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The retinal pigment epithelial cells modulate phagolysosome activation in macrophages through neuropeptides, α-MSH and NPY

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Thesis

THE RETINAL PIGMENT EPITHELIAL CELLS MODULATE PHAGOLYSOSOME ACTIVATION IN MACROPHAGES THROUGH NEUROPEPTIDES, A-MSH AND NPY

by

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B.A., University of California, San Diego, 2009

Submitted in partial fulfillment of the requirements for the degree of
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DEDICATION

To my family and friends:

Our truest life is when we are in dreams awake.

-Henry David Thoreau
ACKNOWLEDGMENTS

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Dr. Taylor, I thank you for the research opportunity, your expertise, and teaching me a small bit of what it is to be a researcher.
THE RETINAL PIGMENT EPITHELIAL CELLS MODULATE PHAGOLYSOSOME ACTIVATION IN MACROPHAGES THROUGH NEUROPEPTIDES, A-MSH AND NPY

YOONA CHOE

ABSTRACT

Objective: The main function of the human eye is to detect light, motion, and color from our surroundings. This information is then processed and translated in the brain as vision. However, what is less known about the eye is its ability to regulate immune function. It is this ocular immune privilege that maintains the eye’s ability collect visual information. The degeneration of immune privilege causes inflammation, which can cause damage to the eye, an increased susceptibility of eye disorders such as autoimmune uveitis (inflammation of the uvea), and may lead to vision impairment. Research in ocular immune privilege can open up potential clinical applications for maladies such as uveitis, septic shock, hypersensitivity, multiple sclerosis and allograft survival. Previous research has shown the importance of the retinal pigment epithelial (RPE) cells for maintenance of ocular immune privilege, and has identified the use of neuropeptides to suppress inflammatory responses in macrophages. This project aims to study the role and mechanism of the RPE cells in phagolysosome activation in macrophages that mediate inflammation.

Methods: Posterior eyecups were prepared from eyes of healthy, EAU immunized, or post-EAU mice. Eyecups, which consisted of the sclera, choroid, and a single layer of RPE, were cultured in serum-free media (SFM). During a 24-hour incubation period,
peritoneal macrophages were collected intraperitoneal (IP) and cultured. Conditioned media (CM) was applied to the collected macrophages along with pHrodo-red opsonized bioparticles and were incubated for 24-hours in 37°C. After incubation, cells were examined by fluorescent microscopy for phagolysosome activation. Also, this was also done with RPE CM depleted of α-MSH, NPY, and α-MSH + NPY. A viability assay was performed on macrophages treated with depleted RPE CM to investigate the possibility that removing the neuropeptides will induce cell death. To examine the cytokines involved in RPE CM—from healthy, EAU and post-EAU mice—modulation of phagolysosome activation, a mouse cytokine array was performed that assessed for twenty different mouse cytokines.

**Results:** Results from fluorescent microscopy showed that healthy RPE CM caused significant suppression of phagolysosome activity in macrophages. The RPE CM depleted of α-MSH, NPY, and α-MSH + NPY showed a significant suppression of phagolysosome activity in macrophages. However, these results may have been misrepresented as the macrophages treated with depleted RPE CM were non-viable. RPE CM from EAU mice showed the inability to down-regulate phagolysosome activity while RPE CM from post-EAU mice recovered its ability to down-regulate phagolysosome activity. The mouse cytokine array of RPE CM from healthy, EAU and post-EAU mice identified keratinocyte-derived cytokine (KC), high concentrations of interleukin-6 (IL-6), trace amounts of vascular endothelial growth factor (VEGF), and no other pro or anti-inflammatory cytokines.
Conclusions: Healthy RPE cells suppress phagolysosome activation in activated macrophages. In contrast, RPE cells from mice with active EAU lose its ability to regulate phagolysosome activation, but regain this ability when the disease resolves following α-MSH treatment. RPE CM from post-EAU mice treated with MC5r agonist did not recover suppression of phagolysosome activation which suggests that α-MSH causes suppression of phagolysosome activation through other melanocortin receptors or that this suppression requires other mechanisms in conjunction to MC5r stimulation. Cytokine IL-6 may be involved in RPE suppression of phagolysosome activation; however, further study will have to be done. The results demonstrate that part of the mechanisms of ocular immune privilege is the tight control by RPE of the phagocytic process in macrophages. It is possible that this contributes to ocular immune privilege minimizing the potential of processing and presenting self-proteins, and to allow for clearance of harmful materials while suppressing the activation of inflammation.
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12 Mouse cytokine KC concentration in RPE CM of EAU and post-EAU mice

13 Mouse cytokine VEGF concentration in RPE CM of EAU and post-EAU mice
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>α-MSH</td>
<td>α-Melanocyte Stimulating Hormone</td>
</tr>
<tr>
<td>AC</td>
<td>Anterior Chamber</td>
</tr>
<tr>
<td>ACAID</td>
<td>Anterior Chamber-Associated Immune Deviation</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund Adjuvant</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin Gene-Related Peptide</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned Medium</td>
</tr>
<tr>
<td>CTCF</td>
<td>Corrected Total Cellular Fluorescence</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-Type Hypersensitivity</td>
</tr>
<tr>
<td>EAU</td>
<td>Experimental Autoimmune Uveitis</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobin</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IRBP</td>
<td>Interphotoreceptor Retinoid Binding Protein</td>
</tr>
<tr>
<td>KC</td>
<td>Keratinocyte-Derived Cytokine</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin Hormone</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal Pigment Epithelium</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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</tbody>
</table>
SFM .............................................................. Serum Free Medium
TGF-β2 .......................................................... Transforming Growth Factor-β2
T_{reg} .............................................................. T Regulatory Cells
VEGF ........................................................... Vascular Endothelial Growth Factor
VIP .............................................................. Vasoactive Intestinal Peptide
INTRODUCTION

The immune system is essential to help identify and remove pathogens in all living organisms and tissues. However useful to health, complications to the immune system may cause disease or pathology (Streilein, 1997). Ocular pathology caused by immunity is infrequent because immune responses are highly controlled (Streilein, 1997). By studying the intrinsic mechanisms behind ocular regulation of immunity would not only help remedy autoimmune diseases, but also advance clinical outcomes to other immune mediated diseases (A.W. Taylor, 2009).

The Importance of Immune Privilege

The term “immune privilege,” first conceived by Sir Peter Medawar in the 1940s (Medawar, 1945; A. W. Taylor & Kaplan, 2010), is defined as a site or tissue where a foreign organ or tissue graft can exist in a prolonged or an unlimited amount of time (J. W. Streilein, 2003). While immune regulation occurs in all organs and tissues, there exist several sites that possess immune privilege (Table 1). Medawar observed skin allografts positioned in the ocular anterior chamber (AC) of rabbits. He found that even though these rabbits were first immunized to reject skin allografts, these allografts experienced an extended survival time (Medawar, 1945, 1948). It was also Medawar who assigned this privilege to be an attribute of a specific tissue or site and hypothesized that immune privilege may be a consequence of an “immunologic ignorance” due to blood:tissue barriers that exist around the brain and eyes (Streilein, Okamoto, Sano, & Taylor, 2000; A. W. Taylor & Kaplan, 2010). Since then, researchers have found that while the blood:tissue barrier partially constitutes a passive or innate part of the immune privilege,
there is also an active or adaptive part of immune privilege (Kaplan & Streilein, 1977; Streilein et al., 2000; A. W. Taylor, 2003).

**Table 1. A Reduced List of Immune Privileged Sites and Tissues**

<table>
<thead>
<tr>
<th>Sites</th>
<th>Tissues</th>
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<tbody>
<tr>
<td>Eye: cornea, anterior chamber, vitreous cavity and subretinal space</td>
<td>Eye: cornea, lens, pigment epithelium and retina</td>
</tr>
<tr>
<td>Brain: ventricles and striatum</td>
<td>Brain and spinal cord</td>
</tr>
<tr>
<td>Pregnant uterus</td>
<td>Placenta</td>
</tr>
<tr>
<td>Ovary</td>
<td>Ovary</td>
</tr>
<tr>
<td>Testis</td>
<td>Testis</td>
</tr>
<tr>
<td>Adrenal cortex</td>
<td>Liver</td>
</tr>
<tr>
<td>Hair follicles</td>
<td></td>
</tr>
<tr>
<td>Hamster cheek pouch</td>
<td>Hamster cheek pouch</td>
</tr>
<tr>
<td>Certain tumors</td>
<td>Certain tumors</td>
</tr>
</tbody>
</table>


**Immune Privilege of the Eye**

The human eye functions to detect light, motion, and color that is processed and translated in the brain as visual information. What is less known about the eye is its capability to regulate immune function. This ability is referred to as the ocular immune privilege. Immune privilege largely contributes to the eye’s maintenance of an anti-inflammatory microenvironment while constantly exposed to outside elements (Strauss, 2005). The degeneration of immune privilege has been known to cause damage to the eye and leads to an increased susceptibility to eye disorders such as uveitis (inflammation of the uvea) (A. W. Taylor & Kaplan, 2010). The main structures that contribute to the eye’s immune privilege are the epithelium at the edge of the cornea, vessels of the iris, ciliary body and retina. This constitutes the blood:ocular barrier and strictly regulates the molecules and cells that enter the eye. What is also unique about the eye is the absence of
direct lymphatic drainage pathways. Because lymph vessels are the main pathway by which most antigens first come across T and B cells, the absence of lymphatic drainage shields ocular antigens from the immune system (Streilein, 1997). Medawar hypothesized that this, along with the blood:ocular barrier, was the reason the eye is immune privileged; however, while the lack of lymphatic drainage is a significant portion of its passive privilege, it does not entirely explain the reduced immune reaction to antigens in or from the eye (Streilein, 2003b).

**Adaptive Immune Privilege**

In the 1970’s, researchers found that injecting allogeneic lymphoid cells into the anterior chamber of rat eyes caused an abnormal immune response. Rather than cause a rejection of foreign substances and tissues, the injection stunted the ability to reject orthotopic skin grafts with identical alloantigens. More specifically, it induced a suppressor immunity of antigen-specific efferent suppressor CD8 T cells and afferent suppressor CD4 T cells, now established as T regulatory (T\textsubscript{reg}) cells (Kaplan & Streilein, 1977; Streilein, 2003a). In 1981, researchers discovered that injecting allogeneic tumor cells into the AC of mice eyes allowed the growth of tumors in the eyes; more importantly, the mice were not able to reject orthotopic donor-specific skin grafts with the same allogeneic antigen expression as the tumor cells (Niederkorn, Streilein, & Shadduck, 1981). These experiments not only confirmed that ocular immune privilege existed but also paved the way for other researchers to explore ocular immune privilege (Streilein et al., 2000).
In the past forty years, more adaptive mechanisms of ocular immune privilege have come to light. In a normal immune response, when inoculated with antigens, the host would develop a donor-specific delayed-type hypersensitivity (DTH). However, the placement of antigens in an immune privileged site failed to produce such hypersensitivity response. More specifically, the placement of antigens in the anterior chamber failed to induce DTH (Streilein, Niederkorn, & Shadduck, 1980). This distinctive suppressor immunity response is called Anterior Chamber-Associated Immune Deviation (ACAID) (Streilein, Ksander, & Taylor, 1997).

ACAID can be instigated by the placement of antigens in the AC, subretinal space, or vitreous cavity. The instigation of ACAID requires that the antigen be injected into a specific ocular compartment, the spleen be intact for the first seven days, and the eye be intact for the first four days (Kaplan & Streilein, 1977). We also understand that ACAID is mediated by antigen presenting cell (APC), F4/80 macrophage, that presents the antigen to CD4 and CD8 T cells, B cells, and NKT cells in the spleen (D’Orazio & Niederkorn, 1998; Lin et al., 2005, p. 4; Sonoda & Stein-Streilein, 2002). Later studies showed that there are an abundance of immunosuppressive molecules and modulatory factors in the aqueous humor that impact the action of immune cells including affecting the presentation of antigens on APCs to produce a suppressor immunity (A. W. Taylor, 2002).

The aqueous humor, which fills the AC, is a fluid that was found to suppress immune activity when applied to macrophages (Wilbanks & Streilein, 1992). It is also known to suppress T-cell activation and proliferation (Kaiser, Ksander, & Streilein,
1989), inhibit IFN-γ production by activate CD4 T cells (Andrew W. Taylor, Alard, Yee, & Streilein, 2007), and even impede NK cell-mediated lysis (Apte, Sinha, Mayhew, Wistow, & Niederkorn, 1998).

Neuropeptides and Ocular Immunity

The aqueous humor consist of neuropeptides, complement factors and their inhibitors, proteins, and other molecules that all compose an ocular microenvironment that allows the maintenance and regulation of the constant potential inflammation and necrosis of the eye (A.W. Taylor, 2009). A large portion of the array of regulating and immunosuppressive factors is neuropeptides. A few of these neuropeptides include vasoactive intestinal peptide (VIP) (A. W. Taylor, Streilein, & Cousins, 1994), α-melanocyte stimulating hormone (α-MSH) (A. W. Taylor, 2005), somatostatin (Andrew W. Taylor & Yee, 2003), and calcitonin gene-related peptide (CGRP) (A. W. Taylor, Yee, & Streilein, 1998).

α-MSH was the first reported immunomodulating neuropeptide in the eye (A. W. Taylor, Streilein, & Cousins, 1992). It is a thirteen amino acid long peptide that is released from proopiomelanocortin hormone (POMC) through endoproteolytic cleavage and posttranslational modifications. It is mainly known for its function in inducing melanin. However, what is less known is that α-MSH has a chief role in regulating defense mechanisms in mammals. α-MSH along with CGRP and cytokine transforming growth factor-β2 (TGF-β2) has been shown to inhibit inflammatory activation in macrophages. They have been shown to inhibit inflammatory activity by suppressing endotoxin-induced inflammatory activity, and also promote an anti-inflammatory
cytokine production by the macrophages (A. W. Taylor et al., 1998). α-MSH with Fas ligand and TGF-β2 may also control the recruitment of macrophages and neutrophils (Catania et al., 1996; Griffith, Brunner, Fletcher, Green, & Ferguson, 1995; Masli, Turpie, Hecker, & Streilein, 2002). Also, α-MSH has been found to induce its own synthesis as well as its melanocortin receptors on macrophages, and thus further contribute to the suppression of inflammation in macrophages through a sustained autocrine loop (Rajora et al., 1996).

While these findings imply that α-MSH can regulate suppression of inherent-mediated inflammation, α-MSH is also involved in the regulation of adaptive immune-mediated inflammation. α-MSH modulates T-cells activities. For example, α-MSH inhibits the synthesis of interferon-γ (IFN-γ) by effector T-cells. It is through α-MSH, with the help of TGF-β2, which aqueous humor modulates T-cell response from pro-inflammatory to non-inflammatory activity (Nishida & Taylor, 1999). Interestingly, α-MSH also alternatively activates macrophages consequently promoting suppression and tolerance when they act as APCs (Lau & Taylor, 2009; T. A. Luger, Kalden, Scholzen, & Brzoska, 1999). The constant growth of new information about α-MSH and its regulation of immunity continue to prove its importance.

Another important neuropeptide involved in anti-inflammatory mechanisms is neuropeptide Y (NPY). NPY is a 36 amino acid long sympathetic neurotransmitter peptide that innervates immune organs as well as leukocytes (Bedoui, von Hörsten, & Gebhardt, 2007; Dimitrijević & Stanojević, 2013; Petitto, Huang, & McCarthy, 1994). Studies have found that in vitro NPY increased different tasks of inflammatory cells such
as chemotaxis, production of nitric oxide and reactive oxygen metabolites, secretion of cytokines as well as simple adherence (Mitić, Stanojević, Kuštrimović, Vujić, & Dimitrijević, 2011). NPY has also been reported to have inhibitory effects on inflammatory cell activity. Specifically, NPY reduces phagocytosis (Bedoui et al., 2007) and granulocyte oxidative burst (Dimitrijević et al., 2006). What is important about NPY in regards to ocular immunity, however, is that researchers have discovered that survival and function of macrophages is reliant on not just NPY but also α-MSH, both produced by the retinal pigment epithelium (RPE) (Kawanaka & Taylor, 2011).

Retinal Pigment Epithelium as an Immune-Privileged Tissue

One of the primary roles of the RPE is visually related. It is made of cuboidal epithelium composed of microvilli on the apical side thus projecting out to meet with the rod and cone photoreceptors. The RPE also functions in metabolism and transportation which is crucial to maintain homeostasis and ultimately visual capabilities of the eye (Bok, 1993). Because the RPE forms an epithelium of tight junctions between the photoreceptors and choroicapillaris (Figure 1), it acts as part of a blood-retinal barrier isolating the inner retina from the rest of the systemic circulation while also controlling which nutrients and metabolites are allowed to cross the barrier. The RPE also mediates phagocytosis of photoreceptors tips that have been shed as well as regeneration of the retinoids (Bok, 1993; Steinberg, 1985; Strauss, 2005). In fact, the apical surface of just one RPE cell supports about 30 to 50 photoreceptors outer segments. The basal surface of the RPE, which is attached to Bruch’s membrane, specializes in exchanging nutrients and
metabolites with the choroid (Bonilha, 2008). As discussed earlier, such features of the RPE structure helps to sustain the passive ocular immune privilege.

Figure 1. Diagram of the blood-retinal barrier locating the RPE in relation to the photoreceptors and choroid. Adapted from Science Of AMD, n.d., Retrieved March 12, 2015, from http://www.scienceofamd.org/learn/. Copyright 2015 The Angiogenesis Foundation.

The RPE also supports ocular immune privilege by its ability to interact with the immune system with the function of suppressing or activating immune response in the healthy eye. More specifically, RPE cells have been proven to down-regulate T-cell activation by direct cell contact as well as production of immunosuppressive factors (Ishida, Panjwani, Cao, & Streilein, 2003; Sugita et al., 2009). Additionally, as previously mentioned, the RPE produce neuropeptides such as α-MSH and NPY. These neuropeptides, along with other immunomodulating factors, are known to affect immune cell functions (Kawanaka & Taylor, 2011). Specifically, it was discovered that α-MSH and NPY control proinflammatory signals and signal the alternative activation mechanisms of macrophages. What is more, it has recently been discovered that α-MSH with NPY prompts myeloid suppressor cell-like activity in macrophages at rest and also helps monitor the same activity in retinal microglial cells (Kawanaka & Taylor, 2011).
Past research indicates that there are four melanocortin receptors $\alpha$-MSH binds to in the body. In the neural retina of the eye, MC3r, MC4r, and MC5r are expressed. The RPE expresses melanocortin receptors MC1r and MC5r (Lindqvist, Näpänkangas, Lindblom, & Hallböök, 2003). In fact, the hypothesis that $\alpha$-MSH prompts $T_{\text{reg}}$ cells in vivo was via MC5r knocked-out mice. After being re-immunized post-recovery from the first incidence of experimental autoimmune uveitis (EAU), these mice not only had a second incidence of EAU but with rapid onset and increased severity. The failure for MC5r knockout mice to establish a post-EAU suppressive immunity, as seen in wild type mice, proved this failure was due to the inability to produce EAU regulatory APCs (Lee & Taylor, 2011).

**Murine EAU as a Model for Intraocular Inflammation**

Uveitis is an intraocular inflammatory disease that is evoked by a large array of etiologies (A. W. Taylor & Kaplan, 2010). The most observed animal model of intraocular inflammation is EAU. EAU can be induced by immunizing in rodents with specific retinal proteins like interphotoreceptor retinoid binding protein (IRBP), phosducin, or rhodopsin (Gery et al., 1986; Schalken et al., 1988). For EAU to be induced, the ocular-autoantigen specific effector T cells must expand either through knocking out tolerance in the eye or adjuvant-mediated immunization (A. W. Taylor & Kaplan, 2010). When EAU does occur, it is apparent that the immunosuppressive ocular microenvironment is no longer prevalent. Studies of the effects from EAU have shown that even before inflammation is observed, pro-inflammatory cytokines are already present and the aqueous humor no longer possess immunosuppressive capabilities. The
loss of immunosuppression is brief, however, and ocular immune privilege is restored
when intraocular inflammation ceases (Ohta, Wiggert, Yamagami, Taylor, & Streilein,
2000).

Specific Aims

Studies have demonstrated that neuropeptides, α-MSH and NPY suppress
phagolysosome activity in macrophages. Researchers also discovered that both
neuropeptides are produced by the RPE. Therefore, there is potential that the RPE,
through the synthesis of α-MSH and NPY, may elicit immunosuppression in
macrophages. Therefore, it is our objective to determine:

1. Does RPE suppress phagolysosome activation in primary macrophages; therefore, inhibit innate immunity

2. Whether neuropeptides α-MSH and NPY are factors in phagolysosome suppression;

3. In EAU immunized mice, does RPE suppress phagolysosome activation in primary macrophages;

4. In post-EAU mice, does RPE suppress phagolysosome activation in primary macrophages;

5. Does treating EAU with α-MSH and/or MC5r agonist restore RPE suppression of phagolysosome activity in primary macrophages;

6. What cytokines may be released by the RPE in EAU that could neutralize suppression of phagolysosome activation.
METHODS

Preparation of Posterior Eyecups

Eyecups were prepared as described previously (Lau & Taylor, 2009; Zamiri, Masli, Streilein, & Taylor, 2006). Eyes from euthanized mice were extracted and placed on ice in phosphate buffered saline (PBS) (Lonza, Walkersville, PA) for approximately 30 minutes. After removal of the connective tissue, muscles, and conjunctiva, a circumferential incision posterior the ciliary body was conducted. The anterior portion of the eye, which includes the ciliary body, lens, cornea, and iris, was discarded. The neural retina was then removed from the monolayer of RPE by microsurgical forceps leaving the final product—the posterior eyecup. Each posterior eyecup, consisting of the choroid, sclera, and a single layer of RPE, were placed into separate wells of a 96-well round-bottom culture plate. The eyecups were submerged in 100 μL of serum-free media (SFM) containing DMEM (Lonza, Walkersville, PA), 0.1 M HEPES, NEAA, 4.5 g/L Glucose, L-glutamine, sodium pyruvate, 1% gentamicin, 0.1% bovine serum albumin (Sigma Chemical, St. Louis, MO), and supplemented with 0.1 x ITS+ solution (Sigma Chemical, St. Louis, MO). The conditioned media (CM) from within the eyecup was removed 24 hours after incubation. The CM was then used in the phagocytosis assays.

Phagolysosome Activity Assay

The macrophages were collected by injecting and then recovering 5ml PBS directly from the non-irritated peritoneal cavity. Upon extraction, the peritoneal macrophages were centrifuged at 1200 RPM for 10 minutes at 4°C, and suspended in 400μl Roswell Park Memorial Institute (RPMI)-1640 with L-Glutamine (Lonza,
Walkersville, PA) and 10% FBS to assess cell purity and viability. An amount of 500µl of peritoneal macrophages at a concentration of 2.5x10^5 cells/ml was distributed into each well of an 8-welled glass slide. The cultures were incubated for 1-2 hours at 37°C in 10% CO₂. The slides were washed with SFM before the resting macrophage cultures were finally cultured in 200µl of CM for 24 hours at 37°C, 10% CO₂ humidified incubator. The macrophages were treated with SFM containing α-MSH and NPY at 1 ng/ml each. This is the concentration of the neuropeptides in conditioned media produced in 24 hours by organotypic healthy RPE monolayer cultures. Also added 30 minutes into the incubation period were 1-2µl of opsonized Escherichia coli pHrodo-red fluorescent bioparticles, which increase in fluorescence at acidic pH, such as the internal compartment of an activated phagolysosome. Opsonized pHrodo bioparticles were prepared with 1:1 mixture of pHrodo red E.coli bioparticles conjugate (Life Technologies-Molecular Probes, Grand Island, NY) and E.coli bioparticles opsonizing reagent (Life Technologies-Molecular Probes, Grand Island, NY).

**Depletion of Neuropeptides**

To characterize the effects of individual neuropeptides, α-MSH, NPY, or both α-MSH and NPY were depleted from the RPE CM before the CM was used to treat the macrophages. To deplete α-MSH and NPY, antibodies against α-MSH, or NPY from Peninsula Laboratories (San Carlos, CA) were added to the CM. For controls, we used an irrelevant immunoglobin (IgG) MC3-R rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The CM with added antibody was incubated at 4°C for 1 hour, protein-G coated beads (Santa Cruz Biotechnology) were added to the CM and incubated for an
additional 1 hour 4°C. The beads were centrifuged down at 2000 RPM for 2 minutes and the neuropeptide-depleted supernatant (absorbed CM) was used to complete the phagocytosis assays. Antibody concentrations were used at 10 - 100x their neutralizing dose (2µl/100µl of CM).

Viability Assay

Once CM or absorbed CM were incubated for a 24-hour period, a fluorescent probe, CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) (Life Technologies, Grand Island, NY), was used to measure and quantify viability of macrophages. The CellTracker fluorescent probe was designed to passively cross the cell membranes and will only fluoresce at physiological pH. The fluorescent probe was diluted to a final working concentration of 10µM. The probe and CM was incubated at 37°C in 10% CO₂ for 30 minutes before fluorescence was measured.

Immunization and Treatment of EAU Mice

Mice were immunized with EAU by 200µl injections of 1:1 Complete Freund Adjuvant (CFA) (DIFCO Laboratories, Detroit, MI) with interphotoreceptor retinoid binding protein (IRBP) (GenScript, Piscataway, NJ) emulsions (2mg/ml) followed by 200µl injections of pertussis toxin (Sigma-Aldrich, St. Louis, MO). All injections were subcutaneous. EAU manifestations were tracked and scored based on a 0-5 scale. An eye with a 0 score showed no signs of EAU while eyes with a score of 5 showed massive scarring and inflammation. When all mice showed an EAU score of 3 or 4, injections of α-MSH (30µg/ml), MC5r agonist (1µg/ml), or PBS were given IP. Mice were sacrificed study only after they were given a score of 0 or 1.
Fluorescent Microscopy for Phagolysosome Activation

To image the macrophages by fluorescent microscopy, after a 24-hour incubation period, the cultured macrophages were washed once with 0.01M PBS and 200µl of PBS was added to each well in the 8-welled glass slide. The macrophage cultures were digitally imaged with the FSX100 digital fluorescent microscope (Olympus, Center Valley, PA) using a 40x objective lens. A range of 1/50-1/200 second exposure time was used for phase contrast. For red fluorescence (pHrodo bioparticles), a range of 1/1.5-1/2 second exposure time was used. For green (viability probe) fluorescence, a 1/2-1/2.5 second exposure time used. These images were corrected for background and overlaid to make the presented images using the FSX100 software. The corrected total cellular fluorescence (CTCF) was calculated and used to quantify the degree of phagolysosome activation.

Mouse Cytokine Array Assay

In order to identify and measure which cytokines were present in RPE CM, the Quantibody Mouse Cytokine Array 1 (RayBiotech, Inc., Norcross, GA) was used to quantify 20 different mouse cytokines. This cytokine array was performed in a two day period allowing for a 24-hour incubation period at 4°C for blocking. Images were taken at an exposure time of 50.05 seconds. Images and concentration analysis were determined using Quantity One 4.6.9 software (Bio-Rad Laboratories, Hercules, CA).

Data and Statistical Analysis

All values were stored in an Excel Spreadsheet (Microsoft, 2014). However, statistical analysis calculations were performed using Prism software (GraphPad
Software Inc., La Jolla, CA). Statistical differences for fluorescence were calculated by non-parametric one-tailed t-test (Mann-Whitney) and transformed for relative CTCF based off of mean CTCF of resting cells. The concentrations for mouse cytokines were determined by non-linear regression curves for best fit. Concentrations were found by interpolation from this standard curve. All significant differences were found at $P \leq 0.05$. 
RESULTS

RPE CM Effect on Phagolysosome Activation

To determine if there was an effect of the RPE CM on the activation of phagolysosomes in macrophages, the macrophages were treated with a 1:1 mixture of E.coli opsonizing agent and E.coli bioparticles to phagocytize. Once phagocytized, bioparticles were designed to fluoresce red as the pH dropped to acidic conditions. Using digital fluorescence microscopy, live images were taken after a 24-hour incubation period (Figure 2).

A. Image of primary macrophages at rest without pHrodo-red bioparticles. B. Image of primary macrophages with pHrodo-red bioparticles and untreated by RPE CM.

Figure 2. Images of macrophages untreated and treated by RPE CM. (A) Image of primary macrophages at rest without pHrodo-red bioparticles. (B) Image of primary macrophages with pHrodo-red bioparticles and untreated by RPE CM.
Figure 2C. Image of primary macrophages with RPE CM.

The untreated macrophages phagocytized and moved the bioparticles to active phagolysosomes, and thus were bright red (Figure 2B). Very little red fluorescence was seen in RPE CM treated macrophages (Figure 2C). This suggests that RPE CM may be suppressing phagolysosome activation in the macrophages. To quantify the effects of the RPE CM on macrophages activation or suppression of phagolysosome activity, cells were

Figure 3. The effects of RPE CM on phagolysosome activity in macrophages. The macrophages were treated with RPE CM and opsonized *E.coli* pHrodo-red-conjugated bioparticles. After 24-hours of
incubation at 37°C, macrophages were washed with 0.01M PBS and analyzed by fluorescent microscopy. Shown is the relative CTCF ± SEM of pHrodo-red expression in the RPE CM treated macrophages compared to the pHrodo-red expression in untreated macrophages. Data is from two separate experiments, N=8. *This group of treated to untreated macrophage was significantly different (P<0.05, P<0.0001).

After the 24-hour incubation period, the macrophages were assayed for fluorescent intensity and relative corrected total cellular fluorescence (CTCF) was calculated. The relative CTCF was compared for phagolysosome activity (Figure 3). There was a significant difference between the relative CTCF expressed by untreated macrophages with relative CTCF expressed by macrophages treated with RPE CM. These results indicated that the macrophages treated by RPE CM significantly suppressed macrophage phagolysosome activation.

**Absorbed RPE CM Effect on Phagolysosome Activation**

To see whether neuropeptides α-MSH and NPY are factors in phagolysosome suppression, the RPE CM was depleted of α-MSH, NPY, and both α-MSH + NPY. A rabbit polyclonal IgG against an irrelevant protein was used as a control. The primary macrophages were then treated with the now absorbed RPE CM. After incubation, digital microscopy was again used for live images showing fluorescence (Figure 4A-F).
Figure 4. Images of macrophages treated with absorbed RPE CM. (A) Image of untreated primary macrophages at rest without pHrodo-red bioparticles (B) Image of primary macrophages with pHrodo-red bioparticles and untreated by absorbed RPE CM. Images are taken from two different experiments with same exposure settings.

Figure 4C-D. (C) Image of primary macrophages treated with –IgG absorbed RPE CM. (D) Image of primary macrophages treated with –αMSH absorbed RPE CM. Images are taken from two different experiments with same exposure settings.
Figure 4E-F. (E) Image of primary macrophages treated with –NPY absorbed RPE CM. (F) Image of primary macrophages treated with –αMSH –NPY absorbed RPE CM. Images are taken from two different experiments with same exposure settings.

The untreated macrophages demonstrated phagolysosome activation again showing a bright red fluorescence (Figure 4B). Macrophages treated by RPE CM depleted using an irrelevant IgG showed little fluorescence (Figure 4C). This demonstrated that the procedure to absorb the neuropeptides did not interfere with usual RPE CM activity. The absorbed RPE CM depleted of α-MSH (Figure 4D), NPY (Figure 4E) or α-MSH + NPY (Figure 4F) showed even less fluorescence than those macrophages treated by irrelevant IgG, and were even comparable to macrophages that were not treated with any pHrodo bioparticles (Figure 4A). Macrophages that were treated with RPE CM absorbed of α-MSH, NPY and α-MSH + NPY exhibited signs of lost viability.
Figure 5. The effects of absorbed RPE CM on phagolysosome activity. The macrophages were treated with RPE CM –αMSH, -NPY, or -αMSH -NPY. After 24-hours of incubation at 37°C, macrophages were analyzed by fluorescent microscopy. Shown is the relative CTCF ± SEM of pHrodo-red expression in the absorbed RPE CM treated macrophages compared to the pHrodo-red expression in untreated macrophages. Data is from 5 separate experiments, N=5. *The group of untreated to RPE CM –MC3r treated macrophage were significantly different, P<0.05. **The groups of RPE CM –MC3r to RPE CM –αMSH, -NPY, -αMSH –NPY and untreated to RPE CM –αMSH, -NPY, -αMSH –NPY were significantly different, P<0.0001).

To quantify the effects of phagolysosome activity of the macrophages, the cells treated with absorbed RPE CM were assayed for CTCF. The relative CTCF was compared for phagolysosome activity (Figure 5). There was a significant difference between the relative CTCF expressed by macrophages untreated with relative CTCF expressed by macrophages treated with relative CTCF of macrophages treated with RPE CM depleted with irrelevant IgG as well as relative CTCF of untreated macrophages with relative CTCF of macrophages treated with RPE CM depleted of α-MSH, -NPY, and α-MSH + NPY. There was no significant difference between CTCF expression of
macrophages treated α-MSH, NPY or α-MSH + NPY. This further suggested that the macrophages treated with the depleted CM lost viability.

**Viability of Absorbed RPE CM and its Effect on Phagolysosome Activation**

Macrophages treated with absorbed RPE CM depleted of α-MSH, NPY and α-MSH + NPY showed no or very little fluorescence and abnormal appearance. This suggested potential loss of viability. To assess whether the viability of the macrophages, the previous study was repeated; however, after RPE CM or absorbed CM were incubated for a 24-hour period and prior to viewing results under fluorescent microscopy, a fluorescent probe, CellTracker Green CMFDA, was added to treated macrophages. The CellTracker fluorescent probe was designed to fluoresce green at physiological pH. The probe and macrophages treated with absorbed RPE CM were incubated at 37°C for 30 minutes before fluorescence was measured (Figure 6A-F).

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**Figure 6. Images of macrophages treated with absorbed RPE CM and assayed for viability.**

(A) Image of untreated macrophages at rest with viability probe without pHrodo-red bioparticles. (B) Image of untreated macrophages with viability probe and pHrodo-red bioparticles.
The macrophages untreated by absorbed RPE CM or pHrodo bioparticles (Figure 6A) showed no red fluorescence as it is null of pHrodo bioparticles, but were bright green fluorescence that indicated viable cells. Macrophages untreated by absorbed RPE CM but fed with pHrodo bioparticles (Figure 6B) show both red and green fluorescence. Areas that fluoresce both red and green manifest as an orange or yellow color. Looking at Figure 6C, which showed the macrophages treated with RPE CM depleted with an
irrelevant IgG, there were the same clear bright green fluorescence indicating viability. However, as was seen in the previous study, there was a decreased amount of red fluorescence demonstrating suppression of macrophage phagolysosome activation. The macrophages that were treated with RPE CM depleted of α-MSH (Figure 6D), NPY (Figure 6E) or α-MSH + NPY (Figure 6F) showed very little to no red fluorescence as well as little to no green fluorescence. This suggested that the RPE produce a soluble factor that promotes cell death that is blocked by α-MSH and NPY.

**Post-EAU RPE CM Effect on Phagolysosome Activation**

To see if there is a change in RPE suppression of phagolysosome activation in EAU, and to see if therapies that suppress EAU promote RPE suppression of phagolysosome activation, mice were immunized to induce EAU and then treated with PBS, MC5r agonist, or α-MSH. Extraction and preparation of eyecups began only after the treated mice, excluding PBS treated mice, were fully recovered from EAU. Phagolysosome activity assay was assayed, as done in the previous studies, and macrophages were assessed for fluorescence via fluorescent microscopy (Figure 7).
Figure 7. Images of macrophages treated with RPE CM from EAU and post-EAU mice. (A) Image of untreated macrophages at rest without pHrodo bioparticles. (B) Image of untreated macrophages with pHrodo-red bioparticles. Images are taken from two different experiments with same exposure settings.

Figure 7C-D. (C) Image of macrophages with RPE CM from non-immunized mice. (D) Image of macrophages with RPE CM from PBS treated EAU mice. Images are taken from two different experiments with same exposure settings.

Figure 7E-F. (E) Image of macrophages with RPE CM from MC5r Agonist treated EAU mice. (F) Image of macrophages with RPE CM from α-MSH treated EAU mice.

The untreated macrophages showed phagolysosome activation as indicated by a bright red fluorescence (Figure 7B). Macrophages treated by RPE CM from the non-
immunized mice, therefore did not develop EAU, showed suppression of macrophage phagolysosome activation (Figure 7C). Although EAU mice treated with MC5r agonist seemed to fully recover, macrophages treated with RPE CM from mice treated with MC5r agonist did not show a significant suppression (Figure 7D). This was also statistically confirmed (Figure 8) as there was not a statistical difference between the relative CTCF expressed from this group and relative CTCF expressed in macrophages with RPE CM from mice who were treated with PBS (the mice that had not recovered from EAU). However, macrophages treated with RPE CM from EAU mice treated with α-MSH did show suppression of phagolysosome activation (Figure 7E). This is also confirmed as there was a statistical difference, $P<0.05$, between the relative CTCF expressed by macrophages with RPE CM from EAU mice treated with PBS and the relative CTCF expressed by macrophages with RPE CM from EAU mice treated with α-MSH (Figure 9).
Figure 8. The effects of RPE CM from EAU and post-EAU mice on phagolysosome activity. Shown is the relative CTCF ± SEM of pHrodo-red expression in the RPE CM from EAU mice treated with PBS or MC5r agonist. Data is from two separate experiments, N=4. There were no significant difference between relative CTCF expressed by macrophages with RPE CM from PBS treated EAU mice and MC5r Agonist treated EAU mice. *There was significant difference between relative CTCF expressed by macrophages with RPE CM from healthy mice and PBS Treated EAU mice (p<0.0001) as well as MC5r agonist treated EAU mice (p<0.0001).

Figure 9. The effects of RPE CM from EAU and post-EAU mice on phagolysosome activity. Shown is the relative CTCF ± SEM of pHrodo-red expression in the RPE CM from EAU mice treated with PBS or α-MSH. N=3. There was no significant difference between α-MSH treated EAU mice and non-immunized mice. *There was significant differences between untreated mice and non-immunized mice, non-immunized mice and PBS treated EAU mice, PBS treated EAU mice and α-MSH treated post-EAU mice, and untreated and α-MSH treated post-EAU mice (p<0.0001).
Cytokines Involved in Phagolysosome Activation

To identify and measure the cytokines that may be involved in RPE CM ability to down regulate phagolysosome activation in primary macrophages, a mouse cytokine array assay was used on RPE CM extracted from non-immunized mice, PBS treated EAU mice, MC5r agonist treated post-EAU mice, and α-MSH treated post-EAU mice. The Quantibody Mouse Cytokine Array kit was used to assess for twenty different mouse cytokines and have similar detection sensitivity as a traditional ELISA. Array kit included a glass chip with embedded array support. After addition of RPE CM to this glass chip and respective incubation periods, a cocktail of Biotin-Ab is added to detect the RPE CM that had interacted with the cytokine array. Following incubation, labeled streptavidin-labeled Cy3 was added so that fluorescence may be detected only with the cytokine-antibody-biotin complex. After washing the glass chip thoroughly, images were taken at 50.05 seconds exposure and analyzed using the Quantity One software from Bio-Rad Laboratories (Figure 10).
Figure 10. Image of cytokine array glass chip. Image was taken at an exposure time of 50.05 seconds.

The top row is the cytokine standards at various dilutions—the standards becoming less dilute from left to right and the first being the negative control (#1). The first two dilutions (#2, 3) were unable to be analyzed due to low density. Standards were used to convert density (INT/mm$^2$) to concentration (ng/ml).

The top row of the array glass chip shows the cytokine standards at multiple dilutions. The least diluted standard, array #8, had standard concentration of 4,000 pg/ml of IL-6, 2,000 pg/ml of KC and 4,000 pg/ml of VEGF. These concentrations correlated to a density of 309142.17 INT/mm$^2$ of IL-6, 178166.12 INT/mm$^2$ of KC and 219308.92 INT/mm$^2$ of VEGF (INT: Intensity). The most diluted standard that could be detected, fourth from the top left, had concentrations of 49 pg/ml of IL-6, 25 pg/ml of KC and 49 pg/ml of VEGF. These more dilute concentrations correlated to a density of 162555.78 INT/mm$^2$ of IL-6, 159512.89 INT/mm$^2$ of KC and 163296.42 INT/mm$^2$ of VEGF.

Fluorescence indicated that all RPE CM consisted of high concentrations of IL-6. There is also evidence of keratinocyte-derived cytokine (KC) and trace amounts of VEGF.

Table 2. Mouse Cytokine Densities and Concentrations

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cytokine</th>
<th>Density (INT/mm$^2$)</th>
<th>Standard Conc (pg/ml)</th>
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<tbody>
<tr>
<td>Non-Immunized</td>
<td>IL-6</td>
<td>587561.8873</td>
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</tr>
<tr>
<td></td>
<td>KC</td>
<td>201356.4456</td>
<td>NA*</td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
<td>185471.423</td>
<td>0.995</td>
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<tr>
<td>Non-Immunized</td>
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<tr>
<td></td>
<td>KC</td>
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</tr>
<tr>
<td></td>
<td>VEGF</td>
<td>175565.1733</td>
<td>1.25</td>
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<tr>
<td>PBS Treated</td>
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<tr>
<td></td>
<td>KC</td>
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<tr>
<td></td>
<td>VEGF</td>
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<tr>
<td></td>
<td>KC</td>
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<td>4.153</td>
</tr>
<tr>
<td>Treatment</td>
<td>Cytokine</td>
<td>VEGF Value</td>
<td>SD Value</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------</td>
<td>------------</td>
<td>----------</td>
</tr>
<tr>
<td>MC5r Antagonist Treated</td>
<td>IL-6</td>
<td>618916.9884</td>
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<td></td>
<td>KC</td>
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<tr>
<td></td>
<td>VEGF</td>
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<td></td>
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<td>α-MSH Treated</td>
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<td>KC</td>
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<tr>
<td></td>
<td>VEGF</td>
<td>169334.8164</td>
<td>1.468</td>
</tr>
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</table>

*Could not be determined; value exceeded standard curve.

These conversions of cytokine standards to the densities analyzed from the glass chip were then interpolated into individual concentrations based off of the best-fit standard curve (Table 2). IL-6 showed an average concentration of 26,489 ng/ml in RPE CM of EAU and post-EAU mice. Graph shows average concentration levels ±SEM. There was an average IL-6 concentration of 26,489±1,4765 ng/ml in

![Graph showing cytokine concentration](image_url)
RPE CM of non-immunized mice, 112,951±94,053 ng/ml in RPE CM of PBS treated EAU mice, 16,572±11,576 ng/ml in RPE CM of MC5r agonist treated post-EAU mice, and 1,067±517.5 ng/ml in RPE CM of α-MSH treated post-EAU mice.

Figure 12. Mouse cytokine KC concentration in RPE CM of EAU and post-EAU mice. Graph shows average concentration levels ±SEM. There was an average KC concentration of 9.391±0 ng/ml in RPE CM of non-immunized mice, 14.34±0.1091 ng/ml in RPE CM of PBS treated EAU mice, 5.872±1.576 ng/ml in RPE CM of MC5r agonist treated post-EAU mice, and 3.166±1.724 ng/ml in RPE CM of α-MSH treated post-EAU mice.
Figure 13. Mouse cytokine VEGF concentration in RPE CM of EAU and post-EAU mice. Graph shows average concentration levels ±SEM. There was an average VEGF concentration of 0.01383±0.003955 ng/ml in RPE CM of non-immunized mice, 0.01008±0.004114 ng/ml in RPE CM of PBS treated EAU mice, 0.02075±0.004219 ng/ml in RPE CM of MC5r agonist treated post-EAU mice, and 0.02616±0.003229 ng/ml in RPE CM of α-MSH treated post-EAU mice.

CM of non-immunized mice, 112,951 ng/ml in RPE CM of PBS treated EAU mice, 16,572 ng/ml in RPE CM of MC5r agonist treated post-EAU mice, and 1,067 ng/ml in RPE CM of α-MSH treated post-EAU mice. KC showed average concentrations of approximately 9.39 ng/ml in RPE CM of non-immunized mice, 14.34 ng/ml in RPE CM of PBS treated EAU mice, 5.87 ng/ml in RPE CM of MC5r agonist treated post-EAU mice, and 3.17 ng/ml in RPE CM of α-MSH treated post-EAU mice. Finally, VEGF showed average concentrations of approximately 0.01 ng/ml in RPE CM of non-immunized mice, 0.01 ng/ml in RPE CM of PBS treated EAU mice, 0.02 ng/ml in RPE CM of MC5r agonist treated post-EAU mice, and 0.03 ng/ml in RPE CM of α-MSH treated post-EAU mice. These results are shown in Figures 11-13. Though VEGF
concentrations seem to be very miniscule, cytokine IL-6 and KC may be worth further investigation particularly because others have suggested that IL-6 may be an important mediator of inflammation in the eye (Ohta et al., 2000).
DISCUSSION

The results further demonstrated that RPE play an important part in the regulation of macrophage activity, and thus overall maintenance of immunosuppression in the eye. RPE CM significantly reduced, P<0.0001, the activation of phagolysosomes in macrophages that phagocytized opsonized bacterial bioparticles. This suppression was mediated by RPE cells producing soluble neuropeptides α-MSH and NPY.

Depletion of RPE CM of certain neuropeptides helped look into mechanisms behind RPE reduction of phagolysosome activation. The depletion of α-MSH, NPY and α-MSH + NPY caused an almost complete lack of fluorescence. A result like this would signified that the phagolysosome activation in macrophages was suppressed; but, these macrophages also displayed an altered appearance. The results showed that macrophages treated with RPE CM depleted of α-MSH and NPY were predominately dead. This is supported by previous studies that established the importance of these neuropeptides to macrophage viability (Kawanaka & Taylor, 2011; Phan & Taylor, 2013; A. W. Taylor, 2013). Because viability was compromised in these macrophages, α-MSH and NPY role on the down-regulation of macrophage phagolysosome activity could not be fully assessed in the current study.

The most used model of intraocular inflammatory disease is the murine EAU model (Gery et al., 1986; Schalken et al., 1988). The murine models have shown to be valuable in many ways. One major reason is that pathologically, the nature and progression of uveal disease is very similar to human ocular inflammatory disease. Further, once immunized with EAU, the active stage of the disease is rather long in
duration. This allows for more freedom for therapeutic experimentation (Caspi et al., 1988). As such many have studied mechanisms before and during disease but few have looked at the mechanisms post-recovery.

The next part of the current study thus looked into the effects of RPE CM collected from EAU and post-EAU mice on phagolysosome activation of primary macrophages. Results revealed that while RPE CM from non-immunized mice caused suppression of phagolysosome activation in macrophages as we have previously seen, RPE CM from PBS treated mice did not cause a significant suppression of phagolysosome activity. This indicated that during active EAU, RPE CM lost its ability to down-regulate phagolysosome activity. What is more interesting is that RPE CM from α-MSH treated post-EAU mice did recover its ability to suppress phagolysosome activity in the macrophage; however, RPE CM from MC5r agonist treated mice—fully recovered post-EAU mice—did not cause a significant suppression in macrophage phagolysosome activity. This implied that, even though the mice may fully recover from EAU, MC5r agonist treatment does not recover RPE’s ability to down-regulate phagolysosome activity in the macrophage. It also suggested that α-MSH, though also acts as a MC5r agonist, may cause reduction of phagolysosome activity through other melanocortin receptors or may even require mechanisms in addition to MC5r stimulation. The results direct us to think that α-MSH may be required for the recovery of inflammation and further study would be beneficial.

Past research has shown that during active uveitis in humans, there is a significant amount secretion of pro-inflammatory cytokines and chemokines in the intraocular
environment (Curnow & Murray, 2006). Similar cytokines are seen in EAU murine model. However, we know little about the cytokines involved when immunosuppression is lost. To further comprehend the components of immunosuppressive activity of the macrophage by RPE before, during and post-EAU, a mouse cytokine array assay was performed on RPE CM taken from non-immunized mice, EAU, and post-EAU mice.

Results showed that out of the twenty cytokines that the assay could have detected, IL-6, KC, and VEGF were the only mouse cytokines that were identified in all RPE CM regardless of EAU stage. Among the three cytokines that were detected, it is interesting to note that IL-6 was present in extreme concentrations, particularly in the RPE CM from EAU mice that were treated with PBS that showed little to no suppression of phagolysosome activation. This is congruent with past findings that suggests that IL-6 acts as a pro-inflammatory factor in the intraocular environment and is present in high concentrations in the aqueous humor during active EAU and suppression of inflammation from an anti-inflammatory agent also showed decreases in IL-6 concentration (Qin et al., 2014).

Cytokine KC (also known as keratinocyte chemoattractant) was also present in all RPE CM although not as high in concentration as IL-6. Results also showed that KC was in higher concentration in RPE CM extracted from EAU mice treated by PBS. This may be because KC is a chemokine that is involved in chemotaxis and neutrophil activation. This is not unusual as KC has been reported to be involved with the recruitment and activation of neutrophils into areas of high inflammation (Huang, Paulauskis, Godleski, & Kobzik, 1992). VEGF was also detected but at very minimal
concentrations. Perhaps further study can elucidate IL-6 as well as KC and VEGF involvement in the immunosuppressive response of macrophages.

Past study has demonstrated the specific importance of neuropeptides α-MSH and NPY on phagocytosis and phagolysosome activity to the resident macrophage (Phan & Taylor, 2013). It has also been discovered that the RPE, among other parts of the retina such as amacrine cells of the inner nuclear and ganglion cell layer, is an important source of both these neuropeptides (Kawanaka & Taylor, 2011). The current study has further illustrated the importance of the RPE in phagolysosome regulation of the primary macrophage. Also found was that both α-MSH and NPY produced by the RPE are essential to RPE regulation of phagolysosome activation.

Further studies into ocular immune privilege should reveal other mechanisms for regulating immunity that may find clinical value. There exists multiple publications showing the appeal in implementing α-MSH as a form of peptide or gene therapy to suppress maladies such as uveitis, septic shock, hypersensitivity, multiple sclerosis and allograft survival (Gatti S, Colombo G, Buffa R, et. al., 2002; Thomas A Luger & Brzoska, 2007; Mirotti, Castro, Costa-Pinto, & Russo, 2010; A. W. Taylor & Lee, 2010). Most of these current treatments are restricted to steroids; however, with the use of an immunomodulating neuropeptide such as α-MSH, perhaps a remedy with efficacy and less side effects is achievable. It would be interesting to see what clinical applications arise with these current discoveries.
REFERENCES


Medawar, P. B. (1948). Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *British Journal of Experimental Pathology, 29*(1), 58–69.


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phagolysosome activity in primary macrophages and neuropeptides α-MSH and
NPY possible contribution to this immunosuppression.

Jose M. Roque, M.D., Inc. (July 2013 – August 2013)
Front & Back Office Receptionist and Procedure Scheduler
Responsible for setting appointments and procedures, insurance verification and
referral requests, procedure registration and authorization requests.

Nakamoto/Chou, LLP (August 2009 – October 2010; February 2012 – March 2013)
Legal Clerk
Duties include setting up incoming files into the filing and billing system, scanning
and inputting legal and medical records into filing system, scheduling and preparing
for depositions, cross-examinations, and medical appointments, taking dictation and
composition of notices and correspondence.
Laser Medi Spa (March 2011 – January 2012)
Front Office Receptionist & Coordinator
Responsible for duties including insurance verification, appointments, managing office and medical supplies. Also responsible for patient care, implementing new technology and nurse training sessions, advertising/marketing, interviewing and training new staff.

Independent Study and Research in Epidemiology with Dr. David P. Phillips (January 2009 – March 2009)
Study on the relationship between suicide rates and presidential elections
Research showed a spike in suicide rates after results of the presidential elections. Data was collected on media references to “phrases of depression” to show its impact on suicide rates.

The Scripps Research Institute (December 2007 - September 2008)
Responsible for genotyping, cloning, preparing solutions and plates. Introduced mutations via PCR mutagenesis and prepared Xenopus oocytes for injection.

Beth Israel Deaconess Medical Center (Summer 2005)
Responsible for extracting DNA from animal cells, gel electrophoresis, preparing cultures for the analysis of proteins in tumor blood vessels.

VOLUNTEER EXPERIENCE
Serve The People Health Center and Food Pantry (2011 - 2013)
A health center dedicated to the low-income community. Volunteering as a front office receptionist and clerk by accepting patients and setting up charts as well as back office work such as taking vitals.

West Anaheim Medical Center (2012)
Volunteer at the Performance Improvement department dedicated to the improvement and maintenance of patient and health provider safety. Responsibilities included keeping records of the quality of patient care and work place environment and writing reviews on abnormal mortalities at the hospital.

Crossway Community Church (2012-2013)
Taught young toddlers of ages 2-4 years and learned about the extra precautions associated with young children and the importance of a supportive and edifying environment.

Paint Your Heart Out (2011)
Non-profit organization dedicated to helping repair homes for low-income, disabled, senior and veterans.

Giving Children Hope (2011)
Non-profit organization that provides other “front line” organizations with basic
resources for children such as school and medical supplies. I helped sort and pack donated medical supplies that was eventually sent to Japan for earthquake and tsunami relief.