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

F Willermain, L Caspers-Velu, B Nowak, P Stordeur, R Mosselmans, I Salmon, T Velu, C Bruyns

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 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. 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. 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. Liverdisge et al first showed that RPE cells could inhibit lymphocyte proliferation. From then on, three main pathways have been proposed to explain this RPE immunosuppression. 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. 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 cytokine secretion in macrophages. 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. 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.

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. Briefly, cells were trypsinised (0.25%) from Bruch's membrane into serum free Dulbecco's Modified Eagle Medium (Gibco BRL, Life Technology, Merselbeke, 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,
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, cytopspun, and visualised under ultraviolet light. On the photomicrograph RPE cells (large dark blue nucleus and large cytoplasm) have phagocyted T cells (small light blue nucleus and no cytoplasm). Magnification ×400.

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 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 FACSscan flow cytometer and a CELLQuest software (Becton-Dickinson, Mountain View, CA, USA). RPE cells having phagocyted 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) (Sigma, St Louis, MO, USA) for 30 minutes at 37°C.

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.24 Serial and ultrathin sections were examined in a Philips 300 electron microscope after staining with uranyl acetate and lead citrate.

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

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 RPE cells. Thereafter, the collected cells were washed, cytopspun, and visualised under ultraviolet light. On the photomicrograph RPE cells (large dark blue nucleus and large cytoplasm) have phagocyted T cells (small light blue nucleus and no cytoplasm). Magnification ×400.
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.

<table>
<thead>
<tr>
<th>RPE</th>
<th>RPE/T Ly (0 hour)</th>
<th>RPE/T Ly (24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp1</td>
<td>239</td>
<td>240</td>
</tr>
<tr>
<td>Exp2</td>
<td>274</td>
<td>217</td>
</tr>
<tr>
<td>Exp3</td>
<td>290</td>
<td>245</td>
</tr>
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</table>

*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 cells were recovered by trypsinisation which destroys the RPE cells. We found engulfed lymphocytes without any morphological apoptotic changes (Fig 2 left) as well as others with apoptotic features (Fig 2 middle and right). Since RPE cells phagocytose T cells: immunobiological relevance
RPE cells phagocytose T cells of T lymphocytes is an active process.
In order to test if phagocytosis wasmediated 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. Results

<table>
<thead>
<tr>
<th>Time of culture (hours)</th>
<th>Exp number</th>
<th>RPE/T Ly</th>
<th>RPE/T Ly apo</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&lt;1</td>
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<tr>
<td></td>
<td>3</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td></td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

| 2                       | 1          | 19       | 14.4         |
|                         | 2          | 7.3      | 11           |
|                         | 3          | 12.9     | 13.1         |
| Mean (SD)               |            | 12.8 [1.7]|              |

| 6                       | 1          | 34.8     | ND           |
|                         | 2          | ND       |              |
|                         | 3          | 16.2     | 22.5 [9.3]   |
| Mean (SD)               |            |          |              |

| 24                      | 1          | 62.7     | ND           |
|                         | 2          | 31.9     |              |
|                         | 3          | 43.6     |              |
| Mean (SD)               |            | 46 [15.5]*|              |

*Statistically significant p <0.01 versus time 2 hours.
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 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 naïve 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.13 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.14 Yet, RPE phagocytosis capabilities are not limited to ROS, since they are able also to phagocytose latex beads, red blood cells, algae, yeasts, and bacteria.15 However, to our knowledge, nothing was known about their potential to phagocytose T cells. Here we demonstrate, 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 immunobiology.20-22 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 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
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diseases, inflammatory cell apoptosis occurs during uveitis, but it is not yet clear how they are removed. 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 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.

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Authors’ affiliations

REFERENCES

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