

# Retinal Pigment Epithelial Cells Suppress Phagolysosome Activation in Macrophages

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**PURPOSE.** The eye is an immune-privileged microenvironment that has adapted several mechanisms of immune regulation to prevent inflammation. One of these potential mechanisms is retinal pigment epithelial cells (RPE) altering phagocytosis in macrophages.

**METHODS.** The conditioned media of RPE eyecups from eyes of healthy mice and mice with experimental autoimmune uveitis (EAU) were used to treat primary macrophage phagocytizing pHrodo bacterial bioparticles. In addition, the neuropeptides were depleted from the conditioned media of healthy RPE eyecups and used to treat phagocytizing macrophages. The conditioned media from healthy and EAU RPE eyecups were assayed for IL-6, and IL-6 was added to the healthy conditioned media, and neutralized in the EAU conditioned media. The macrophages were treated with the conditioned media and assayed for fluorescence. The macrophages were imaged, and the fluorescence intensity, relative to active phagolysosomes, was measured. Also, the macrophages were assayed using fluorescent viability dye staining.

**RESULTS.** The conditioned media from healthy, but not from EAU RPE eyecups suppressed phagolysosome activation. Depletion of the neuropeptides alpha-melanocyte-stimulating hormone and neuropeptide Y from the healthy RPE eyecup conditioned media resulted in macrophage death. In the EAU RPE eyecup conditioned media was  $0.96 \pm 0.18$  ng/mL of IL-6, and when neutralized the conditioned media suppressed phagolysosome activation.

**CONCLUSIONS.** The healthy RPE through soluble molecules, including alpha-melanocyte-stimulating hormone and neuropeptide Y, suppresses the activation of the phagolysosome in macrophages. In EAU, the IL-6 produced by the RPE promotes the activation of phagolysosomes in macrophages. These results demonstrate that under healthy conditions, RPE promotes an altered pathway of phagocytized material in macrophages with implications on antigen processing and clearance.

**Keywords:** immune privilege, retinal pigment epithelial cells, macrophages, phagocytosis, experimental autoimmune uveitis, IL-6, alpha-melanocyte stimulating hormone, neuropeptide Y

The retinal pigment epithelial cells (RPE) contribute to the mechanisms of ocular immune privilege through contact and soluble molecules that suppress inflammation mediated by innate and adaptive immune cells.<sup>1-8</sup> The RPE suppress effector T cells and promote T-regulatory (Treg) cell activity, activate latent TGF- $\beta$ 2, and through soluble factors, like neuropeptides, suppress inflammatory activity of monocytes.<sup>5-7,9,10</sup> Two neuropeptides, alpha-melanocyte stimulating hormone ( $\alpha$ -MSH) and Neuropeptide Y (NPY), produced by RPE, suppress endotoxin-induced inflammatory activity in macrophages.<sup>6</sup> Moreover, they induce anti-inflammatory IL-10 production by the macrophages. In addition, the presence of  $\alpha$ -MSH and NPY counters an unidentified apoptotic signal from RPE to macrophages.<sup>5,11,12</sup>

These two neuropeptides induce coexpression of nitric oxide synthase 2 and Arginase-1 in macrophages.<sup>5</sup> This is characteristic of suppressor cells, often found as tumor-associated macrophages, and retinal microglial cells also coexpress both enzymes.<sup>5,13</sup> In addition, the neuropeptides regulate the phagocytic potential of macrophages. Macrophages treated with  $\alpha$ -MSH and NPY are suppressed in their phagocytic uptake of unopsonized gram-negative and gram-positive bacterial bioparticles.<sup>14</sup> This may be related to how

these neuropeptides alter innate-immune receptor signals by the bacteria.<sup>15-18</sup> The neuropeptides have no effect on Fc-mediated phagocytosis.<sup>14</sup> The opsonized bioparticles are readily taken up by macrophages treated with both  $\alpha$ -MSH and NPY, but there is suppression of phagolysosome activation.<sup>14</sup> Therefore, the effects of the two neuropeptides is to suppress scavenger pathways of phagocytosis, but not Fc-receptor (FcR)-mediated phagocytosis; however, processing of the phagocytized material would have to go through a pH-neutral degradation.<sup>19</sup> Because the FcR-mediated phagocytic pathways are directly linked to activation of proinflammatory cytokines,<sup>20,21</sup> it suggests that the RPE through these two neuropeptides permit monocyte phagocytic activity within the retina, but it is with an altered processing of material to prevent innate-mediated inflammation, and prevent the possibility of autoantigen presentation.

These previous studies into ocular regulation of phagocytic activity used cell lines and in vitro neuropeptide treatment of macrophages.<sup>12,14</sup> It not known if RPE mediate this suppression of FcR-mediated phagocytosis. In addition, it is not understood if there is a change in an eye with uveitis. In autoimmune uveitis, there has to be presentation of autoantigens by monocytes that are recognized by autoantigen-specific effector



T cells. This would require the monocytes to process phagocytized material through a conventional phagolysosome pathway to generate T cells recognizing peptides for presentation. Using RPE eyecups and primary macrophages, we demonstrate that RPE did suppress phagolysosome activation, and that the production of IL-6 by RPE in uveitic eyes promotes phagolysosome activation.

## MATERIALS AND METHODS

### Preparation of Mouse RPE Eyecups

All mouse procedures described in this study were approved by the Boston University Institutional Animal Care and Use Committee, and adheres to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME), and housed in the Boston University Animal Science Center.

The RPE eyecups were made as described by Kawanaka and Taylor.<sup>5</sup> Eyes from euthanized mice were enucleated and dissected in ice-cold serum-free media (SFM), RPMI 1640 (Lonza, Walkersville, MD, USA) supplemented with 10 µg/mL gentamycin (Sigma-Aldrich Corp., St. Louis, MO, USA), 10 mM HEPES, 1 mM sodium pyruvate, and nonessential amino acids from Lonza with 0.2% ITS+1, and 0.1% BSA from Sigma-Aldrich Corp. The connective tissue and optic nerve were excised from the eyeball. Then a circumferential cut was made just below the ciliary body to remove the entire anterior portion of the eye, including the cornea, iris, ciliary body, and lens. The neural retina was gently lifted off the RPE monolayer using microsurgical forceps and discarded. The remaining posterior segment of the eye (RPE eyecups) containing the RPE monolayer, choroid, and sclera was placed into the well of a 96-well round bottom tissue culture plate (Corning, Corning, NY, USA) containing 200 µL SFM. The cultures were incubated for 24 hours at 37°C in 5% CO<sub>2</sub>, and a 100-µL aliquot of conditioned media (CM) was isolated from each well, centrifuged at 2100g for 1 minute, and the supernatant was used in the assays as RPE eyecup CM.

### Depletion of $\alpha$ -MSH and NPY

Depletion of  $\alpha$ -MSH and NPY from the RPE eyecup CM was done by adding antibodies specific to  $\alpha$ -MSH and NPY (Peninsula Labs, San Carlos, CA, USA).<sup>5,6</sup> To control for the absorption, an irrelevant rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used instead of the specific antibodies. Antibody concentrations were 2 µg/mL, that were far in excess of what is needed to deplete the neuropeptides.<sup>5</sup> The antibody-CM mixture was incubated 1 hour at 4°C. To the mixtures, Protein-A/G-coated magnetic-beads (Biotool.com, Houston, TX, USA) were added, and the mixture was incubated for an additional 30 minutes at 4°C. The tubes with the mixture were placed on a Dynal magnetic particle concentrator for microtubes (ThermoFisher, Grand Island, NY, USA), for 3 minutes and the supernatant (depleted RPE CM) was collected, and used to treat the macrophages.

### Experimental Autoimmune Uveoretinitis (EAU)

Mice were immunized for EAU as previously described.<sup>22</sup> Briefly, an emulsion of complete Freund's adjuvant with 5 mg/mL desiccated *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI, USA) and 2 mg/mL interphotoreceptor retinoid binding protein peptide amino acids 1–20 (Genscript, Piscataway, NJ, USA) was used to immunize mice for EAU. A volume of 100 µL of the emulsion was injected subcutaneously

at two sites in the lower back followed by an intraperitoneal injection of 0.3 µg pertussis toxin (Sigma-Aldrich Corp.). Every 3 to 4 days, the mice iris was dilated with 1% tropicamide (Akorn, Lake Forest, IL, USA) and the cornea was numbed with 0.5% proparacaine (Akorn), and the retina was examined using a slit lamp microscope through a cornea flattened with a glass coverslip. The severity was scored on a standard 5-point scale based on the clinical signs of observable infiltration and vasculitis in the retina as previously described.<sup>22</sup> When all the mouse eyes reached a score of 3 (21 days after immunization), the maximum possible score under our current animal facility conditions, the mice were euthanized and the eyes enucleated for RPE eyecups.

### Macrophage Cultures and Phagolysosome Assay

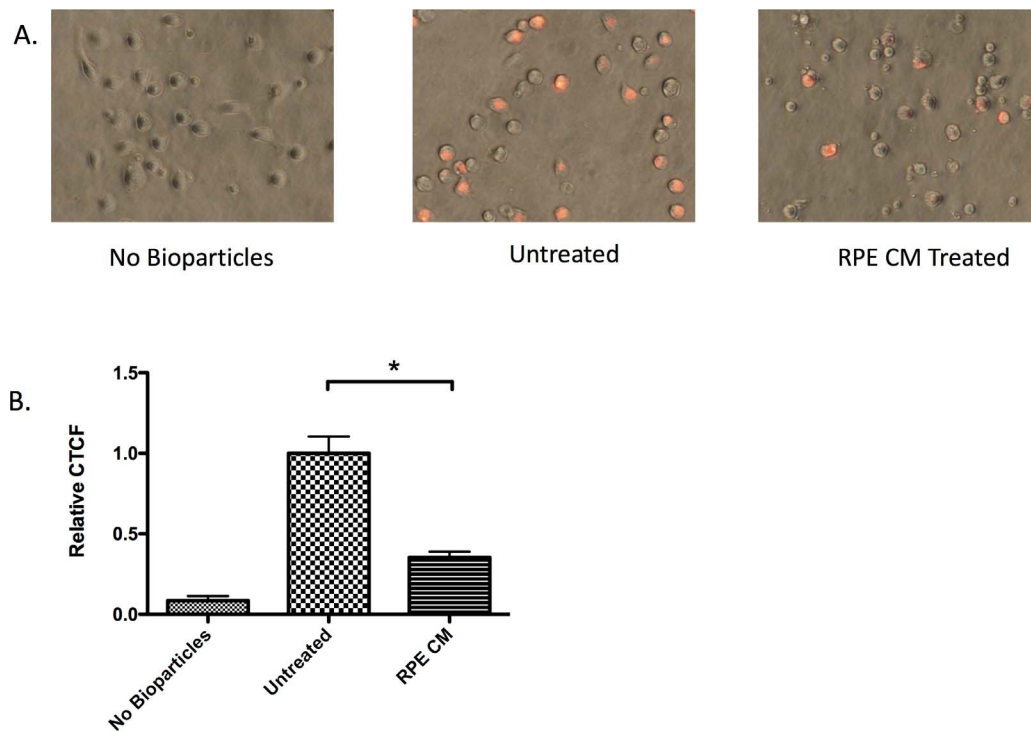
The assay for phagosome activation was done using primary resting macrophages obtained from a peritoneal lavage of naive mice. The cells were collected by recovering 5 mL 0.01 M PBS (Lonza) injected directly into the peritoneal cavity. The cells were centrifuged at 400g for 5 minutes, then mixed with 1 mL red blood cell lysing buffer (Sigma-Aldrich Corp.), iced for 5 minutes, and then centrifuged again at 400g for 5 minutes. The cells were suspended in RPMI 1640 with 10% fetal bovine serum (Hyclone, Logan, UT, USA), and seeded into the wells of a Nunc Lab-TekII 8-chamber slide (ThermoFisher) at  $1.25 \times 10^5$  cells per well. The cultures were incubated for 2 hours at 37°C in 10% CO<sub>2</sub>. Each well was washed once with 200 µL SFM. Into chambers of the macrophage cultures was added 200 µL of SFM, or a 1:2 dilution of RPE eyecup CM from healthy or EAU eyes, diluted CM of EAU RPE eyecups with 1 µg/mL of neutralizing anti-IL-6 antibody (R&D Systems, Minneapolis, MN, USA), or 1 ng/mL IL-6 (R&D Systems) added to the diluted CM of healthy RPE eyecups, or the neuropeptide-depleted CM. The cultures were incubated for 30 minutes at 37°C in 5% CO<sub>2</sub>. To each well was added opsonized pHrodo-*Staphylococcus aureus* beads (0.25 µg) from Invitrogen (ThermoFisher), and incubated at 37°C in 5% CO<sub>2</sub> for 24 hours. The wells were washed twice with 200 µL phenol-red-free SFM before imaging. Fluorescent microscopy was performed using a FSX100 inverted fluorescent imaging-microscope (Olympus, Center Valley, PA, USA). Five images were captured from each well at a constant exposure and time for all the wells in the experiment. The fluorescence minus the background was calculated for each image, and the relative fluorescence was calculated using the mean fluorescence of the untreated macrophages as the maximum value of activity.

### Viability Assay

The macrophages in the eight-well chambered slides treated with RPE CM, or neuropeptide-depleted RPE CM were incubated for 24 hours at 37°C in 5% CO<sub>2</sub>. Into each of the wells was added 10 mM 5-chloromethylfluorescein diacetate (CellTracker Green CMFDA; Molecular Probes, Eugene, OR, USA) in the last 30 minutes of the incubation. The cells were imaged using the FSX100 microscope. Five images were captured from each well at a constant exposure and time for all the wells in the experiment. The fluorescence minus the background was calculated for each image, and the relative fluorescence was calculated using the mean fluorescence of the untreated macrophages as the maximum value of viability.

### Quantification of RPE Cytokines and IL-6

The quantification of cytokines in the CM of RPE eyecups from healthy and EAU eyes were assayed by a Quantibody Mouse Cytokine Array 1 (RayBiotech, Inc., Norcross, GA, USA). This



**FIGURE 1.** The effects of RPE CM on phagolysosome activation. Conditioned media were collected from 24-hour cultures of healthy RPE eyecups (RPE CM). Primary resting macrophages were treated with the RPE CM, and fed opsonized pHrodo-bioparticles. The cells were incubated for 24 hours, imaged by microscopy, and fluorescent intensity measured. (A) Presented are representative images of the pHrodo-bioparticle expression in the macrophages. (B) The relative fluorescent intensity was calculated versus untreated macrophage cultures, and presented are the mean  $\pm$  SEM of three independent experiments. Significant suppression,  $*P \leq 0.001$ , of phagolysosome activation was seen in cells treated with RPE CM.

array assays for mouse IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, IL-13, IFN $\gamma$ , IL-3, IL-5, IL-9, IL-12, IL-17, TNF- $\alpha$ , macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, keratinocyte-derived chemokine, macrophage inflammatory protein-1, regulated on activation normal T-cell expressed and secreted, and VEGF. The CM was further assayed for a single cytokine IL-6 by ELISA (R&D Systems).

### Statistics

Statistical significance between the relative fluorescence was calculated using a nonparametric Mann-Whitney *t*-test. All experiments were independently repeated three times with each condition duplicated in each experiment. The presented data are a pooling of the results from all three independent experiments. Cytokine concentrations were analyzed by 1-sample *t*-test for being significantly different from a value of 0. Differences were considered significant when  $P \leq 0.05$ .

## RESULTS

### Suppression of Phagolysosome Activation by RPE

The CM of established confluent monolayers of ARPE-19 cell cultures suppress the activation of phagolysosomes within macrophages.<sup>12</sup> This was detected by treating the macrophages with the CM, and feeding the macrophages with opsonized pHrodo-bioparticles that become fluorescent in the pH 4 activated lysosome. The CM of cultured healthy RPE eyecups was assayed to see whether primary RPE suppress phagolysosome activation (Fig. 1). The fluorescent micrographs showed a noticeable suppression in phagolysosome activation in the RPE CM-treated macrophages. When this was quantified, there

was a significant 75% suppression in phagolysosome activation. Therefore, as suggested by the CM of confluent ARPE-19 cell line cultures, primary RPE do produce soluble factors that suppress activation of the phagolysosome.

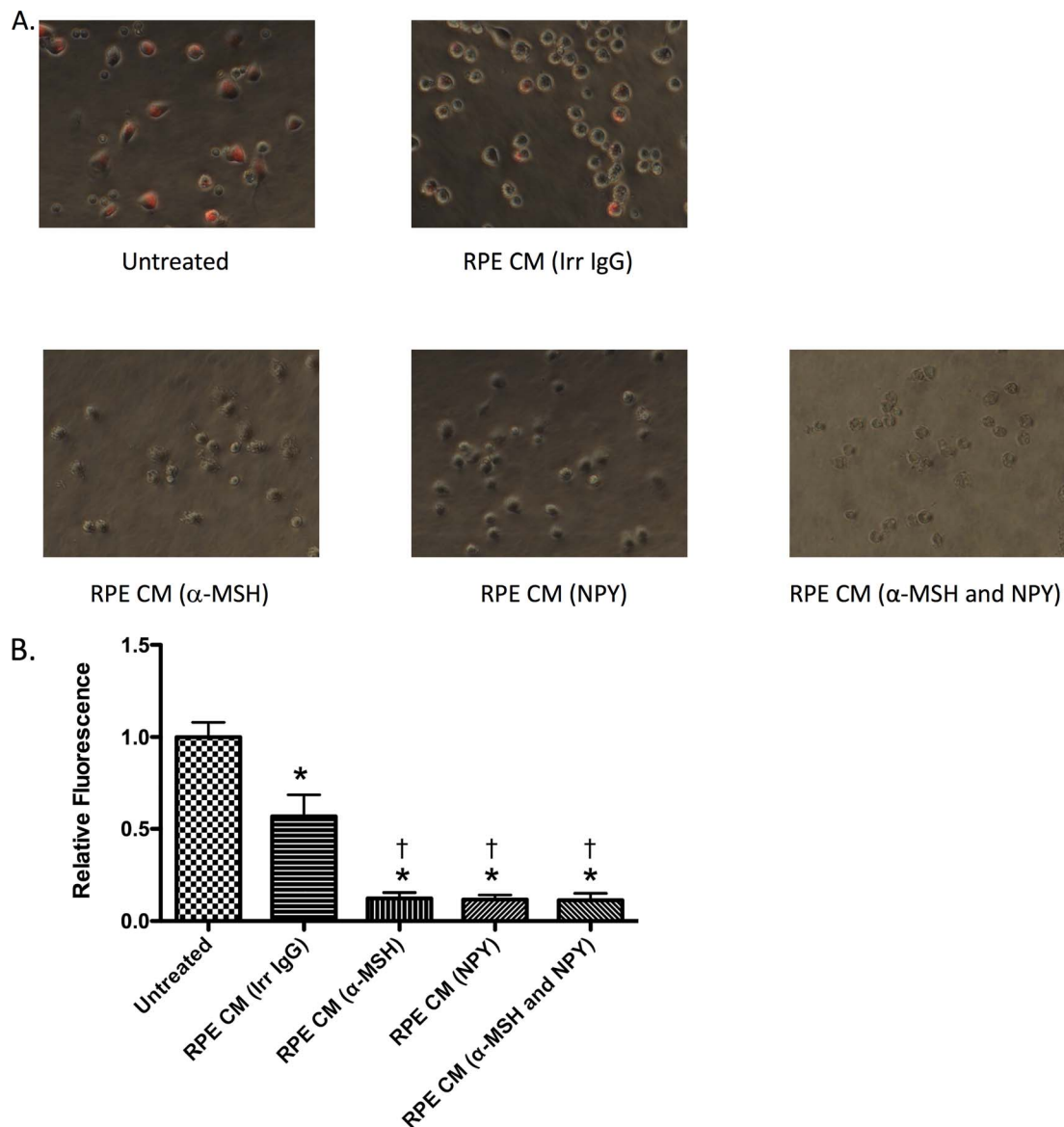
### The RPE $\alpha$ -MSH and NPY Act as Survival Signal for Macrophages

Previous work demonstrated that  $\alpha$ -MSH and NPY suppress phagolysosome activation while promoting suppressor activity by the macrophages.<sup>5,14,23</sup> The CM of the healthy RPE eyecups were depleted of  $\alpha$ -MSH and NPY before treating the macrophages fed with the opsonized pHrodo-bioparticles (Fig. 2). The results demonstrated that removing these neuropeptides from the CM had no effect on the RPE CM suppression of phagolysosome activation; however, the micrographs show the depleted RPE CM-treated macrophages do not look healthy. Using a viability stain, depleted RPE CM-treated macrophages were significantly reduced in viability (Fig. 3). This showed as we had previously reported that RPE release a soluble death signal that is neutralized by the presence of  $\alpha$ -MSH and NPY.<sup>5</sup> Our previous work showed that the individual neuropeptides  $\alpha$ -MSH and NPY suppressed the phagocytic pathway in macrophages. Therefore, in the RPE CM,  $\alpha$ -MSH and NPY are functioning in a similar manner as we have seen before.

### Uveitis Permits Activation of the Phagolysosome

To see whether the regulation of phagolysosome activation is changed in uveitic eyes, RPE CM from eyes of mice at maximum EAU (21 days after immunization) were used to treat macrophages fed the opsonized pHrodo-bioparticles (Fig. 4). There was no statistical difference in the phagolysosome





**FIGURE 2.** The effects of depleting  $\alpha$ -MSH and NPY from RPE CM on phagolysosome activation. Conditioned media from healthy RPE eyecup cultures were treated with antibodies to  $\alpha$ -MSH and NPY, and absorbed with Protein A/G-coated beads. An irrelevant rabbit IgG (irr IgG) was used as a control. The primary resting macrophages were treated with the absorbed CM and opsonized pHrodo-bioparticles. After 24 hours, the cells were imaged and fluorescent intensity was measured. The presented results are (A) representative images of the treated macrophages, and (B) the mean  $\pm$  SEM of the relative fluorescent intensity of three independent experiments. Depletion of  $\alpha$ -MSH and NPY had no effect on detecting suppressed phagolysosome activation by the RPE CM. \*Significantly different  $P \leq 0.001$  to untreated macrophages, and † $P \leq 0.001$  with RPE CM (irr IgG)-treated macrophages.

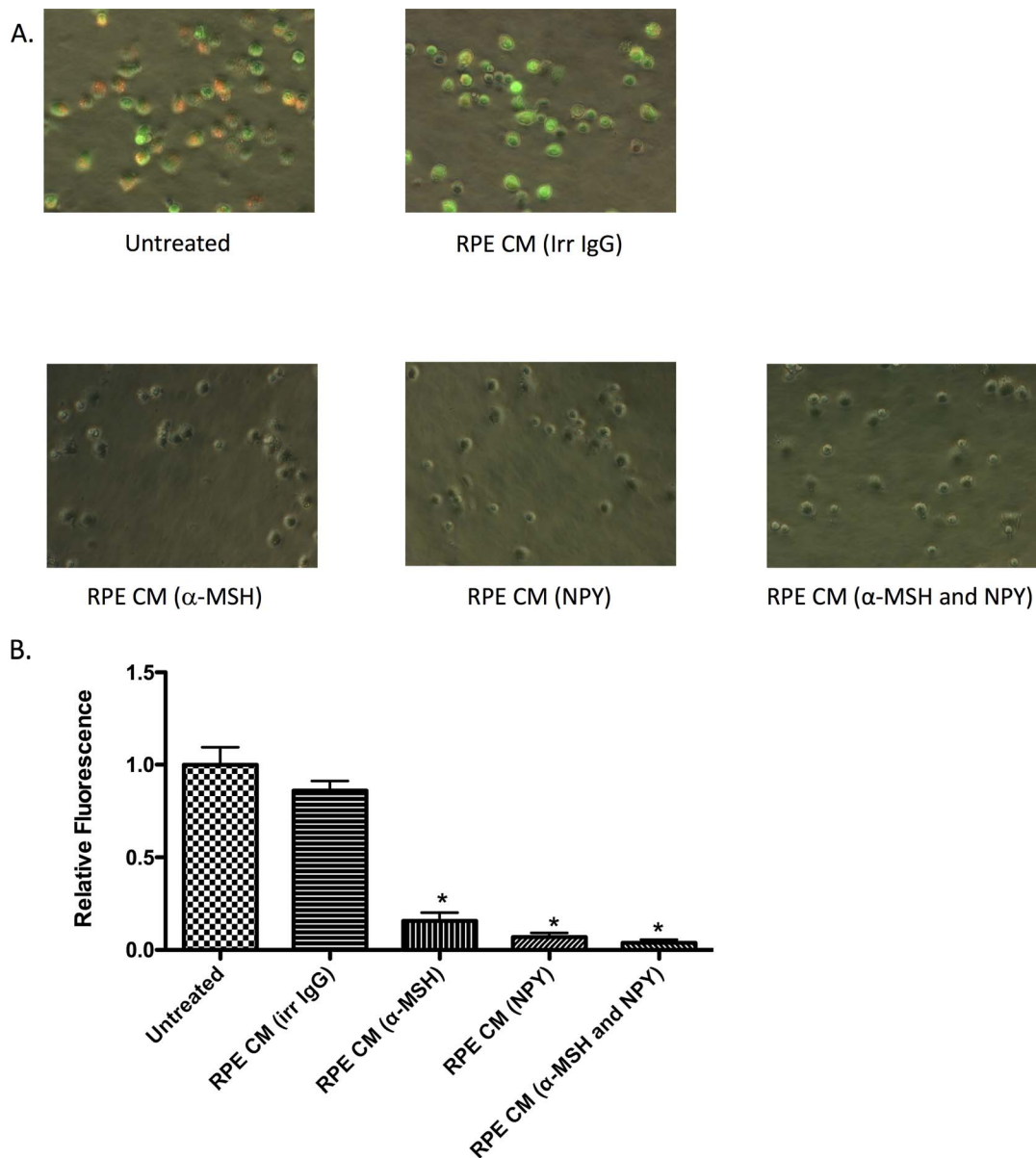
activation between untreated macrophages, and the macrophages treated with EAU RPE CM. In contrast, there was a statistically higher activation of phagolysosome activation in the EAU RPE CM-treated macrophages than in the healthy RPE CM-treated macrophages. These results demonstrate a change in RPE during EAU that no longer alters the phagocytic pathway, and possibly promotes the conventional pathway of antigen processing.

### The Effects of IL-6 in EAU RPE CM and Phagolysosome Activation

The CM of RPE eyecups from healthy and EAU eyes were assayed for 20 mouse cytokines by a multi-array ELISA assay (not shown). Of the cytokines assayed, only three were

detected: IL-6, KC, and VEGF. Of the three cytokines, only the concentration of IL-6 was qualitatively different between the RPE eyecup CM from healthy and EAU eyes. To quantitatively measure the difference in IL-6 production, the CM were assayed by ELISA for IL-6 (Fig. 5). The CM of healthy RPE had no detectable amounts of IL-6. In contrast, there was  $0.96 \pm 0.18$  ng/mL of IL-6 in the EAU RPE CM. Therefore, IL-6 is a soluble factor produced by the RPE of EAU eyes.

Because IL-6 was present in the EAU RPE CM, and that it is known to play an important role in EAU by inhibiting immune privilege,<sup>24,25</sup> the IL-6 in the EAU RPE CM was neutralized with an anti-IL-6 antibody. The antibody-treated CM was assayed for suppression of phagolysosome activation. With IL-6 neutralized, the EAU RPE CM significantly suppressed the phagolysosome activation (Fig. 6). The addition of 1 ng/mL of IL-6 into



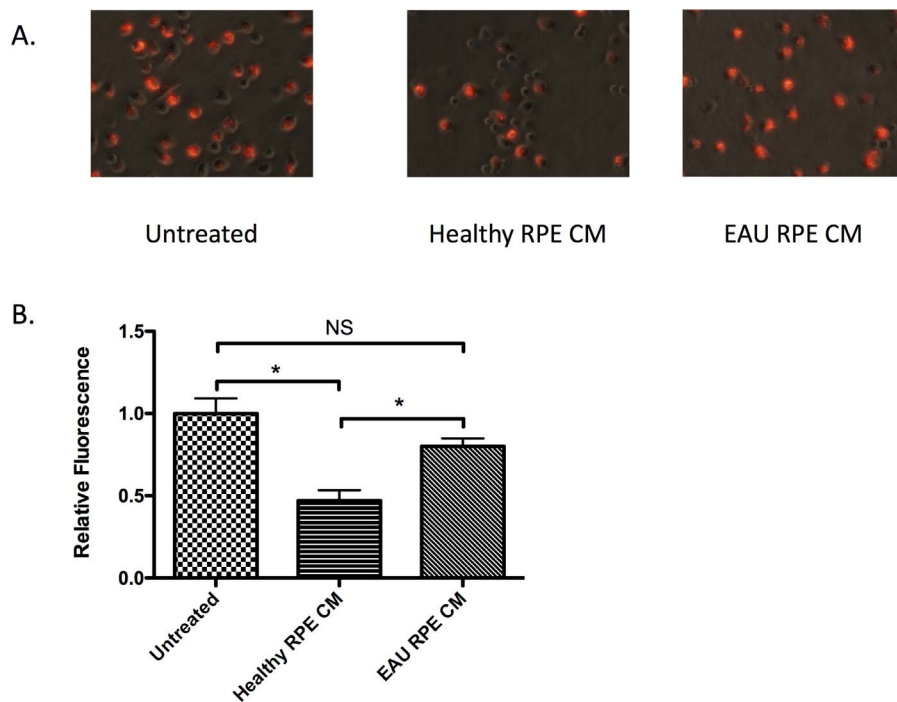
**FIGURE 3.** The effects of depleting  $\alpha$ -MSH and NPY from RPE CM on macrophage viability. Conditioned media from healthy RPE eyecups were depleted of  $\alpha$ -MSH and NPY and macrophages treated as in Figure 2. In addition, cell tracker viability dye was added in the last hour of the 24-hour incubation. The cells were imaged (A) and green fluorescent intensity was measured (B). The presented results are representative images of the treated macrophages, and the mean  $\pm$  SEM of the relative fluorescent intensity of three independent experiments. Depletion of  $\alpha$ -MSH and NPY had a significant effect on macrophage viability. \*Statistical differences,  $P \leq 0.001$ , to untreated macrophages. No statistical differences were seen between RPE CM with or without irr IgG treatment.

healthy RPE CM had no significant effect on healthy RPE CM suppression of phagolysosome activation (Fig. 6). These results demonstrate that the activation of phagosomes by the EAU RPE CM is through RPE-derived IL-6. Without IL-6, there are still suppressors of phagolysosome activation produced by the RPE. Because addition of IL-6 to the healthy RPE CM had no effect, it suggests that the factors produced by the EAU RPE may not be necessarily the same factors produced by healthy RPE suppressing the phagolysosome.<sup>5,14,26</sup>

## DISCUSSION

Consistent with our studies into the effects of  $\alpha$ -MSH and NPY on phagocytic activity of macrophages, primary RPE eyecup

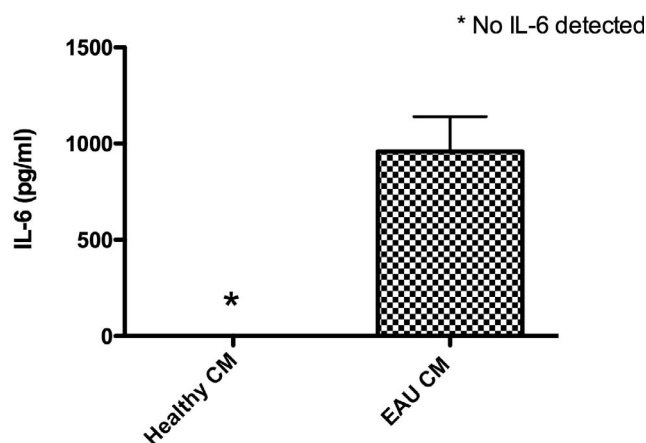
CM suppressed phagolysosome activation in macrophages. Previously, we identified that  $\alpha$ -MSH and NPY are present in the RPE eyecup CM, and their depletion from the CM made the CM induce apoptosis in the macrophages.<sup>5</sup> The CM of the primary RPE with  $\alpha$ -MSH and NPY depleted also induced cell death, and in this case in primary macrophages. Although the mechanisms of how RPE-soluble factors can induce apoptosis in macrophages is to be determined, it has been shown that  $\alpha$ -MSH rescues cells from apoptosis after caspase 3 activation.<sup>11</sup> The CM of RPE eyecups from EAU mice did not suppress activation of the phagolysosomes in macrophages and expressed IL-6. Although the neutralization of IL-6 in the EAU RPE CM recovered suppression of phagolysosome activation, the addition of IL-6 into healthy RPE CM did not promote phagolysosome activation. The results demonstrate that the



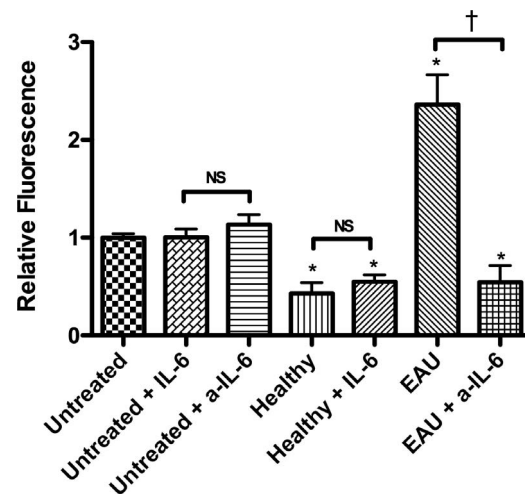
**FIGURE 4.** The effects of RPE CM from uveitic eyes on phagolysosome activation. Conditioned media were collected from 24-hour cultures of RPE eyecups (RPE CM) from mice with active EAU. Primary resting macrophages were treated with the RPE CM and fed opsonized pHrodo-bioparticles. The cells were incubated for 24 hours, imaged by microscopy, and the fluorescent intensity measured. (A) Presented are representative images of the treated macrophages. (B) The relative fluorescent intensity was calculated versus untreated macrophage cultures and presented as the mean  $\pm$  SEM of three independent experiments. Significant suppression,  $*P \leq 0.001$ , of phagolysosome activation was seen in cells treated with healthy RPE CM, but not (NS) with RPE CM from EAU mice.

RPE through soluble factors do regulate the phagocytic pathway and survival of macrophages, and in uveitis this regulation is altered and RPE-generated IL-6 promotes conventional phagocytic pathways in macrophages.

The presence of IL-6 in the EAU ocular microenvironment has been known for some time.<sup>24,25</sup> Its concentration increases in aqueous humor before cellular infiltration begins. At its uveitic concentration, IL-6 inhibits the antigen-presenting cells from mediating activation of regulatory T cells in the anterior



**FIGURE 5.** Interleukin-6 in EAU RPE eyecup CM. Conditioned media of RPE CM from healthy eyes and EAU eyes were assayed for IL-6 by ELISA. No IL-6 was detected in the healthy RPE CM, and  $0.96 \pm 0.18$  ng/mL was detected in the EAU RPE CM. This is the mean  $\pm$  SEM ng/mL of IL-6 from four RPE CM.



**FIGURE 6.** Effects of IL-6 on RPE CM regulation of phagolysosome activation. Added to healthy RPE CM 1 ng/mL of IL-6, and to EAU RPE CM was added neutralizing IL-6 antibody. Macrophages were treated with CM and fed opsonized pHrodo-bioparticles. After 24 hours, the cells were imaged and fluorescent intensity was measured. The presented results are the mean  $\pm$  SEM of the relative fluorescent intensity of three independent experiments. Although neutralization of IL-6 recovered significant suppression of phagolysosome activation, the addition of IL-6 into healthy RPE CM did not enhance phagolysosome activation. \*Statistical differences,  $*P \leq 0.001$  to untreated macrophages, and  $\dagger P \leq 0.001$  with EAU RPE CM-treated macrophages, and NS, not statistically significant.

chamber-associated immune deviation (ACAID) phenomena, which is antagonized by IL-6.<sup>24,25</sup> This suggests that when we neutralize IL-6 in the CM generated by EAU RPE cells it could well be a TGF- $\beta$ -mediated suppression and not the neuropeptide suppression of healthy RPE, because we know that  $\alpha$ -MSH production is significantly suppressed in eyes with EAU.<sup>5,6,26</sup> If the ACAID phenomenon is dependent on the antigen being processed through a highly regulated pathway within the macrophages, then our findings suggest that IL-6 promotes conventional processing and presentation of antigen by the macrophages. The early expression of IL-6 in EAU<sup>24</sup> may be promoting conventional antigen presentation within the retina by microglial cells and migrating macrophages that is needed to promote the activation of effector T cells.

There was no effect of adding IL-6 to the healthy RPE CM on the suppression of phagolysosome activation. In healthy CM is  $\alpha$ -MSH, and we have shown before that its concentration is diminished in EAU aqueous humor and in the CM of stressed RPE monolayers and RPE eyecups.<sup>5,6,26</sup> Adding  $\alpha$ -MSH back into the EAU CM of RPE eyecups restores suppressive activity.<sup>5</sup> Treating mice with  $\alpha$ -MSH also restores suppression of inflammation within the uveitic eye<sup>26-28</sup>; however, depleting  $\alpha$ -MSH from the CM of healthy RPE eyecups reveals a soluble signal from the RPE to induce apoptosis in the macrophages.<sup>5</sup> The apoptotic signal is not present in the uveitic RPE CM. This demonstrates that the RPE under uveitic or stressed conditions have different patterns of secreted molecules, which can include changing its production of  $\alpha$ -MSH for IL-6.<sup>5,6,29</sup> The change could be in response to the immunization used to induce EAU, or to infiltration of active inflammatory cells. Also, it means that the induction of autoimmune uveitis requires three events. First, there is the peripheral expansion of autoantigen-specific effector T cells with or without loss of Treg cell control. The second event would have to be a change in the RPE regulation of antigen processing possibly through reduced  $\alpha$ -MSH production with induced IL-6 production to generate T-cell-cognate autoantigen-peptides within retinal antigen-presenting cells. Along with this change is a third event that has to be associated with the loss of the ocular microenvironment to mediate immune privilege that would permit activation of effector T cells. There is little understood of the mechanisms that would promote these events or how they are interrelated.

The implications of the results suggest that within the immune-privileged ocular microenvironment there is suppression of conventional pathways of antigen uptake and processing. The pathways of phagocytosis proceed through steps that lead to the formation and activation of the phagolysosome.<sup>20</sup> The healthy RPE through the neuropeptides  $\alpha$ -MSH and NPY suppress this process, which means that any antigen processing will not be done through an active phagolysosome. Endocytic vesicles of phagocytized proteins can merge with the major histocompatibility complex (MHC) class II compartment, where the processed protein peptides are loaded onto MHC class II molecules for presentation.<sup>19</sup> Processing the proteins under neutral pH is not only inefficient, it would generate different peptides than proteins processed under acidic conditions of the activated phagolysosome. Therefore, within the healthy retina, the RPE are modulating antigen presentation or promoting different peptides for presentation. This would mean that T cells expanded in the periphery, through conventional pathways of antigen presentation, will either not receive a strong enough signal for activation, or not see cognitive antigen within the retina. This could be considered a type of antigen sequestration that contributes to preventing autoantigen-specific effector T-cell activation in the immune-privileged eye. Also, it suggests that by using RPE mechanisms to alter the processing of

antigen within the retina it should prevent autoimmune disease.

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