Inhibition of B-Cell Activation by Retinal Pigment Epithelium

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PURPOSE. To determine whether retinal pigment epithelial (RPE) cells can inhibit B-cell activation in vitro.

METHODS. Primary cultured RPE cells were established from normal C57BL/6 mice. Activated target B cells were established from splenic B cells stimulated with anti-mouse CD40 antibody and lipopolysaccharide (LPS) in the presence of recombinant interleukin 4 (rIL-4). B-cell activation was assessed by examining proliferation through [3H]-thymidine incorporation or carboxyfluorescein succinimidyl ester dilution, and antibody production was determined by ELISA. Expression of costimulatory molecules and the receptors on B cells was evaluated by flow cytometry. Neutralizing anti–TGFβ antibodies were used in the assay.

RESULTS. Addition of primary cultured RPE cells suppressed B-cell proliferation in response to anti-CD40, LPS, and rIL-4 stimulation. Similarly, antibody production by these activated B cells was suppressed. Suppression of B-cell activation was mediated by a soluble factor because supernatants from cultured RPE cells were sufficient to inhibit B-cell responses. Moreover, TGFβ was identified as the soluble mediator given that RPE-supernatants failed to suppress B-cell activation if pretreated with neutralizing anti–TGFβ antibodies.

CONCLUSIONS. Cultured RPE cells suppress the activation of B cells in vitro. These data support the hypothesis that retinal pigment epithelium has immunosuppressive properties that are capable of suppressing B-cell activation. (Invest Ophthalmol Vis Sci. 2010;51:5783–5788 DOI:10.1167/iovs.09-5098

Cognate interactions between B cells and T cells have critical roles during immunologic responses. Activated T cells are able to induce B-cell proliferation and immunoglobulin secretion in a major histocompatibility complex (MHC)-restricted and cell contact-dependent manner. Moreover, B cells play an important role in antigen presentation. Activation of B cells results in higher expression of MHC class II and antigen presentation that can elicit proliferation and cognate help from antigen-specific T cells. These cognate interactions between B and T cells elicit reciprocal activation of both cells, resulting in a potentially inflammatory microenvironment. Generation of an inflammatory response in the eye can be detrimental because it may lead to the destruction of vulnerable intraocular tissues.

More than a decade ago, there were many reports of the detection of infiltrating B cells within inflamed eyes. For example, granulomatous lesions containing T-helper and B lymphocytes were observed in patients with sympathetic ophthalma. Histopathologically, most intraocular lymphomas are classified as diffuse large B-cell lymphomas (a B-cell-type, high-grade malignant non-Hodgkin lymphoma) that produce IL-10. These abnormal infiltrating B cells can affect the retina, choroid, or vitreous or the optic nerve. In animal models of herpes simplex keratitis, immunohistochemical analysis revealed large numbers of infiltrating B cells. These results suggest that intraocular B cells, as well as T cells, have an important role in the generation of inflammatory responses during infectious uveitis, noninfectious uveitis, and intraocular lymphoma.

Recently, several investigators reported that resident ocular tissues/cells can suppress effector T cells that have been activated by cross-linking of CD3. Primary cultured cells and cell lines established from various intraocular tissues greatly suppress the activation of bystander effector T cells in vitro. The immunosuppressive properties of intraocular cells may create immune tolerance to avoid adverse consequences of intraocular inflammation, such as blindness. However, the mechanisms by which resident ocular cells can suppress infiltrating B cells are not yet elucidated.

Therefore, we designed experiments to investigate whether cultured ocular cells are able to suppress the activation of B cells in vitro. To evaluate this, we used primary cultured retinal pigment epithelial (RPE) cells, which have powerful immunosuppressive properties and create immune tolerance in the posterior segment of the eye. Activated target B cells were generated using stimulators such as anti-mouse CD40 antibody and lipopolysaccharide (LPS) in the presence of rIL-4.

MATERIALS AND METHODS

Cultured Retinal Pigment Epithelial Cells

Adult C57BL/6 mice, purchased from CLEA Japan Inc. (Tokyo, Japan), were used as donors for ocular RPE cells. RPE cells were cultured as described previously. Eyes were enucleated and cut into halves along a circumferential line posterior to the ciliary process, creating a ciliary body-free posterior eyecup. The eyecup was incubated in 0.2% trypsin (BioWhittaker, Walkersville, MD) for 1 hour. The RPE tissues were triturated to make a single cell suspension and then resuspended in Dulbecco’s modified Eagle medium (DMEM) complete medium, placed in 24-well plates, and incubated for 2 weeks. DMEM complete medium containing 20% fetal bovine serum (FBS) was used for the primary RPE cultures. We also prepared other ocular PE, iris pigment epithelial (IPE), and ciliary body pigment epithelial (CBPE) cells, as described previously. As determined by flow cytometry, the primary RPE cultures were found to be greater than 98% cytokeratin positive.
Preparation and Activation of Purified B Cells

Purified B cells were prepared separately from donor spleens using isolation kits (MACS Cell Isolation Kits; Miltenyi Biotec, Auburn, CA). These B cells, which were purified by a single immunomagnetic depletion step using MACS magnetic beads, proved to be >95% CD40 positive. Purified B cells (1 x 10^6 cells/well in a 24-well plate) were stimulated with anti–mouse CD40 antibody (clone 1C10; eBioscience, San Diego, CA), LPS (Sigma Chemical Co., St. Louis, MO), and recombinant mouse IL-4 (100 U/mL; eBioscience). Depending on the individual experiment, the concentration of the soluble anti–CD40 in these cultures ranged from 0.01 to 10 μg/mL; for LPS concentrations, it ranged from 0.1 to 100 ng/mL. Proliferation assays lasted from 1 to 5 days. Cultured RPE cells (5 x 10^5 cells/well) were added to B cells at the initiation of oCD40, LPS, and IL-4 stimulation.

Evaluation of B-Cell Activation

After 72- or 96-hour incubation, the cultures were assayed for uptake of [3H]-thymidine (1 μCi/mL for the terminal 8 hours of culture), as a measure of B-cell proliferation. Incorporated radioactivity was measured by liquid scintillation counting and expressed as counts per minute (cpm).

Labeling of B cells with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) was performed as described in previous reports. Briefly, 5 x 10^6 B cells were diluted in 1 mL serum-free HBSS; 1 μM CFSE was added, and the cell suspension was incubated for 8 minutes at room temperature. Purified B cells (5 x 10^6) were labeled or were not labeled with CFSE at a final concentration of 1 μM (controls) and then were added (1.5 x 10^6/well in 24-well plates) to wells containing anti–CD40 mAb, LPS, and rIL-4 in the presence or absence of RPE cells. After 96 hours, the B cells exposed to RPE were washed and analyzed by flow cytometry.

The amounts of IgG in the supernatant of B cells exposed to RPE were measured by ELISA (mouse IgG quantitation kit; Bethyl Laboratories, Inc., Montgomery, TX).

Flow Cytometry

Flow cytometry was used to analyze the expression of surface molecules/receptors such as CD40, CD80, CD86, CD152, and TGFβ receptor on B cells. The following antibodies were used to stain the RPE-exposed B cells and control B cells: FITC-conjugated anti–mouse CD40 (clone 1C10; eBioscience). PE-conjugated anti–mouse CD152 (clone RIIB; BD Biosciences), PE-conjugated anti–mouse MHC class II antibody (clone M5/114.15.2; eBioscience), and PE-conjugated anti–mouse CD80 (clone 2.4G2; BD Biosciences). To confirm the capacity of RPE cells, we used different concentrations of anti–CD40 antibody or LPS. First, B cells were stimulated with anti–CD40, 0.01 to 1.0 μg/mL (LPS 10 ng/mL and rIL-4 100 U/mL) in the presence or absence of RPE. As revealed in Figure 1C, RPE cells significantly suppressed the activation of B cells with 1 or 10 μg/mL anti–CD40, whereas B cells stimulated with 0.01 or 0.1 μg/mL anti–CD40 were not suppressed. Next, we titrated the amount of LPS, ranging from 0.1 to 100 ng/mL LPS (anti–CD40 1 μg/mL and rIL-4 100 U/mL). RPE cells significantly suppressed activated B cells over the entire range of LPS concentrations tested, especially 10 to 100 ng/mL (Fig. 1D). In further experiments, we used target B cells stimulated with 1 μg/mL anti–CD40, 10 ng/mL LPS, and 100 U/mL rIL-4 because these were optimal conditions for RPE suppression.

To determine the duration of RPE cell suppression, B-cell proliferation was followed over time. As can be seen, B-cell proliferation peaked on day 4 (Fig. 1E), whereas RPE cells had little effect on the initial burst of B-cell proliferation on day 1, and RPE cells were able to significantly suppress proliferation over the full duration of the time course examined.

Next, we investigated whether other PE cells located on the anterior segment of the eye, IPE, and CBPE could suppress the activation of B cells. As expected, both types of PE cells significantly suppressed B-cell proliferation, as did RPE cells (Fig. 1F).

Next we examined whether RPE cells could suppress antibody production by activated B cells. As revealed in Figure 1G, IgG production by activated B cells was significantly suppressed. Together, these results indicate that cultured RPE cells have the capacity to suppress the activation of B cells in vitro.
Inhibition of B-Cell Proliferation by RPE Cells via Soluble Inhibitory Factors

To elucidate the role of cell contact in the suppression of B-cell activation by RPE cells, we physically separated the cells (Transwell; Corning, Inc., Corning, NY). As expected, in the absence of an appropriate insert (Transwell; Corning, Inc.), activated B-cell proliferation was greatly suppressed, as shown in Figure 2. Interestingly, RPE cells significantly suppressed B-cell proliferation across the membrane (Transwell; Corning, Inc.). To confirm that cell contact was not required, RPE cell supernatants were added, and B-cell proliferation was suppressed by the RPE supernatants in a dose-dependent manner (Fig. 2). These results suggest that RPE cells are able to suppress B-cell activation by soluble factors.

Expression of Costimulatory Molecules by RPE-Exposed B Cells

B cells constitutively express CD40,17 and B cells stimulated by anti–CD40 antibody and IL-4 upregulate MHC class II and costimulatory molecules, such as CD80 and CD86.18 We evaluated the expression of costimulatory molecules (ligand/receptors) on B cells activated by anti–CD40 antibody, LPS, and IL-4 in the presence of retinal pigment epithelium. Activated B cells in the presence or absence of RPE cells were stained with anti–mouse CD40, CD80, and CD86 antibodies and were analyzed by flow cytometry. B cells stimulated with anti-CD40, LPS, and IL-4 showed high CD40, CD80, and CD86 expression (Fig. 3). In contrast, B cells exposed to RPE showed low expression compared with control cultures, indicating that the expression was downregulated when RPE cells were cocultured with activated B cells. The expression of CD152 costimulatory receptor, which binds to CD80 or CD86, was poor in both B cells (Fig. 3). MHC class II expression was upregulated, but similar levels were observed on B cells from both culture conditions (data not shown). In contrast, RPE-exposed B cells expressed TGFβ receptor type II (TGFβ RII) at much higher levels than did control B cells (Fig. 3). These studies demonstrate that though exposure to RPE cells results in the downregulation of costimu-
B cells positively regulate immune responses through antibody production and optimal helper T-cell activation. Murine B cells express Toll-like receptor-4 (TLR-4), which binds LPS, and, therefore, can be activated in response to LPS stimulation. B cells constitutively express CD40, which is another important stimulatory receptor for B-cell function. In humans and mice, activated T cells highly express CD40 ligand (CD40L; CD154), and cognate B-cell activation requires CD40-CD40L signals. Activation of B cells by stimulation of both LPS and CD40 leads to upregulated expression of MHC class II and costimulatory molecules, such as CD80 and CD86. In addition, CD4<sup>+</sup> T cell–produced IL-4 is necessary for B-cell activation and differentiation. Under CD40 and IL-4 signals, B cells differentiate into antibody-producing cells. Therefore, optimal B-cell activation can be achieved by TLR activation with CD40-CD40L signals in the presence of IL-4. We found that cultured RPE cells fully and significantly suppressed anti-CD40, LPS, IL-4–treated B-cell responses, and we also showed the suppression of immunoglobulin secretion of B cells in the presence of RPE cells. B cells exposed to ocular resident cells or soluble factors such as TGFβ produced by the ocular cells were suppressed.

Ocular pigment epithelia of the retina have been identified as important participants in helping to create and maintain immune tolerance. Previously, several investigators demonstrated that cultured RPE cells suppress T-cell activation through the production of immunosuppressive factors. Moreover, cultured RPE cells established from healthy mice can suppress the activation of T cells by secreting TGFβ. Thus, RPE cells produce and secrete immunoregulatory soluble factors. RPE cells, might be a critical mechanism by which activated B cells fail to proliferate in the presence of rTGFβ.
such as CD40, CD80, and CD86 in DCs. In addition, TGFβ plays an important role in maintaining immune tolerance. The TGFβ receptor in the presence of RPE (Fig. 3) and that B-cell suppression by RPE was dependent on soluble TGFβ. For example, B cells proliferated significantly in the presence of RPE supernatants pretreated with anti-TGFβ blocking antibody (Fig. 4C). However, B-cell proliferation was only partially restored if the supernatants were pretreated with anti-TGFβ alone, suggesting that other soluble inhibitory factors or other cell surface molecules expressed on RPE are involved in the suppression of B-cell activation. Optimal activation of both B and T cells requires the receipt of reciprocal stimulatory signals during the cognate interaction between B and T cells. Given that RPE cells have been shown to regulate T cells and now B cells, ocular pigment epithelium makes important contributions to maintaining immune tolerance in the eye.

TGFβ has potent immunoregulatory effects that act on both T cells and B cells. It strongly inhibits immunoglobulin secretion in human and murine activated B cells and can induce apoptosis in human B cells. Recombinant TGFβ significantly suppresses B-cell function and antigen-specific T-cell responses in vitro, suggesting that this factor may play an important role in the regulation of the antigen-dependent immune responses. In antigen-dependent immune responses, B cells and dendritic cells (DCs) function as antigen-presenting cells. DCs, which are well-known for their T cell-stimulatory properties, strongly affect B-cell growth and immunoglobulin secretion. TGFβ can suppress DC functions and interfere with DC maturation, indicating its ability to suppress the expression of costimulatory molecules such as CD40, CD80, and CD86 in DCs. In addition, TGFβ plays critical roles in T-cell suppression and regulatory T-cell induction in the inflamed eye. Thus, TGFβ is a common denominator in various suppressive mechanisms elicited by cultured RPE cells.

In conclusion, cultured RPE cells, through soluble inhibitory factors, inhibit B cells that have been simultaneously activated through CD40, TLR-4, and the IL-4 receptor. The TGFβ/IL-10 pathway, known to be a powerful inhibitory factor within the eye, is one of the important immunomodulatory mechanisms by which infiltrating B cells are suppressed in the eye during inflammation.

Acknowledgments

The authors thank Ikuyo Yamamoto for expert technical assistance.

References


