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Tissue-specific production of MicroRNA-155 inhibits melanocortin 5 receptor-dependent suppressor macrophages to promote experimental autoimmune uveitis

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Abstract

Tissue-specific immune regulation is an important component of the immune response relevant to many areas of immunology. The focus of this study is on tissue-specific mechanisms that contribute to autoimmune uveitis. Precise gene regulation is necessary for the proper expression of an inflammatory or regulatory response. This precision gene regulation can be accomplished by microRNA at the level of the mRNA transcript. miR-155, in particular, has a complicated role in the immune response with positive and negative inflammatory effects. In this work, we identify a decrease in miR-155 in suppressor macrophages and further examine how tissue-specific production of miR-155 impacts experimental autoimmune uveitis. Importantly, we show that eliminating miR-155 expression by the target tissue before initiation reduces disease severity, but elimination of miR-155 after the onset of inflammation does not alter the course of disease. Additionally, expression of miR-155 by the target tissue before initiation is necessary for the induction of regulatory immunity that protects from further autoimmune disease, but not after the onset of inflammation. In summary, we find a MC5r-dependent decrease in miR-155 in postexperimental autoimmune uveitis APC, miR-155 production by the target tissue is necessary for the initiation of autoimmune uveitis, and may have a role in establishing protective regulatory immunity.

Keywords

Autoimmunity; Experimental autoimmune uveitis; Macrophages; miR-155

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Small noncoding RNA or microRNA (miRNA) have been identified as regulatory elements that allow for an additional level of gene regulation by binding the complementary mRNA to either stabilize the transcript or aid in the degradation of the transcript [1, 2]. Roles for miRNA were initially discovered in cancer biology [3] and more recently, in physiological development and function, and in immunology [1, 4–9]. In particular, roles for miR-155 in cancer and immunology have been demonstrated [6, 10–14]. Interestingly, the immunological role of miR-155 can vary depending on the cell type. For example, expression of miR-155 in macrophages promotes an inflammatory state [15, 16]. In contrast, miR-155 has a role in the induction of Tregs to suppress inflammation [17–19]. As such, it is of interest to determine the roles of miR-155 in the initiation and after the onset of autoimmune disease.

Experimental autoimmune uveitis (EAU) is a widely used neuroinflammatory mouse model in which the uveoretina is the target of an inflammatory immune response [20]. This is clinically relevant because uveitis is a leading cause of blindness, with 25.6–122 new cases per 100 000 a year, and a prevalence of 69–623 cases per 100 000 [21–24]. While corticosteroids are an effective anti-inflammatory treatment that works well for acute uveitis, the myriad of undesirable side-effects make them unsuitable as a long-term treatment option [25–27]. Therefore, chronic uveitis patients are transitioned to immunosuppressive medications with the goal of sustained remission [28–33]. Unfortunately, not all the immunosuppressive medications are effective and recalcitrant uveitis cause patients to fail multiple treatment regimens. As such, a better understanding of the immunobiology of autoimmune uveitis is necessary to develop additional immunosuppressive treatment options for the treatment of autoimmune uveitis. The most widely used model of human autoimmune uveitis, EAU, is used to study the immunobiology of autoimmune uveitis. EAU can be divided into different phases, the onset of EAU is 2–4 weeks after immunization, followed by a chronic phase for 1–2 months, and resolution spontaneously occurs without relapse at 2–3 months after immunization [20, 34, 35]. When EAU resolves (post-EAU), regulatory immunity emerges in the spleen [36] and provides resistance to EAU during reimmunization and when adoptively transferred to mice that are immunized for EAU [36–39].

We have previously demonstrated that the melanocortin 5 receptor (MC5r), a receptor for the potent immunosuppressive neuropeptide, α -melanocyte stimulating hormone (α -MSH), is necessary for the emergence of suppressor macrophages in the spleen of post-EAU mice [37, 38, 40]. These post-EAU MC5r-dependent macrophages are important because they are necessary to activate post-EAU regulatory immunity. In addition to expression on macrophages, MC5r is expressed in retinal pigmented epithelial cells (RPE) [41]. Therefore, we asked if there is a role for miR-155 in MC5r-stimulated macrophages and the role that tissue-specific RPE production of miR-155 has on EAU. In this report, we show that miR-155 is downregulated in suppressor macrophages, and has a role in the initiation of EAU, but not in the progression of EAU. We further demonstrate that miR-155 has a role in the induction of post-EAU regulatory immunity during the initiation of EAU, but not after the onset of EAU.

Results

miRNA expression in α -MSH-treated macrophages

We have previously demonstrated that stimulation of MC5r on macrophages with the immunosuppressive neuropeptide, α -MSH, results in a suppressor macrophage that can activate regulatory T cells [39, 40, 42]. Therefore, to identify miRNA that may have an inflammatory role in macrophages, we asked what miRNAs were significantly downregulated in a suppressor macrophage population when treated with α -MSH. The top 10 miRNAs we focused on are shown in Table 1. These miRNA were first selected based on the condition that FDRq should be less than 0.25, $p < 0.01$, and downregulated with α -MSH treatment. Because it has been demonstrated that the post-EAU suppressor APC expresses PD-L1, CD73, and CD39 [38, 40], we determined the first list of miRNA that target PD-L1, CD73, or CD39 miRNA shown in Table 2. We next focused on miRNA with a fold change of two or greater, leaving six miRNAs to focus on. While miR-17, miR-106, miR-222 were downregulated greater than twofold, miR-155 has been previously shown to be involved in the pathogenesis EAU [12, 43–45] and it is predicted to interact with CD39 and PD-L1 mRNA (Table 2). We, therefore, decided to focus on the role of miR-155 in EAU.

miR-155 Expression in post-EAU macrophages

We have previously demonstrated that deletion of the MC5r does not change the course of EAU [37, 40], and the severity of disease is not significantly different compared to MC5r-sufficient mice (Supporting Information Fig. 1), but is required for the emergence of a post-EAU suppressor macrophage [37, 40]. Therefore, we next determined the miR-155 expression in post-EAU suppressor macrophages from the spleen of WT and MC5r^(-/-) mice to confirm the in vitro expression is similar with in vivo expression. The post-EAU suppressor macrophage is dependent on expression of MC5r and has been previously identified as CD11b⁺F4/80⁺Ly-6C^{lo}Ly-6G⁺ [38], so we sorted the spleen of post-EAU mice and unimmunized age-matched mice. The fold change in post-EAU suppressor macrophages from the spleen relative to the same population from the spleen of unimmunized was determined (Fig. 1). We observed a greater than threefold downregulation of miR-155 in WT mice and a greater than twofold upregulation of miR-155 in MC5r^(-/-) mice. These observations confirm the microarray miRNA analysis and demonstrate a role for miR-155 in macrophages during EAU.

Effect of miR-155 production by retinal pigmented epithelium on EAU

It has been demonstrated that miR-155 functions to promote EAU using a global knock-out mouse [12]. Because microRNAs have an important role in the retina and specifically the RPE [46, 47], we asked if tissue-specific expression of miR-155 is necessary for EAU. A doxycycline inducible RPE-specific Cre (RPE/rtTA) expressing mouse that has been extensively characterized in multiple models [48–50] was crossed with a floxed miR-155 (miR-155^{fl/fl}) mouse to create an inducible RPE-specific miR-155 knock-out. Specific excision of miR-155 in the RPE was confirmed (Supporting Information Fig. 2). Mice carrying the doxycycline inducible RPE-Cre and floxed miR-155 (RPE/rtTA; miR-155^{fl/fl}) were fed doxycycline containing chow for 1 week prior to immunization for EAU. Control mice were also put on the same feeding schedule but were WT mice. The course of EAU in

RPE/rtTA; miR-155^{fl/fl} doxycycline-fed mice showed significantly earlier resolution (Fig. 2A). The severity of disease was also determined with the maximum EAU score for each mouse over the entire course of disease, and was significantly reduced in the RPE/rtTA; miR-155^{fl/fl} doxycycline-fed mice compared to doxycycline-fed WT mice (Fig. 2B). EAU was not significantly different in miR-155^{fl/fl} mice not fed doxycycline compared with WT doxycycline-fed mice (Supporting Information Fig. 3). These observations show that a RPE-specific deletion of miR-155 provides resistance to EAU.

Role of miR-155 production by RPE during EAU

We next asked if miR-155 production by the RPE was important for the progression of EAU. The RPE/rtTA;miR-155^{fl/fl} mice were immunized for EAU and were given doxycycline between the onset and peak of retinal inflammation (day 28 after immunization). We observed no significant change in the course of disease or the severity of EAU (Fig. 3). Importantly, there was also no delay in the resolution of EAU. These observations demonstrate that miR-155 production by the RPE is not necessary for the progression or resolution of EAU.

Effect of miR-155 production by RPE on the induction of post-EAU regulatory immunity

The role of miR-155 is generally proinflammatory, but the role in Treg cells is more ambiguous with evidence that it is involved in Treg development, but not in function [17, 18]. Because post-EAU Treg cells are found in the spleen of EAU-recovered mice, we asked if miR-155 production by the RPE is necessary for emergence of post-EAU Treg cells in the spleen. The spleen from post-EAU RPE/rtTA;miR-155^{fl/fl} doxycycline-fed mice was collected and splenocytes were restimulated with interphotoreceptor retinoid binding protein (IRBP) in vitro, as we have done before [37–40]. The restimulated splenocytes were transferred to recipient mice immunized for EAU and the fundus was monitored for signs of inflammation. We compared the EAU scores of mice that received splenocytes from mice 90 days after immunization with CFA without IRBP with EAU mice that received no transfer of cells and found no significant difference (Supporting Information Fig. 4A). While the course of disease appears lower and the maximum severity trends lower, the differences compared to EAU mice that did not receive an adoptive transfer were not statistically significant (Fig. 4). These observations demonstrate that miR-155 production by the RPE at the initiation of EAU is necessary to promote the generation of post-EAU regulatory immunity in the spleen.

Temporal effect of miR-155 production by RPE on the induction of post-EAU regulatory immunity

Since the role of miR-155 has been demonstrated to be involved in the induction of Treg cells [17, 18], we asked if production of miR-155 by the RPE after the onset of EAU has an impact on the generation of regulatory immunity in the spleen of mice that have recovered from EAU. This is of particular interest because it has been demonstrated in the past that resolution of EAU is independent from the generation of post-EAU Tregs in the spleen [36]. RPE/rtTA;miR-155^{fl/fl} mice were given doxycycline at day 28 of EAU and monitored until resolution. At resolution of EAU, the spleen from the EAU-recovered RPE/rtTA;miR-155^{fl/fl} mice were collected, reactivated in vitro, and transferred to recipient mice immunized for EAU. Mice that received post-EAU RPE/rtTA;miR-155^{fl/fl}, spleen cells had significantly

lower EAU scores and accelerated resolution of disease compared to mice that did not receive an adoptive transfer (Fig. 5) and was also significantly lower compared to EAU mice that received a transfer of cells from splenocytes from mice 90 days after immunization with CFA without IRBP (Supporting Information Fig. 4B). This demonstrates that miR-155 production by the RPE is not necessary for the generation of post-EAU regulatory immunity once EAU has started.

Discussion

Our observations that deletion of miR-155 in the RPE impacts the course of EAU indicates that production by the RPE is necessary for the initiation of tissue-specific inflammatory disease. Because miR-155 in macrophages is proinflammatory, a potential explanation is that the miR-155 produced by the RPE at the initiation of EAU is promoting the inflammation by promoting an inflammatory macrophage. However, because eliminating miR-155 in the RPE after the onset of inflammation had no impact on inflammation and because the progression of an inflammatory disease involves additional mechanisms that extend past antigen presentation by macrophages, these observations demonstrate that miR-155 is not necessary for progression of disease from initiation of inflammation to the chronic phase.

There are conflicting reports regarding the role of miR-155 in uveitis. In the rat model of autoimmune anterior uveitis, miR-155 decreases in the iris and ciliary body at the peak of disease, but increases in leukocytes at the peak [45]. In contrast, STAT3 activates miR-155 to promote EAU by activating Th17 cells [12]. In human studies, upregulation of miR-155 has been observed in PBMCs from Behcet's disease (BD) patients [44] and in BD patients with active disease [10]. However, an earlier report showed a decrease in miR-155 in PBMCs from active BD patients [43]. The immunological role of miR-155 in T cells is the expansion of Th1, Th17, Th2, and Tregs [6, 11, 17, 18, 51], and functions in macrophages and DCs to promote an inflammatory state [7, 16]. As such our observations demonstrate an inflammatory role for miR-155, and additionally show that the tissue-specific expression of miR-155 is also timing specific in the progression of autoimmune disease.

We further show that the generation of systemic regulatory immunity that emerges following the resolution of EAU does not emerge if miR-155 is not produced by the RPE at the initiation of disease. However, emergence of this regulatory immunity does not depend on miR-155 production after the onset of disease. This is important because the absence of this regulatory immunity may contribute to chronic, relapsing autoimmune disease. However, it should be noted that the lack of post-EAU regulatory immunity when the miR-155 production by the RPE was blocked before the initiation of inflammation may be due to the absence of severe EAU and not necessarily because of a specific absence of miR-155 production by the RPE.

The observation that miR-155 production by the RPE is also interesting because it demonstrates the ability of the tissue to modulate the immune response. Furthermore, this tissue-specific modulation of the immune response occurs through production of miR-155 by the RPE. This could be relevant to other retinal diseases with an inflammatory component

such as diabetic retinopathy and age-related macular degeneration. As such, retinal neovascularization is attenuated by miR-155 through the PI3K/Akt pathway [52]. Therefore, targeting miR-155 could have potential therapeutic benefit to these other diseases, as well.

It should be noted that there are potential drawbacks to the EAU model. Because it is a posterior autoimmune model, it does not represent that majority of uveitis cases. Infectious uveitis accounts for the majority of cases with geography as a factor [53]. Among autoimmune uveitis cases, anterior idiopathic uveitis accounts for the majority of cases [53]. While there are some susceptibility loci, such as HLA-B27, HLA-B51, and HLA-B5 [54–56], there is also likely another environmental trigger as well. While autoimmune uveitis can manifest with the eye as the only target, it can be the target in addition to other organs such as the joints in ankylosing spondylitis, the gut in colitis, and brain as in MS [57, 58]. Because not all autoimmune uveitis patients respond to the same therapy, even if it is the same type, this suggests that there are multiple etiologies. This is supported with our observation that not all the mice are fully protected from EAU. The EAU model is limited in that it is posterior uveitis whereas clinical uveitis includes anterior, intermediate, scleritis, and conjunctival involvement. However, the most devastating type of uveitis is posterior uveitis. While these observations indicate that miR-155 has a role in the initiation of EAU, it is likely that it is only part of a larger pathway that is not completely understood. Additional factors that are involved in the initiation of EAU include the induction of Th1 and Th17 cells that are specific for retinal antigen [59], and this response is mediated through induction of other factors such as TNF- α and IL-6. Others have reported that miR-155 promotes EAU by augmenting the Th17 response through STAT3 induction [12]. However, because these observations indicate that miR-155 production by the RPE is involved in the initiation of EAU, neutralizing miR-155 with a complementary ssDNA or RNA could be used as a localized therapeutic if a sufficient delivery system is designed to deliver it directly to the eye. Phase 1 clinical trials are underway to test cobomarsen (MRG-106), a miR-155 inhibitor, that is sponsored by miRagen Therapeutics, Inc. (Boulder, CO) in patients diagnosed with lymphoma or leukemia ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02580552) Identifier: NCT02580552).

Materials and methods

Mice

The University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (OUHSC IACUC) approved of all mouse procedures described in this study and were carried out in accordance with the relevant guidelines approved by the OUHSC IACUC. C57BL/6J mice and miR-155^{fl/fl} mice with a floxed exon 2 that encodes the hairpin region of the miRNA (026700) were purchased from Jackson Laboratories. Mice containing a doxycycline inducible RPE-specific (human 2.9-kb *VMD2* promoter associated with Best disease) Cre (RPE/rtTA) was a generous gift from Dr. Yun-Zheng Le. Cre expression in these mice was demonstrated to be specific to the RPE in multiple models [48–50]. We further confirmed that excision of miR-155 was specific for the RPE (Supporting Information Fig. 2). MC5r^(-/-) mice on a C57BL/6J background were obtained from Roger D. Cone (Oregon Health Sciences, Portland, Oregon).

RNA assays

In vitro cultured spleen cells with and without α -MSH were enriched for CD11b⁺ cells and then sorted for Ly-6G⁺ Ly-6C^{lo} cells into Trizol. Sorted cells were then sent to the Boston University Clinical and Translational Science Institute where the RNA was extracted and analyzed using Affymetrix GeneChip miRNA 4.0 arrays. All samples had similar quality metrics, including mean Relative Log Expression and percent Present calls (%P). All arrays were normalized together using Affymetrix Expression Console (version 1.3.0.187) using RMA normalization and Detection Above BackGround (DABG). Normalization was performed only across mouse and control probe sets. The expression values are log₂-transformed by default. Each gene was also assigned a Present or Absent call in each sample, denoting whether its expression was significantly higher than that of a collection of negative control probes.

Post-EAU spleen cells were sorted as described above from WT and MC5r^(-/-) mice. The sorted samples were sent to Anna Trivett at the National Cancer Institute for quantification of miR-155. The miR-155 primers from Qiagen were used (MS00001701 Mm miR-155 1 miScript primer) and SNORD-68, 73, 95 were used as housekeeping genes for normalization.

Experimental autoimmune uveoretinitis

EAU was induced in mice as previously described [39]. Briefly, CFA was emulsified with 5 mg/mL desiccated *Myobacterium tuberculosis* (Difco Laboratories, Detroit, MI) and 2 mg/mL IRBP (peptides 1–20) (Genscript, Piscataway, NJ) was used to immunize mice for EAU. Mice received a volume of 100 μ L of the emulsion injected subcutaneously at two sites in the lower back followed by an intraperitoneal injection of 0.3 μ g pertussis toxin. Retinal inflammation during the course of EAU was evaluated every 3–4 days by fundus examination using a slit lamp microscope. Before examining the retina, the iris was dilated with 1% tropicamide, and the cornea was flattened with a glass coverslip to examine the retina. The clinical signs of observable infiltration and vasculitis in the retina were scored on a 5-point scale, as previously described [60]. Both eyes were scored and the higher score was used to represent the mouse for that day, the average score for the group of mice was then calculated. When maximum scores were calculated, the maximum score for each mouse over the entire course of disease is shown.

Doxycycline administration

Doxycycline containing chow was purchased from Bio-Serv (Flemington, NJ). The doxycycline concentration was 200 mg/kg and mice were fed doxycycline for 1 week, then switched back to the regular chow for the remainder of the experiment.

In vitro α -MSH treatment

The spleens from mice that recovered from EAU (day 85–90 after immunization) or unimmunized mice were collected into 5% FBS in RPMI supplemented with 10 μ g/mL Gentamycin (Sigma, St. Louis, MI), 10 mM HEPES, 1 mM sodium pyruvate (BioWhittaker, Basel, Switzerland), nonessential amino acids 0.2% (BioWhittaker). Spleen cells were made into a single cell suspension that was subsequently depleted of RBCs with RBC lysis

buffer (Sigma, St Louis, MO). The RBC-free spleen cells were then separated to obtain adherent APC that were then cultured in serum-free media (SFM) with 1 ng/mL α -MSH for 48 h at 37°C and 5% CO₂. SFM consisted of RPMI-1640 with 1% ITS+1 solution (Sigma) and 0.1% BSA (Sigma). The cultured APC were then stained and sorted to obtain a pure population of suppressor APC.

Adoptive transfer experiments

Spleen cells from post-EAU mice were collected, made into a single cell suspension, RBC depleted, and reactivated in vitro in SFM with 50 μ g/mL IRBP 48 h at 37°C and 5% CO₂. Following the reactivation, cells were collected and 1×10^6 cells were adoptively transferred to recipient mice intravenously at the time of immunization for EAU.

Cell sorting

Mouse spleen cells were washed with PBS with 1% BSA (staining buffer), blocked with mouse IgG in staining buffer, then stained with conjugated antibodies. Antibodies used were anti-CD11b (clone M1/70, Biolegend, San Diego, CA), anti-Ly-6C (clone HK1.4, Biolegend), and anti-Ly-6G (clone 1A8, Biolegend). Cells were enriched using a mouse CD11b positive selection kit (StemCell Technologies, Vancouver, BC, Canada), then sorted in the BU Flow Cytometry Core.

Statistics

Statistical significance between EAU scores was determined using nonparametric Mann-Whitney U test between groups of mice. Two-way ANOVA was also used to assess significant differences in the course of disease between the groups of treated EAU mice. Statistical significance was determined when $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

α-MSH	α -melanocyte stimulating hormone
BD	Behcet's disease
EAU	experimental autoimmune uveitis
IRBP	interphotoreceptor retinoid binding protein
MiRNA	microRNA

MC5r	melanocortin 5 receptor
RPE	retinal pigmented epithelial cells
SFM	serum-free media

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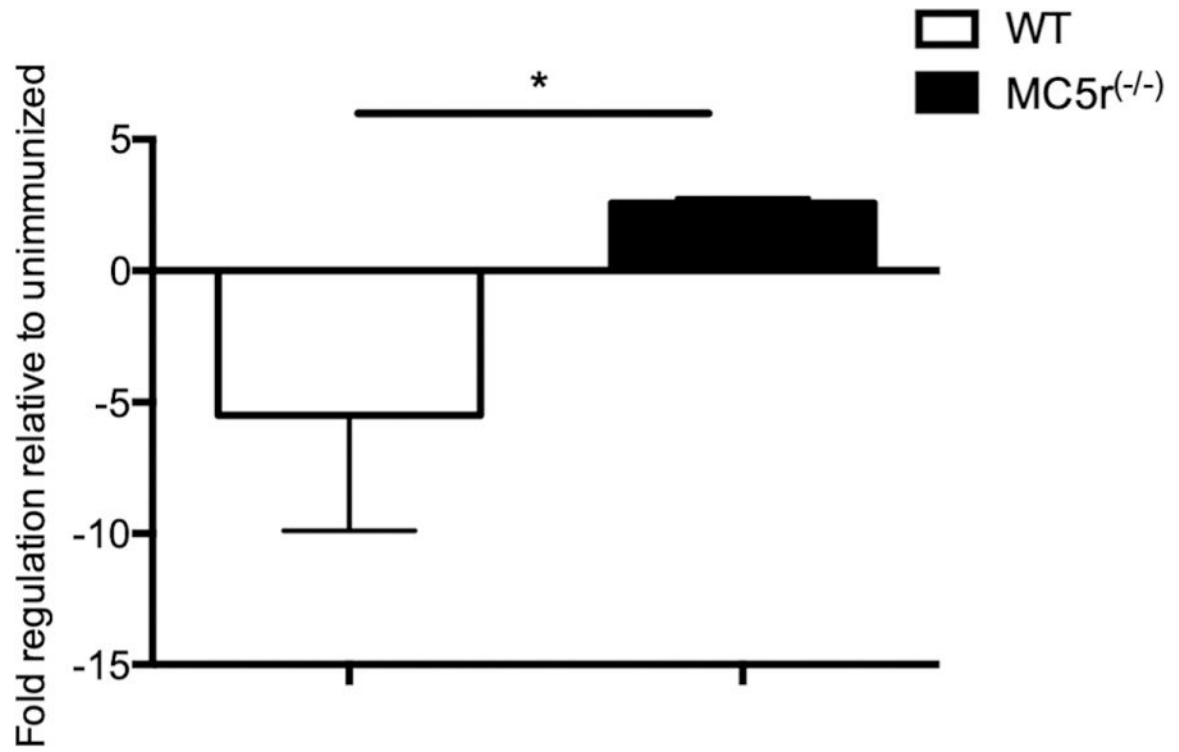


Figure 1.

Expression of miR-155 in post-EAU CD11b⁺ F4/80⁺ Ly-6G⁺ Ly-6C^{lo} cells. Spleen cells were collected from post-EAU C57BL/6J and post-EAU MC5r^(-/-) mice and sorted for CD11b⁺ F4/80⁺ Ly-6G⁺ Ly-6C^{lo} cells. Total RNA was collected and miR-155 was measured by RT-PCR, normalized to housekeeping genes, and the fold regulation of post-EAU to unimmunized was calculated as shown. Results shown are obtained from three independent experiments consisting of one to three mice per experiment. Statistical significance is designated by * when $p < 0.05$ determined by nonparametric Mann-Whitney U test.

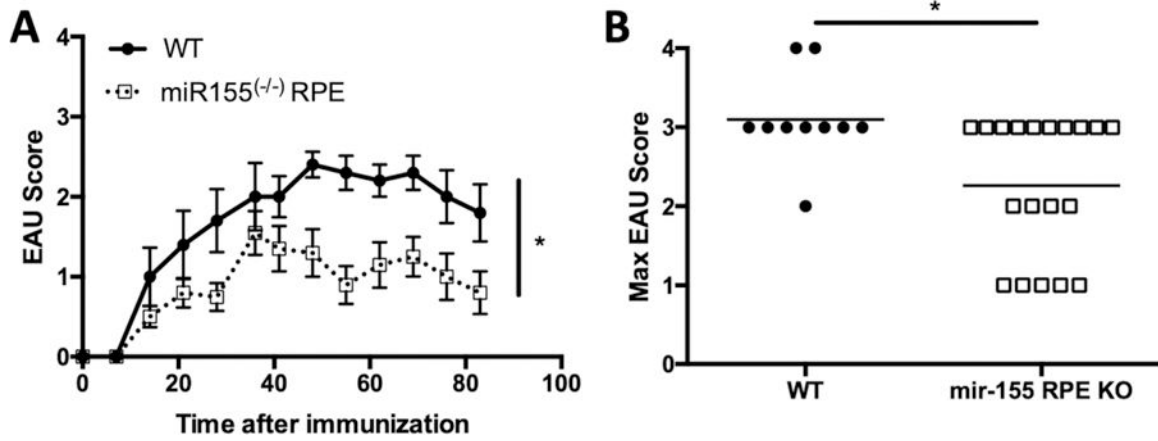


Figure 2. Effect of eliminating miR-155 production by RPE before EAU induction. Mice with flox sites at the miR-155 locus and a doxycycline inducible RPE-specific Cre (RPE/rtTA; mir-155^{fl/fl}) were fed doxycycline before immunization for EAU. Mice were monitored for clinical signs of retinal inflammation one to two times per week. The course of EAU with the average scores \pm SEM is shown (A). The closed circle and solid line represent the scores of WT mice ($n = 10$) and the open square and dashed line represent scores of RPE/rtTA; mir-155^{fl/fl} mice ($n = 10$). The highest clinical score for each mouse over the entire course of disease was also determined and is shown with the line representing the mean for each group (B). Each experiment consisted of four to five mice per group, and was repeated three times. Statistical significance is designated by * when $p < 0.05$ determined by two-way ANOVA for the course of disease and nonparametric Mann-Whitney U test for the maximum EAU scores.

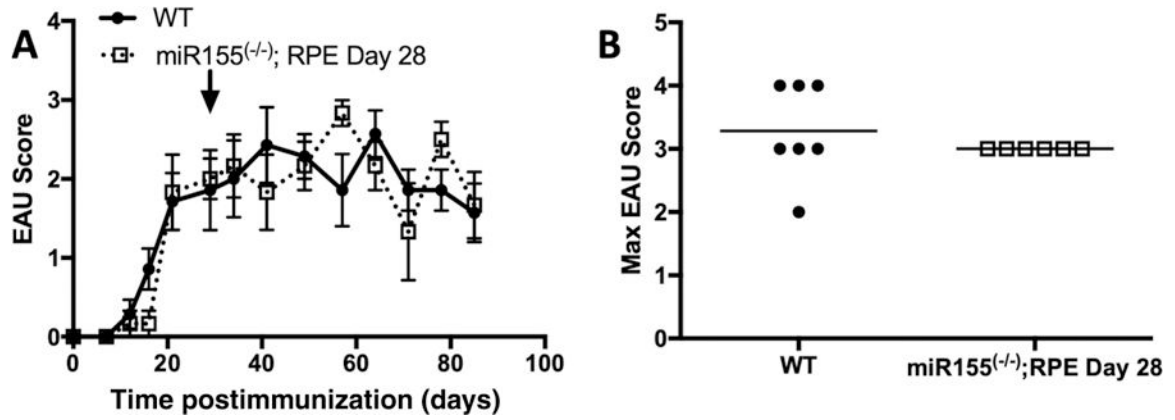


Figure 3. Effect of eliminating miR-155 production by RPE between the onset and peak of EAU. Mice with flox sites at the miR-155 locus and a doxycycline inducible RPE-specific Cre (RPE/rtTA; mir-155^{fl/fl}) were fed doxycycline at the day 28 of EAU (indicated by arrow). Mice were monitored for clinical signs of retinal inflammation one to two times per week. The course of EAU with the average scores ± SEM is shown (A). The closed circle and solid line represent the scores of WT mice ($n = 7$) and the open square and dashed line represent scores of RPE/rtTA; mir-155^{fl/fl} mice ($n = 6$). The highest clinical score for each mouse over the entire course of disease was also determined and is shown with the line representing the mean for each group (B). Each experiment consisted of three to five mice per group, and was repeated two times. Statistical significance is designated by * when $p < 0.05$ determined by two-way ANOVA for the course of disease and nonparametric Mann-Whitney U test for the maximum EAU scores.

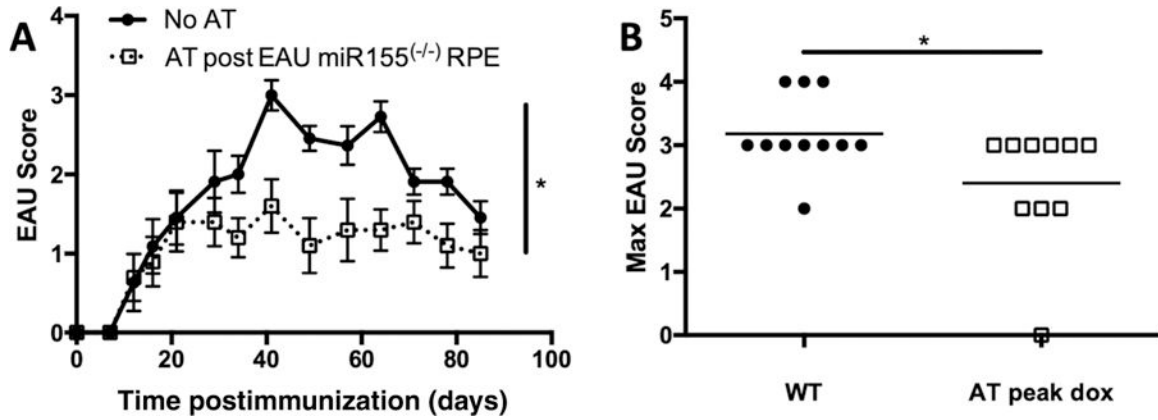


Figure 5. Effect of eliminating miR-155 production by RPE between the onset and peak of EAU on induction of post-EAU regulatory immunity. Spleen cells were collected from post-EAU mice with flox sites at the miR-155 locus and a doxycycline inducible RPE-specific Cre (RPE/rtTA; mir-155^{fl/fl}) that were fed doxycycline at day 28 of EAU as in Figure 3. Spleen cells were reactivated with IRBP and transferred to recipient mice immunized for EAU. Recipient mice were monitored for clinical signs of retinal inflammation one to two times per week. The course of EAU with the average scores ± SEM is shown (A). The closed circle and solid line represent the scores of mice that received no adoptive transfer ($n = 11$) and the open square and dashed line represent scores of recipient mice ($n = 10$). The highest clinical score for each mouse over the entire course of disease was also determined and is shown with the line representing the mean for each group (B). Each experiment consisted of two to five mice per group, and was repeated three times. Statistical significance is designated by * when $p < 0.05$ determined by two-way ANOVA for the course of disease and nonparametric Mann-Whitney U test for the maximum EAU scores.

Table 1.

Top 10 downregulated transcripts from miRNA microarray

Transcript ID	Fold change	p	Ly-6G α-MSH 1	Ly-6G α-MSH 2	Ly-6G α-MSH 3	Ly-6G untreated 1	Ly-6G untreated 2	Ly-6G untreated 3
mmu-miR-17-3p	-5.5	1.30×10^{-4}	1.7	2.3	2.2	3.9	4.4	5.1
mmu-miR-106b-5p	-4.1	2.50×10^{-5}	3.9	3.6	4.1	5.9	5.6	6.3
mmu-miR-222-3p	-4.1	2.60×10^{-3}	1.5	2.5	1.6	3.6	3.2	4.7
mmu-miR-106a-5p	-3.7	1.60×10^{-3}	3.2	2.8	2.2	4.7	3.9	5.2
mmu-miR-17-5p	-2.9	2.30×10^{-5}	5.3	5.3	5.5	7	6.7	7.1
mmu-miR-155-5p	-2	7.00×10^{-2}	4.7	5.8	2.9	6	5.7	5.8
mmu-miR-296-3p	-1.6	1.50×10^{-2}	1.7	2.2	1.4	2.7	2.5	2.3
mmu-miR-485-3p	-1.6	8.80×10^{-2}	2.6	1.6	2.4	2.7	3.4	2.5
mmu-miR-16-5p	-1.5	2.10×10^{-2}	8.1	8.3	7.9	8.7	8.3	9.1
mmu-miR-181a-5p	-1.5	5.00×10^{-3}	6.8	6.6	6.6	7.1	7.2	7.4

The fold change and p value are shown for each miRNA, FDRq values are less than 0.25 for all of the miRNA transcripts shown.

Table 2.

Predicted mRNA target transcripts of selected miRNA

Transcript ID	Fold change	Predicted target: PD-L1	Predicted PD-L1 Alignment	PD-L1 alignment score	Predicted target: CD39	Predicted CD39 alignment	CD39 alignment score	Predicted target: CD73	Predicted CD73 alignment	CD73 alignment score
mmu-miR-17-3p	-5.5	X								
mmu-miR-106b-5p	-4.1	X	: :	153				X	:	158
mmu-miR-222-3p	-4.1									
mmu-miR-106a-5p	-3.7	X	: :	157						
mmu-miR-17-5p	-2.9	X								
mmu-miR-155-5p	-2.0	X	:	156	X	:	123			
mmu-miR-296-3p	-1.6	X		140				X		121
mmu-miR-485-3p	-1.6				X	: :	152	X	: :	124
mmu-miR-16-5p	-1.5	X	: :	125	X	:	141			
mmu-miR-181a-5p	-1.5	X	:	125				X		145

The predicted target transcripts were indicated on www.microrna.org, fold change and p value are shown for each miRNA, FDRq values are less than 0.25 for all of the miRNA transcripts shown. The predicted alignment is shown, the line indicates complementary base pairing and the alignment score is shown in the next column.