymphocytes

Time of culture (hours)	Exp number	RPE/T Ly	RPE/T apo Ly
0	1	<1	<1
	2	<1	<1
	3	<1	<1
	Mean (SD)	<1	<1
2	1	19	14.4
	2	7.3	11
	3	12.9	13.1
	Mean (SD)	13 (5.8)*	12.8 (1.7)
6	1	34.8	ND
	2	ND	
	3	16.2	
	Mean (SD)	25.5 (9.3)	
24	1	62.7	ND
	2	31.9	
	3	43.6	
	Mean (SD)	46 (15.5)*	

°Statistically significant p <0.01versus time 2 hours.
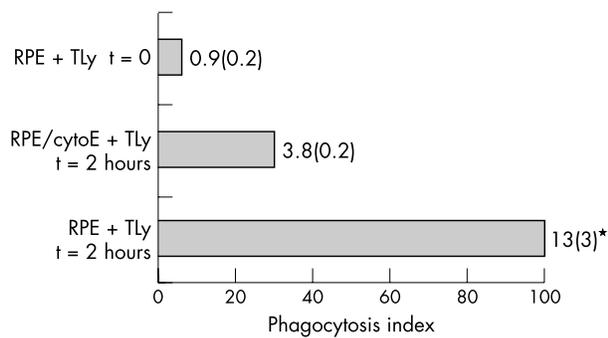
Figure 3 RPE cells phagocytosis of T cells is inhibited by cytochalasin E. 500 000 red labelled T cells were co-cultured for 2 hours at 37°C with overnight plated unlabelled hRPE cells (200 000 cells), resting (RPE) or previously treated for 30 minutes with cytochalasin E (cytoE) at 20 µM. Cells were then prepared for flow cytometry and analysed as described in Table 2. Results are expressed as a phagocytosis index, considering the phagocytosis obtained by 2 hours of co-culture between resting hRPE and T cells as 100. Numbers represent the percentage of phagocytosis, as mean (SD) of three different experiments. *Statistically significant.

Figure 4 RPE cells phagocytose T cells: immunobiological relevance. (A) Role of phagocytosis in RPE cell induced T lymphocyte inhibition of proliferation. T lymphocyte (T Ly) (500 000 cells/well) activated by PHA were co-cultured during 8 days with ARPE-19 cells (500 000 cells/well). In some settings, phagocytosis was blocked before co-culture, by incubating adherent RPE cells with cytochalasin E (20 µM) for 30 minutes at 37°C. T lymphocyte proliferation was measured by ³H thymidine incorporation assay. PHA stimulation of responders T Ly alone was used as positive control. Depicted results are from one representative experiment out of three. (B) Modulation of IL-1β mRNA expression by ARPE-19 cells. Allogeneic purified T lymphocytes (T Ly, 500 000 cells/ml) were co-cultured with ARPE-19 cells (500 000 cells/ml) for 5 hours. In some settings, phagocytosis was blocked before co-culture by incubating adherent RPE cells with cytochalasin E (20 µM) for 30 minutes at 37°C. All cells were collected and submitted to reverse transcription and real time PCR for IL-1β and β-actin mRNA. Results are expressed in IL-1β mRNA copy numbers normalised against β-actin. They are from three independent experiments. *Results are statistically significant, p<0.05.

from one representative experiment out of three (Fig 4A) show that when co-cultures were made with hRPE cells pretreated with cytochalasin, day 8 T cell PHA induced proliferation was somewhat restored. Similar experiments were repeated three times with different order of magnitude but all leading to a restoration of T cell proliferation.
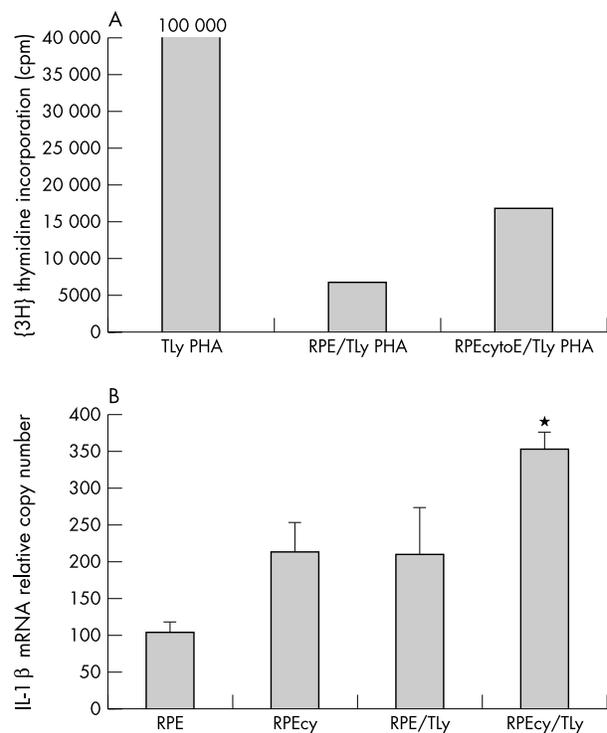
Modulation of IL-1β mRNA expression by ARPE-19 cells

Real time PCR represents a new methodology able to accurately quantify nucleic acids by the use of fluorogenic probes. We applied this methodology to IL-1β and β-actin mRNA quantification in 5 hours co-cultures of RPE/T cells, with or without cytochalasin E pretreatment. Figure 4B shows that blocking T cell phagocytosis by ARPE-19 cells led to an increased IL-1β mRNA expression in ARPE-19 cells. Results are expressed in copy numbers calculated relatively to naive ARPE-19 cells after normalisation against β-actin.

DISCUSSION

It has been known for years that RPE cells phagocytose rods outer segment (ROS), thereby having an essential role in vision.[16] The in vivo importance of ROS phagocytosis is highlighted by the spontaneous degeneration of photoreceptors and blindness of the Royal College of Surgeons rats which results from a defect of such phagocytosis.[17] Yet, RPE phagocytosis capabilities are not limited to ROS, since they are able also to phagocyte latex beads, red blood cells, algae, yeasts, and bacteria.[18] However, to our knowledge, nothing was known about their potential to phagocytose T cells. Here we demonstrated, by three different methods, that RPE cells phagocytose T lymphocytes, in a time dependent manner. In addition, we also found that RPE cells phagocytosed with the same rate apoptotic and non-apoptotic lymphocytes.

Similar phagocytosis of apoptotic and non-apoptotic lymphocytes was described for other cell types having a role in blood-tissue barrier, like oligodendrocytes, astrocytes, and microglia, where it is believed to be an important mechanism regulating CNS inflammation.[19 20] We thus investigated if RPE phagocytosis of T lymphocytes could also have a role in the immune protection of the eye by analysing the modulation in reactivity of each cell partner when RPE phagocytosis was blocked by cytochalasin pretreatment. We first found that inhibition of RPE cell phagocytosis capabilities within RPE-T cell co-cultures resulted in an increased lectin induced T cell proliferation, reflecting a larger remaining number of T cells. Using real time PCR technology, we also detected, in same culture conditions, an upregulation of IL-1β RNA expression

in RPE cells. The latter could result either from a prolonged activation of RPE cells by T cells (more T cells remaining when phagocytosis was blocked), or from a direct downregulation of IL-1β gene expression in phagocytic RPE cells. This last hypothesis is attractive, since it is known that ROS phagocytosis modulates RPE gene expression, notably genes implicated in inflammatory reactions like PPAR and cyclo-oxygenase.[21 22] IL-1β is an important pro-inflammatory cytokine, stimulating the production of other pro-inflammatory cytokines by RPE cells.[23 24] In vivo, its injection into the vitreous of Lewis rats, allows the recruitment of inflammatory cells into the retina and provokes the disruption of the blood-retinal barrier.[25] Hence, our results suggest that if autoaggressive lymphocytes reach the eye through the choroid, they would be phagocytosed by RPE cells, and this phagocytosis would decrease their proliferation rate and the local level of IL-1.

We also found that RPE cells phagocytosed apoptotic lymphocytes. This finding is in agreement with Finneman et al who have shown that RPE cells phagocytosed apoptotic polymorphonuclear cells.[26] Interestingly, the phagocytic clearance of apoptotic lymphocytes, by protecting the neighbour tissues from leakage of toxic contents appears as a key event in the resolution of inflammation. As in other inflammatory

diseases, inflammatory cell apoptosis occurs during uveitis, but it is not yet clear how they are removed.[27] Our results suggest that RPE cells are excellent candidates to achieve this important function in vivo.

In conclusion, we thus postulate that T lymphocyte phagocytosis by RPE cells might, by decreasing the total number of T lymphocytes, removing apoptotic lymphocytes, and down-regulating the expression of IL-1β, participate in vivo in the induction and maintenance of the immune privilege of the eye, preventing the development of intraocular inflammation.

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