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ORIGINAL ARTICLE

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Melanocortin 5 Receptor Expression and Recovery of Ocular Immune Privilege after Uveitis

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ABSTRACT

Purpose: The therapeutic use of the RPE-neuropeptide α -MSH suppresses experimental autoimmune uveitis (EAU). This suppression is partially through the α -MSH melanocortin 5 receptor (MC5r). Therefore, we examined the possible role of MC5r-expression in the recovery of RPE suppression of phagolysosome-activation in macrophages following α -MSH-treatment of EAU.

Methods: The conditioned media of cultured *in situ* RPE-eyecup from α -MSH-treated EAU wild-type and MC5r(-/-) mice were used to treat macrophages to assay for phagolysosome activation.

Results: MC5r(-/-) mice treated with α -MSH recovered from EAU, but with greater retinal damage, and the RPE suppressed phagolysosome activation in wild type but not in MC5r(-/-) macrophages. In addition, α -MSH did not suppress phagolysosome activation in MC5r(-/-) macrophages, and resting-MC5r(-/-) macrophages had augmented phagocytic activity.

Conclusion: α -MSH treatment of EAU mediates a MC5r-dependent recovery of RPE suppression of phagolysosome activation in macrophages possibly altering antigen processing and presentation. Also, MC5r-expression helps protect the retina from inflammatory damage. In addition, MC5r-expression is important in the homeostatic maintenance of phagosome-maturation within macrophages.

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The eye has adapted within its tissue microenvironment several mechanisms, which work to prevent and suppress the activation of inflammation that establishes immune privilege.¹ The mechanisms of immune privilege are mediated in part by neuropeptides produced within the eye. These molecules mediate localized suppression of inflammation, prevent activation of effector T cells, and promote immune tolerance to antigen expressed within the eye. An essential mediator of these immunomodulating mechanisms is the neuropeptide alpha-melanocyte-stimulating hormone (α -MSH).^{1–3} The neuropeptide α -MSH is constitutively expressed within the eye, and retinal pigment epithelial cells (RPE) are a source of α -MSH.^{4,5} In the aqueous humor, α -MSH mediates aqueous humor suppression of effector T cell activation and induces T cell production of TGF- β that further suppresses immune cell activity.⁶ In the conditioned media of RPE-eyecups, α -MSH suppresses pro-inflammatory activity, promotes anti-inflammatory cytokine production, alters the maturation of phagosomes, and induces suppressor cell activity in macrophages.^{5,7–12}

The neuropeptide α -MSH is part of the highly conserved melanocortin family of molecules that includes Adreno corticotrophic hormone (ACTH), and the five G-protein-coupled melanocortin receptors.^{13–15} While ACTH binds all five melanocortin receptors (MCR), α -MSH binds all but MC2r.^{15,16} The MC2r is exclusively expressed on the adrenal glands through which ACTH induces corticosteroid production. The melanocortin receptors MC1r, MC3r, and MC5r are expressed on immune cells, and cells of the retina.^{8,17–23} The literature demonstrates that

there is differential regulation of immune cell activity through the different melanocortin receptors. The neuropeptide α -MSH through MC1r and MC3r suppresses pro-inflammatory activity in activated macrophages.^{21,24–27} Through MC5r, α -MSH promotes the induction of Treg cells and induces suppressor APC with the capacity in an antigen-specific manner to mediate counter-conversion of effector CD4 $^{+}$ T cells into Treg cells.^{8,18,22,28–31}

Immune privilege is an evolutionary adaptation that protects the eye from the collateral damage of inflammation to its delicate and non-replicating light-gathering tissues and reduces susceptibility to autoimmune disease. Mice with experimental autoimmune uveitis (EAU), a well-studied rodent model of human endogenous uveitis, enter a stage of chronic retinitis that resolves without therapeutic interventions.^{32,33} As the untreated EAU mice begin to resolve the retinitis there is an expansion of the suppressor APC in their spleens.^{8,31} These suppressor APC counter-convert retinal-autoantigen effector T cells into inducible Treg cells, which provide long-term protection from reactivation of autoimmune uveitis.^{22,30,31} The expansion of the suppressor APC is dependent on the expression of MC5r, and without MC5r expression, there are no detectable retinal-autoantigen specific Treg cells in the spleen post-EAU. When EAU mice are therapeutically treated with α -MSH there is an accelerated resolution of retinitis, and expansion of the suppressor antigen-presenting cells (APC) in the spleen.^{8,31} Moreover, α -MSH-treated EAU mice have well-preserved retinal structures, and are resistant to re-inducing EAU.^{18,34}

When treated with α -MSH, EAU mice with MC5r knocked-out ($MC5r^{(-/-)}$) have an accelerated resolution of EAU, but without the post-EAU suppressor APC, and without the induction of protective inducible-Treg cells in the spleen.^{22,30,31,33} This suppression of uveitis by α -MSH-treatment is presumably the anti-inflammatory activity mediated by α -MSH through MC1r and MC3r. However, α -MSH-therapy in EAU $MC5r^{(-/-)}$ mice had less preservation of retinal structures.³⁴ Recent reports have demonstrated that α -MSH promotes retinal cell survival through MC1r and MC5r.^{19,20,35} Also, the $MC5r^{(-/-)}$ mice are highly susceptible to an immediate and severe reactivation of EAU.¹⁸

Our previous findings showed that RPE through its production of α -MSH regulates phagocytic maturation within APC that can change with disease.¹¹ This regulation is important since phagocytic activity is linked to both the induction of inflammation and the processing and presentation of antigen, of the innate and adaptive immune interface.³⁶ Phagocytes (macrophages, dendritic cells, and microglial cells) phagocytize proteins, dead cell material, and toxic molecules to prevent them from adversely affecting tissue structure and cell survival. As the phagosome matures it fuses with the lysosome. Within the acidic environment of the activated phagolysosome, there is an efficient degradation of proteins into peptides that can be shuttled into the MHC class II compartment. If the peptides have high affinity to the MHC class II molecule, they are loaded onto MHC class II and presented on the surface of the phagocyte that is now an antigen-presenting cell. Altering this pathway would prevent the activation of autoreactive effector T cells and be beneficial to maintaining a healthy ocular micro-environment. To suppress the phagosome maturation pathway in autoimmune disease would help to rob the autoreactive effector T cells their central driving mechanism of presented autoantigen. Since suppression of EAU by α -MSH-therapy may require MC5r-expression, we assayed whether MC5r expression is needed for α -MSH-therapy to restore RPE-regulation of phagosome maturation in EAU.

Materials and methods

Animals

The mice used in these experiments were 6–8 week old C57BL/6 J and $MC5r^{(-/-)}$ (C57BL/6 J background) mice. The C57BL/6 J mice were purchased from Jackson Laboratories (Bar Harbor, ME), and housed in the Boston University Animal Science Center (ASC). The $MC5r^{(-/-)}$ mice were rederived mice supplied to us from Dr. Robert Cone, Oregon Health Science Center, and bred in the Boston University ASC. All experimental use of animals was in accordance with procedures approved by the Boston University Institutional Animal Care and Use Committee and ARVO statement for the use and care of animals in vision research.

Induction of Experimental Autoimmune Uveitis, and α -MSH Peptide Therapy

The EAU was induced in the mice as previously described.^{8,11,30,34} Human interphotoreceptor retinoid binding

protein peptide (IRBP) spanning amino acid residues 1–20 emulsified in complete Freund's adjuvant was injected subcutaneously. This was followed by intraperitoneal injections of 10 ng of pertussis toxin in 100 μ L of PBS 2 days apart. Every 3 to 4 days, eye fundus was examined and scored for severity of retinal inflammation on a score of 0–5.^{29,37,38} Retinas with no inflammation were scored as 0, with only white focal lesions of vessels were scored as 1, with linear lesions of the vessels within half of retina were scored as 2, with linear lesions of vessels over more than half of the retina were scored as 3, with severe chorioretinal exudates or hemorrhages in addition to the vasculitis were scored as 4, and retinas with subretinal hemorrhage or retinal detachments were scored as 5. No mouse under our housing and care reached a clinical score of 4. Data presented are from 3–5 experiments, and in each experiment, 10 mice per group were used. The α -MSH peptide was purchased from Bachem (Torrance, CA). The α -MSH was reconstituted to a concentration of 500 μ g/mL in sterile PBS carrier. When the uveitis reached the start of its chronic inflammatory plateau, a sustained EAU score of 3, 50 μ g of α -MSH was intraperitoneally injected, and two days later a second injection was given.

Preparation of Mouse RPE Eyecups

After treatment, EAU was scored every 3 to 4 days per week until the mice treated with α -MSH had resolved the inflammation. The eyes were enucleated for the preparation of RPE eyecups. The RPE eyecups were made based on our previously published method.^{5,7,11,39} Eyes were placed in ice-cold 0.01 M PBS (Lonza) and the connective tissue and optic nerve were removed from the eyeball. A circumferential cut was made around the limbus to remove the entire anterior segment including the cornea, iris, ciliary body, and lens. The neural retina was gently removed from the RPE monolayer and discarded. The remaining posterior of the eye (RPE eyecups) contains only the RPE monolayer, choroid, and sclera. The RPE eyecup was placed into the well of a 96-well round-bottom tissue culture plate (Corning, Corning, NY, USA) containing 200 μ L Serum-free Media (SFM): RPMI 1640 supplemented with a 1/500 dilution of ITS+ (Insulin/Transferrin/Selenium + linoleic acid) (Sigma-Aldrich, St. Louis, MO), and 0.1% sterile BSA solution (Sigma-Aldrich). The cultures were incubated for 24 hours at 37°C in 5% CO₂, and the conditioned media (CM) was collected, centrifuged at 2100 g for 5 minutes, and the supernatant was used immediately in the assays as RPE eyecup CM.

Collection of Primary Resting Macrophages

Resting peritoneal macrophages were used to assay for phagolysosome activation. Macrophages were obtained from a peritoneal lavage of naive WT or $MC5r^{(-/-)}$ mice. Five mL 0.01 M PBS (Lonza) was injected into the peritoneal cavity and was recovered after 5 minutes. The lavage was centrifuged at 400 g for 5 minutes and then mixed with 1 mL red blood cell lysing buffer (Sigma-Aldrich) for 5 minutes on ice, centrifuged

at 400 g for 5 minutes. The cells were resuspended in RPMI 1640 with 10% fetal bovine serum (Hyclone, Logan, UT, USA).

Phagolysosome Activation

To assay the effects of RPE CM on phagolysosome activation in macrophages, the peritoneal macrophages were seeded at 1×10^5 cells per well in a Nunc Lab-Tek 8-chamber slide (ThermoFisher, Waltham, MA) and incubated at 37°C for 2 hours. Each well was washed once with 200 µL SFM, and the macrophages were treated with a 1:2 dilution of RPE CM for 30 minutes at 37°C in 5% CO₂. Opsonized pHrodo-Red Staphylococcus aureus bioparticles (0.25 µg) from Invitrogen (ThermoFisher) were added to each well, and cultures were incubated at 37°C in 5% CO₂ for 24 hours. Each culture well was assayed by fluorescence microscopy using an FSX100 inverted fluorescence imaging-microscope (Olympus, Center Valley, PA, USA). Ten images were captured from each well at constant exposure and time for all the wells in an experiment, and at least 100 cells were measured per culture.

Phagocytosis Activities

To investigate the difference between the phagocytosis and phagolysosome activation of WT and MC5r^(-/-) mice, the peritoneal macrophages were cultured the same as above, and a solution of equal weight of opsonized AF488-conjugates of Escherichia coli bioparticles (0.125 µg), and opsonized pHrodo-Red S. aureus bioparticles (0.125 µg) were added to the cultures. The cells were incubated for 2 hours at 37°C in 5% CO₂ and washed with warm serum-free media to remove bioparticles that were not phagocytized. Fresh serum-free media was added, and the cells incubated for a total of 48 hours. The cultures were imaged using the FSX100 inverted fluorescence imaging-microscope at 2, 24, and 48 hours of incubation, and the fluorescent intensity of at least 100 cells was measured per culture.

Effect of α-MSH and Neuropeptide Y (NPY) on Phagolysosome Activation

To find out whether WT and MC5r^(-/-) macrophages responded differently to the neuropeptides of α-MSH and NPY, macrophages were cultured the same as above, and treated with either 1 ng/ml of α-MSH, 1 ng/ml of NPY (1 ng/ml), or a mixture of α-MSH and NPY at 1 ng/ml each.⁹ The cultures were incubated for 30 minutes at 37°C in 5% CO₂, and 2 µl of opsonized pHrodo-Red S. aureus bioparticles (0.25 µg) were added. The cultures were incubated for 30 minutes at 37°C in 5% CO₂. The cells were examined by fluorescence microscopy. Ten images were captured from each well at a constant exposure and time for all wells in an experiment, and at least 100 cells were measured per culture.

Image Analysis

Each fluorescent image was analyzed using a MATLAB program (R2017b) to calculate an objective measure of fluorescence. For experiments analyzing activation of phagolysosome,

the relative intensity was calculated as a ratio of the sum of intensities above the “positive” threshold to the number of pixels occupied by cells. This was done by creating a combination of binary masking techniques and filters using MATLAB’s built-in functions (more information in supplementary). To do the analysis, the image was processed to isolate relevant artifacts in the image such as the cells from noise (i.e. random artifacts). The fluorescence was calculated for each image from this analysis (Sub 1).

Histology

Enucleated eyes from EAU mice with or without treatment were fixed in Davidson’s fixative for 24 hours and then transferred to 10% buffered formalin for an additional 24 hours. The eyes were subsequently embedded in paraffin, after which 5-µm sections were cut and stained with H&E. The images were taken using a CX33 microscope (Olympus) and QColor 5 camera system and software (Olympus). Presented are the retinal sections at two optic disk diameters from the center of the optic nerve. Thickness of the retina and the outer nuclear layer (ONL) were measured using ImageJ software of full-length retinal sections centered on the optic nerve (representative images in Supplemental Figure 2). Measurements were made at 0.5, 1.0, 1.5, 2 mm to the right and left (-0.5, -1.0, -1.5, -2 mm) of the optic nerve head.

Statistical Analysis

Statistical significance was calculated using an ordinary one-way ANOVA with a post-analysis Dunnett’s multiple comparisons test to compare means between test groups and controls. For analysis of EAU scores, a non-parametric Mann–Whitney T-test was used to measure significance. Statistical differences for retinal layer thickness were determined using a two-way ANOVA with a post-analysis Tukey multiple comparisons of the whole curve. In all cases, significance was detected when P ≤ 0.05. The data is presented as the mean ± SEM for each experimental group. Presented micrograph images represent the group mean of each experiment.

Results

The Effects of α-MSH-therapy on EAU MC5r^(-/-) Mice

We have previously demonstrated that the resolution of EAU in mice is in part associated with the α-MSH receptor MC5r. To demonstrate the importance of MC5r in resolving EAU following α-MSH-treatment, we assayed the clinical EAU score, and by histology examined the structure of the retina in EAU MC5r^(-/-) mice treated with α-MSH. In both the wild-type (WT) and MC5r^(-/-) mice, the course of EAU was similar (Figure 1a,b) with both reaching a level of chronic inflammation at a clinical score of 3. The MC5r^(-/-) mice took an extra week to reach the chronic stage of EAU. As we have shown before in WT EAU mice following α-MSH therapy, the inflammation begins to resolve within a week and reaches clearance in 30 days (Figure 1a). In contrast, the α-MSH-treated MC5r^(-/-) EAU-mice took almost 2 weeks for resolution to start, but still

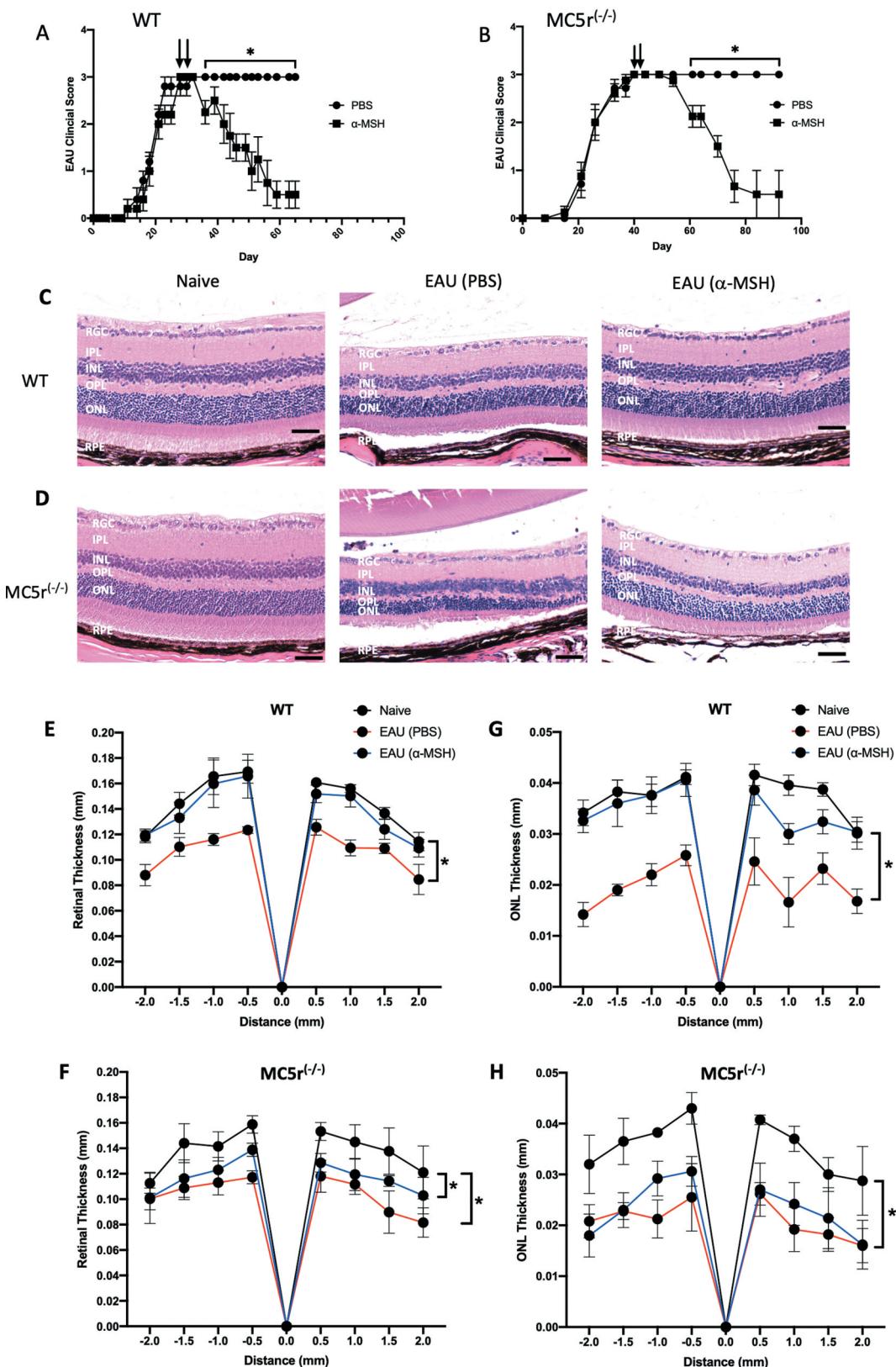


Figure 1. The effects of α-MSH-treatment on EAU in MC5r^(-/-) mice. EAU was induced in Wildtype and MC5r^(-/-) mice and treated with α-MSH when clinical scores reached the chronic phase of inflammation (clinical score = 3) on Day 30 for WT mice and Day 40 for the MC5r^(-/-) with a second injection of α-MSH 2 days later. The mean EAU scores ± SEM of each treated group (N = 10) of (a) WT and (b) MC5r^(-/-) mice are presented over time. Representative retinal sections on Day 65 of WT mice and Day 90 of MC5r^(-/-) mice were stained and presented. (c) The retinas of α-MSH treated EAU mice (EAU (α-MSH)) appeared the same as retinas of naive mouse eyes. The vehicle-injected (PBS) EAU retina was the thinnest with cell loss in both inter-nuclear layer and outer-nuclear layer. (d) Both α-MSH and PBS-injected MC5r^(-/-) EAU mice had retinas that were thinner than the naive mouse retina with about half the photoreceptor outer-segment layer lost in the PBS-injected MC5r^(-/-) EAU mouse retinas. The size bar equals 50 μm. RGC – Retinal Ganglion Cells Layer; IPL – Inner Plexiform Layer; INL – Inner Nuclear Layer; OPL – Outer Plexiform Layer; ONL – Outer Nuclear Layer; RPE – Retinal Pigment Epithelium. The thickness of retina (e, f) and ONL (g, h) (N = 5 each) were measured by ImageJ and presented as mean (mm) of thickness ± SEM at 0.5 mm intervals from the optic nerve head. There is no statistical difference between WT and α-MSH-treated EAU-WT retinal and ONL thickness (e, g); whereas there were (* P ≤ 0.001) significant loss of retinal and ONL thickness in the untreated and α-MSH-treated EAU MC5r^(-/-) mice.

reached clearance in 30 days after treatment (Figure 1b). This suggests that in MC5r^(-/-) mice, EAU is a prolonged process of inflammation both in development and in α-MSH-induced resolution.

To see if there is a difference in the retinal structures of the EAU WT and MC5r^(-/-) mice, the eyes were collected on Day 65 for the WT and Day 90 for the MC5r^(-/-) α-MSH treated. Naive mouse eyes were also sectioned and stained for histological analysis (Figure 1c,d). The retinal structures of Naive WT and MC5r^(-/-) mice were similar (Figure 1c,d Naive), but the retinal structure in untreated-EAU MC5r^(-/-) mice had severe retinal degeneration with loss of photoreceptor outer-segments and nuclear layer thinning compared to the untreated WT EAU mouse retinas (Figure 1c,d EAU(PBS)). The retinas from α-MSH-treated EAU WT-mice retained almost normal retinal layer thickness and structure, and retention of ganglion cells (Figure 1c EAU(α-MSH)). In contrast, the retinas of α-MSH-treated EAU-MC5r^(-/-) mice had detectable thinner nuclear and photoreceptor layers with ganglion cell dropout (Figure 1d EAU(α-MSH)). In eyes with EAU, there is a significant thinning of the retina and the outer nuclear layer (ONL) in both WT and MC5r^(-/-) mice (Figure 1e-h). Treatment with α-MSH preserved the thickness of the retina and ONL in EAU wild-type mice (Figure 1e,g); however, α-MSH-therapy had no effect on preventing retinal and ONL thinning in EAU MC5r^(-/-) mice (Figure 1f,h). These results demonstrate that the resolution of clinical inflammation in EAU following α-MSH-treatment is not fully dependent on MC5r expression; however, MC5r expression is needed for α-MSH-treatment to protect the retinal structure from the damage of inflammation within the retina.

Post-EAU Recovery of RPE Suppression of Phagolysosome Activation in Macrophages after α-MSH-treatment

Since it is considered that tissue damage is collateral to an inflammatory response, we examined the possibility that without MC5r expression there is missing tissue sparing RPE suppression of immune cell activity. Our previous studies demonstrated that RPE suppresses phagosome maturation at an early stage, down-regulate lysosomal-associated membrane protein 1 (LAMP-1) expression needed for phagolysosome formation and activation in macrophages.^{11,12} Therefore, we examined the possibility that α-MSH-therapy promoted RPE recovery of its ability to govern innate immune cell activity by suppressing phagolysosome activation in macrophages. Cultures of RPE eyecups from EAU WT and MC5r^(-/-) mouse eyes were collected on EAU Day 65 for the WT and Day 90 for the MC5r^(-/-) mice. These mice were either untreated or treated with α-MSH. Age-matched naive mouse RPE eyecups were also collected. The conditioned media of cultured RPE-eyecups were used to treat macrophages given opsonized pHrodo-Red bioparticles to phagocytize. The phagocytized pHrodo-Red-bioparticles are weakly fluorescent before they are in an activated phagolysosome where the acidic conditions make the bioparticles fluoresce at their maximum intensity. This allows for quantifying phagolysosome activation.

The RPE from both naive WT and MC5r^(-/-) mice significantly suppressed phagolysosome-activation in WT macrophages (Figure 2a-e (Naive)), and in contrast, the RPE from

untreated EAU WT and MC5r^(-/-) mice did not suppress phagolysosome-activation in the WT macrophages (Figure 2a-e (EAU+PBS)). Treatment of both EAU WT and MC5r^(-/-) mice with α-MSH significantly recovered RPE suppression of phagolysosome-activation in the WT macrophages (Figure 2a-e (EAU+α-MSH)). Since in MC5r^(-/-) mice both the RPE and the macrophages are MC5r^(-/-), we assayed the effects of conditioned media of RPE-eyecups from MC5r^(-/-) mice on phagolysosome activation in macrophages from MC5r^(-/-) mice (Figure 2c,f). While the RPE from Naive MC5r^(-/-) mice suppressed phagolysosome-activation in the MC5r^(-/-) macrophages, the RPE from α-MSH-treated EAU MC5r^(-/-) mice did not suppress phagolysosome-activation in the MC5r^(-/-) macrophages as it did in WT macrophages. These results demonstrated that α-MSH-treatment of EAU promotes recovery of RPE suppression of phagolysosome activation in macrophages whether or not the RPE are in eyes expressing MC5r; however, the benefit is dependent on MC5r expression by the macrophages.

The Effects of α-MSH and NPY on Phagolysosome Activation in MC5r^(-/-) Macrophages

The results suggest that the macrophages from MC5r^(-/-) mice were not affected by the RPE mediated suppression of phagolysosome activation. The neuropeptides α-MSH and NPY are two soluble factors produced by the RPE that suppress phagolysosome activation.^{9,11} Resting macrophages obtained from WT and MC5r^(-/-) mice were treated with α-MSH, NPY, or both, and then assayed for phagolysosome activation with opsonized-pHrodo-Red bioparticles (Figure 3). The phagolysosome activation in WT macrophages was significantly suppressed by α-MSH, NPY, and co-treatment of α-MSH and NPY (Figure 3a,c). In contrast, the two neuropeptides and in combination did not have an effect on phagolysosome activation in MC5r^(-/-) macrophages (Figure 3b,d). Therefore, without expression of MC5r α-MSH does not suppress phagolysosome activation in macrophages. Interestingly, the MC5r^(-/-) macrophages are not suppressed by NPY which does not work through MC5r. This suggests the possibility that resting MC5r^(-/-) macrophages are differently regulated in their phagocytic activity than WT macrophages.

The Phagocytic Activity of MC5r^(-/-) Macrophages

To find out whether the phagocytic activity of MC5r^(-/-) macrophages was different from WT macrophages, we performed a timed assay that traced the maturation of phagocytized bioparticles. This was done by culturing the macrophages with a mixture of opsonized-Alexa488-conjugated-bioparticles and pHrodo-Red-conjugated-bioparticles for 2 hours then washed the cultures of any unphagocytized bioparticles from the cultures. The phagocytized Alexa488-bioparticles move through the maturing phagosomes fluoresce until they are degraded in an active phagolysosome. In contrast, the phagocytized pHrodo-Red bioparticles we used above are weakly fluorescent until they reach the activated-phagolysosome. We took images of the macrophage cultures at 2, 24, and 48 hours after adding the

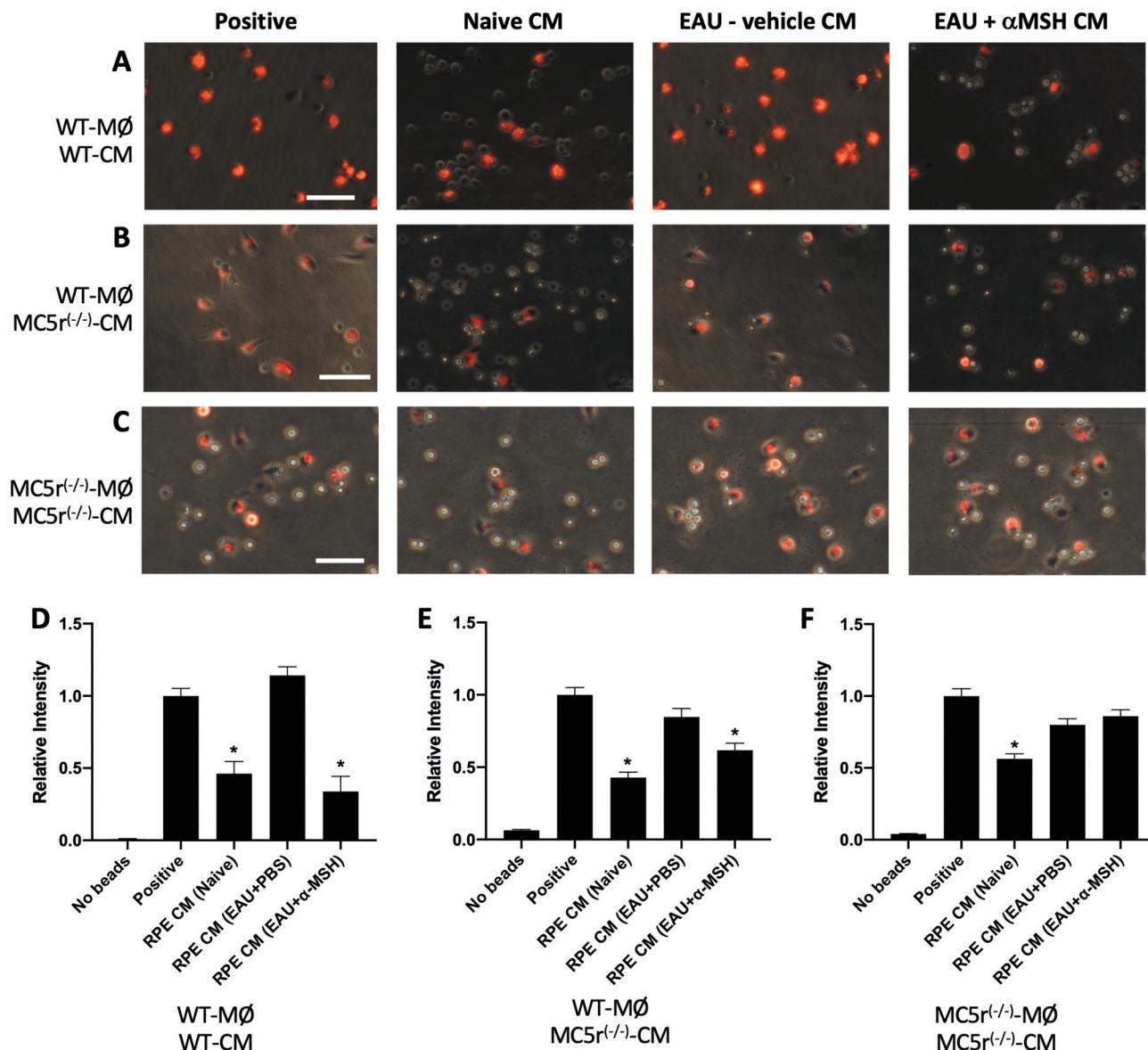


Figure 2. The effects of α-MSH-treatment of EAU on recovery of RPE suppression of phagolysosome activity. RPE-conditioned media (CM) were collected from 24-hr cultures of RPE eyecups from WT (a and b) and MC5r^(-/-) (c) eyes of naive mice, EAU mice injected with PBS-vehicle (EAU+PBS), or EAU mice treated with α-MSH (EAU+α-MSH). Primary resting macrophages from (a) WT mice or b and c) MC5r^(-/-) mice were treated with the CM and given opsonized pHrodoRed-bioparticles to phagocytize. The cells were incubated for 24-hours, imaged and fluorescent intensity was measured. Presented are representative images of the pHrodoRed-bioparticle fluorescence in the macrophages indicating activated phagolysosomes. The size bar equals 50 μm. (d) The relative fluorescent intensity of phagolysosome activation in WT macrophages treated with WT-CM was calculated vs the positive control macrophage cultures and presented are the mean ± SEM of 3 independent experiments (N = 5). Both naive CM and EAU+α-MSH CM (*) significantly (P ≤ 0.001) suppressed phagolysosome activation while vehicle (PBS)-treated EAU-CM showed no suppression. (e) The relative fluorescent intensity of phagolysosome activation in WT macrophages treated with MC5r^(-/-)-CM was calculated vs the positive control macrophage cultures and presented are the mean ± SEM of 3 independent experiments (N = 5). The naive MC5r^(-/-)-CM and the α-MSH-treated EAU MC5r^(-/-)-CM (*) significantly (P ≤ 0.001) suppressed phagolysosome activation. (f) Using primary resting MC5r^(-/-) peritoneal macrophages instead of wildtype-macrophages showed that only the naive MC5r^(-/-)-RPE-CM (*) significantly (P ≤ 0.001) suppressed phagolysosome activation. Presented are the mean ± SEM of 3 independent experiments (N = 5).

opsonized-bioparticle mixture (Figure 4a,b). The phagocytic activity (green fluorescence intensity per pixel) in both WT macrophages and MC5r^(-/-) macrophages was the same showing increased intensity at 24 hours, due to the coalescing of the maturing phagosomes within the macrophages (Figure 4c). This was followed by the expected decrease at 48 hours as the phagosomes further matured into phagolysosomes and degraded the bioparticles. The rate of phagolysosome activation was significantly different between the WT and MC5r^(-/-) macrophages, with the MC5r^(-/-)

macrophages having nearly twofold greater intensity than the WT macrophages (Figure 4c). When the ratio of Red to Green intensity was calculated, indicating the relative progression of phagosome maturation into a phagolysosome, a threefold increase in phagolysosome maturation in the MC5r^(-/-) macrophages was seen at 48 hours (Figure 4d). These results demonstrated a higher level of phagosome maturation in MC5r^(-/-) macrophages, suggesting that the macrophages are more active when stimulated for phagocytosis than WT macrophages.

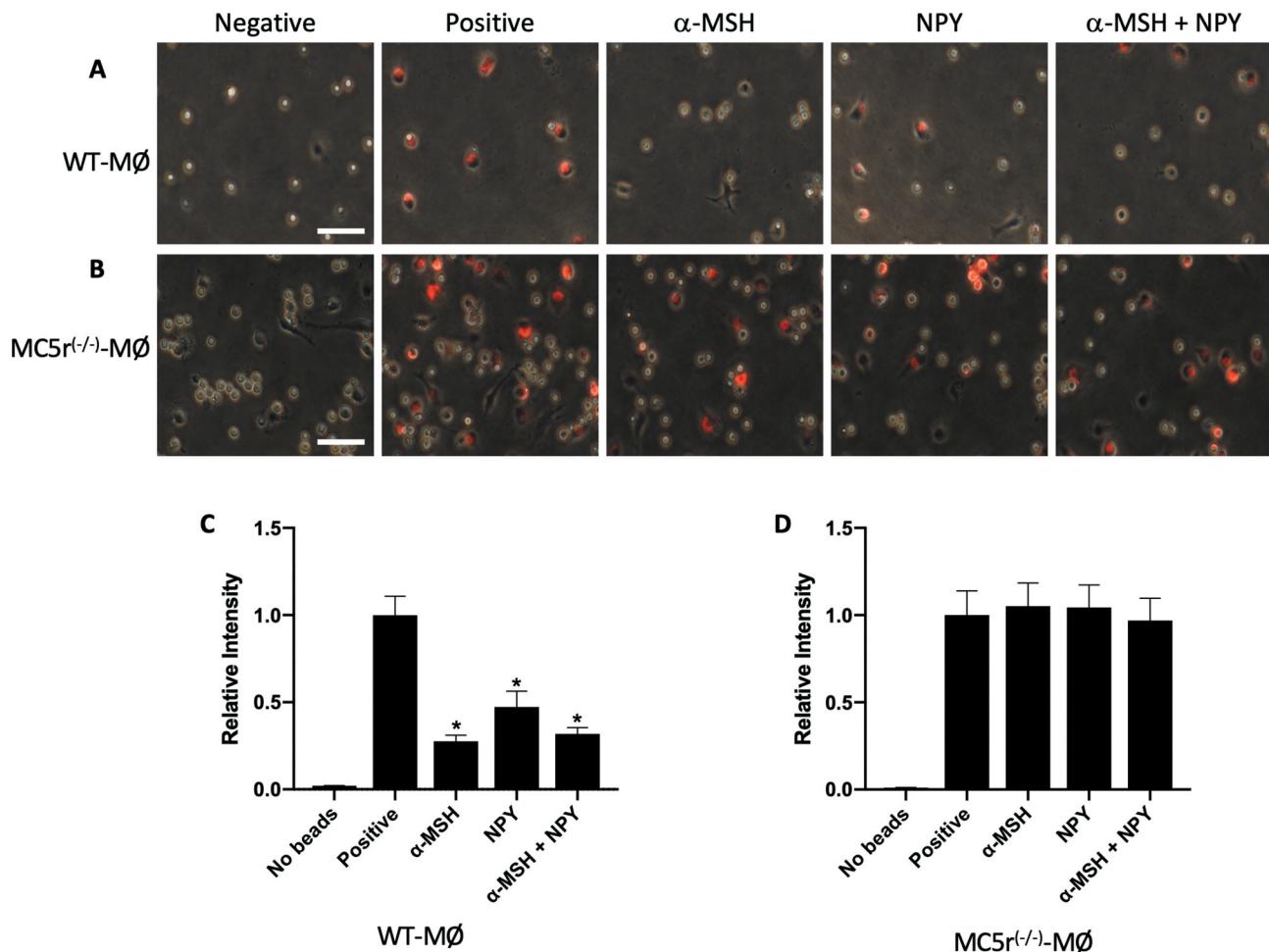


Figure 3. The effects of neuropeptides on phagolysosome activation in MC5r^(-/-) macrophages. Wildtype and MC5r^(-/-) peritoneal macrophages were collected and treated with α-MSH, NPY or both for 30 minutes, and then given opsonized-pHrodoRed-bioparticles. The cells were incubated for 24 hours and imaged by microscopy. Presented are representative images of red-fluorescence expressed in (a) wildtype and (b) MC5r^(-/-) macrophages indicating activated phagolysosomes. The size bar equals 50 μm. (c) The relative fluorescent intensity of the bioparticles in the untreated (Positive) and treated wildtype-macrophages were measured. The relative fluorescent intensity was calculated to the positive control and presented as the mean ± SEM of 3 independent experiments. WT macrophages treated with α-MSH, NPY or α-MSH+NPY were (*) significantly suppressed ($P \leq 0.001$) in phagolysosome activation. (d) The relative fluorescent intensity of the bioparticles in the untreated (Positive) and treated MC5r^(-/-) macrophages. The relative fluorescent intensity was calculated to the positive control and presented as the mean ± SEM of 3 independent experiments. No treatment suppressed phagolysosome activation in the MC5r^(-/-) macrophages. The negative controls are macrophages cultured with no bioparticles.

Discussion

The role of melanocortins in regulating immune cell activity has been well documented, including in our own studies that show the importance of the melanocortins in the mechanisms of ocular immune privilege.^{4–6} Our current results demonstrated that the highly conserved neuropeptide α-MSH, and its equally conserved melanocortin receptors, in a therapeutic manner have the potential to reestablish ocular immune privilege within eyes with autoimmune uveitis. In addition, as we have seen before, there is a differential effect of α-MSH through its melanocortin receptors on immune cell activity with a dependence of macrophage expression of MC5r to have their phagocytic activity regulated by RPE after recovery from uveitis. Our results also demonstrated that there may be a different mechanism of suppressing phagocytic activity of macrophages by RPE in MC5r^(-/-) mice and that without MC5r macrophages had an enhanced level of phagosome maturation. Therefore, α-MSH through MC5r mediates RPE suppression of antigen processing in potential APC and may be a homeostatic moderator of phagosome-maturation within macrophages.

Rodent EAU has been the best model to identify important conserved pathways in immune activity associated with autoimmune disease of the retina. This includes recent findings of the potential for the gut microbiome to induce autoreactive effector T cells to antigens within the eye.⁴⁰ Also, it has revealed the role of Th1 and Th17 cells in the severity and duration of uveitis, and that targeting their cytokines with biologics is effective in suppressing uveitis.⁴¹ A unique feature of EAU in C57BL/6 mice is that these mice resolve autoimmune uveitis without intervention, and are resistant to reactivation of ocular-autoimmune disease.¹⁸ It has been seen that there is at the resolution of EAU a significant increase in Treg cells both within the eye and in the spleen.³³ In the spleen of EAU-recovered mice, there is present a suppressor APC that is dependent on MC5r-expression for its activation, and its presence in the spleen.⁸ These suppressor APC counter-convert effector T cells into Treg cells.³¹ These Treg cells are retinal-antigen specific and express markers of inducible Treg (iTreg) cells.³⁰ The suppressor APC are induced when α-MSH-therapy

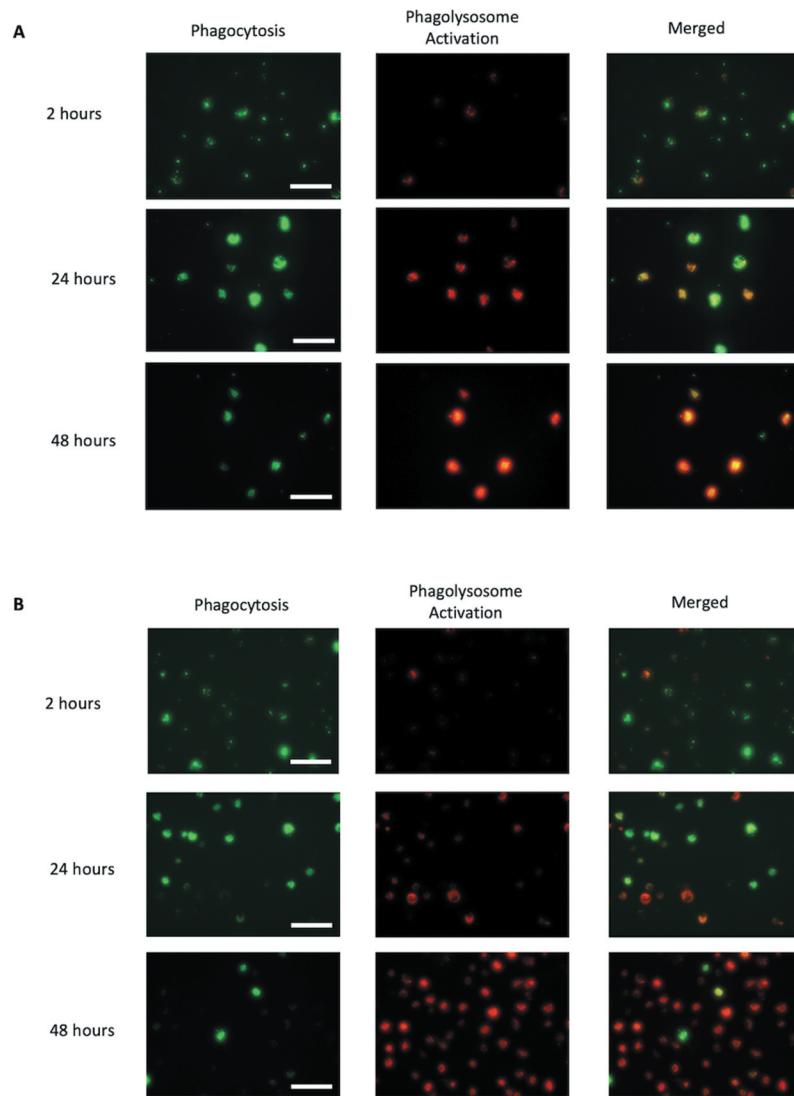


Figure 4. Comparison of phagocytic activity in Wildtype and $MC5r^{(-/-)}$ macrophages. Both wildtype (a) and $MC5r^{(-/-)}$ (b) macrophages were cultured with a mixture of opsonized AF488-bioparticles and pHrodoRed-bioparticles. Two hours later the cultures were washed to remove unphagocytized bioparticles. At 2, 24, and 48 hours fluorescent images were taken and the expression of green-fluorescent (indication of phagocytic uptake), and red-fluorescent (indication of phagolysosome activation) were imaged. The green and red-fluorescent images of (a) WT and (b) $MC5r^{(-/-)}$ macrophages are presented. The size bar equals 50 μ m. (c) From the images of 4A and B the relative green and red-fluorescent intensity, mean \pm SEM of 6 cultures, were calculated relative to 2-hour WT macrophages. A significant difference ($P \leq 0.001$) was seen between relative red-fluorescence intensity between WT and $MC5r^{(-/-)}$ macrophages at 48 hours of incubation. (d) The ratio of red to green fluorescent-intensity was calculated from the images of 4A and 4B and presented as mean \pm SEM to measure the progression of the bioparticles from phagocytosis to phagolysosome activation. There is a significantly ($P \leq 0.006$) greater progression of phagosome maturation seen in the $MC5r^{(-/-)}$ macrophages compared to WT macrophages.

is applied to mice with EAU. And similar to the results in this manuscript, α -MSH-therapy suppresses EAU in $MC5r^{(-/-)}$ mice; however, it does not induce the suppressor APC nor the subsequent retinal-antigen specific iTreg cells. These findings, including the results in this report, demonstrate an importance in MC5r-expression for α -MSH to mediate regulatory immunity.

The ability of RPE to suppress phagolysosome activation provides the immune-privileged ocular-microenvironment with a mechanism to block locally the engine of antigen presentation that drives activation of CD4 $^+$ effector T cell that mediate autoimmune disease. Through the melanocortin pathway, RPE alter the maturation of the phagosome that can result in the presentation of a set of antigens that are not recognized

by effector T cells. Since α -MSH treated APC promote activation of Treg cells, it suggests that any APC still presenting antigen within the retina after α -MSH therapy may well be presenting antigens recognized by Treg cells within the eye.^{8,22,30,31} In addition, α -MSH induces the production of anti-inflammatory cytokines that further suppress the activity of effector T cells. Therefore, α -MSH-treatment of uveitis not only suppresses the retinitis, but also has the potential through MC5r to reestablish immune tolerance to retinal antigens.

In addition, NPY did not suppress phagolysosome activation in the $MC5r^{(-/-)}$ macrophages. We have seen NPY and α -MSH work together to suppress phagocytic activity and promote differentiation of macrophages into suppressor cells expressing both M1 and M2 characteristics.^{5,9,11,12}

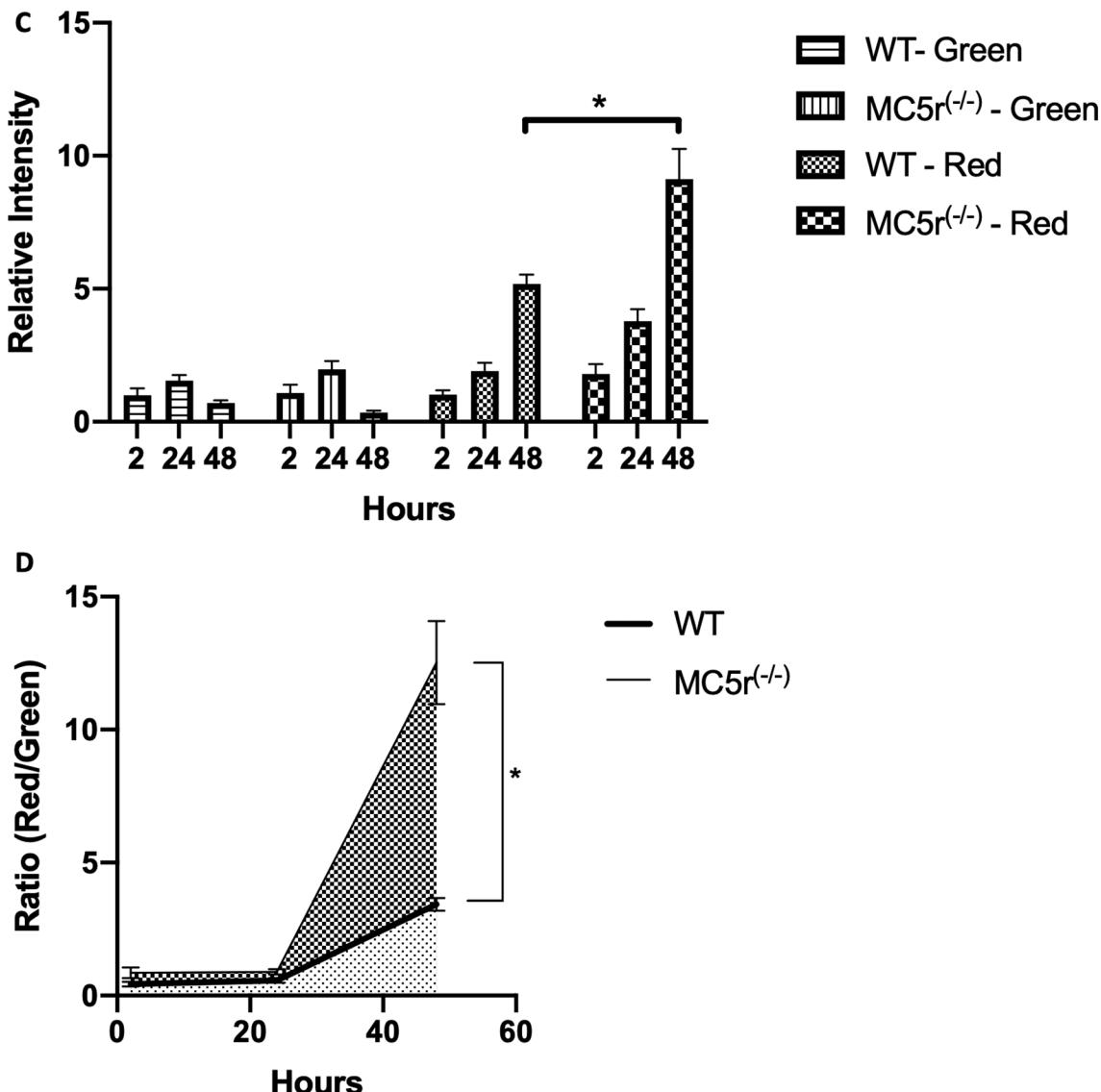


Figure 4. (Continued).

It is not known if there is a dependency of NPY receptor signaling on MC5r expression. While there are only a few reports on NPY suppression of phagocytic activity, it appears that the effects of NPY on macrophage activity, pro or anti-inflammatory, are influenced by the microenvironment, the stimulating agents, and aging.^{42–44} It is possible that since the MC5r^(-/-) macrophages have enhanced phagosome activity, there also is a change in NPY receptors or signaling. Without MC5r the macrophages act like conventional APC and stimulate effector T cells^{8,31}; therefore, it is to be seen if NPY may on the same macrophages induce cytokine production that is proinflammatory.

We demonstrate that α -MSH can suppress EAU without MC5r expression.^{8,30,31} This corresponds with observed differential regulation of immunity by α -MSH with anti-inflammatory activity mediated through MC1r and

MC3r.^{21,25} With MC5r-expression there is a recovery in RPE suppression of phagolysosome activation within macrophages. However, the recovery of RPE regulatory activity may be either a direct effect of α -MSH on the RPE cells, or indirectly from the immunosuppressive actions of α -MSH on the macrophages/microglial cells in the retina. The finding that macrophages in MC5r^(-/-) mice have enhanced levels of phagosome maturation could mean that within retinas of MC5r^(-/-) mice the expected regulatory activity of the RPE is different in response to potentially heightened myeloid cell-activity. Also, it suggests that in MC5r^(-/-) mice their macrophages when stimulated may mediate or prolong inflammation that is more damaging to the retina than in WT mice. Recently, there are reports that retinal cell survival is enhanced by α -MSH through MC1r and MC5r.^{19,35} Severe damage is seen in the retinas of EAU MC5r^(-/-) mice in contrast to the relatively protected retinal structures of α -MSH-treated EAU WT-mice. This

demonstrated that MC5r-expression is needed to fully maintain ocular immune privilege, to provide survival of retinal cells, and to recover immune privilege after uveitis.

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Disclosure statement

In accordance with Taylor & Francis policy and my ethical obligation as a researcher, I (AWT) am reporting that I am a consultant to a company that may be affected by the research reported in the enclosed paper. I have disclosed those interests fully to Taylor & Francis, and I have in place an approved plan for managing any potential conflicts.

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