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Both MC5r and A2Ar are required for protective regulatory immunity in the spleen of post-experimental autoimmune uveitis in mice

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Abstract

The ocular microenvironment uses a poorly defined melanocortin 5 receptor (MC5r)-dependent pathway to recover immune tolerance following intraocular inflammation. This dependency is seen in experimental autoimmune uveoretinitis (EAU), a mouse model of endogenous human autoimmune uveitis, with the emergence of autoantigen-specific regulatory immunity in the spleen that protects the mice from recurrence of EAU. In this new study, it was found that the MC5r-dependent regulatory immunity was an increase of $CD11b^+$ $F4/80^+$ $Ly\text{-}6C^{low}$ $Ly\text{-}6G^+$ $CD39^+$ $CD73^+$ APC in the spleen of post-EAU mice. These MC5r-dependent APC require adenosine 2A receptor (A2Ar) expression on T cells to activate EAU-suppressing $CD25^+$ $CD4^+$ $FoxP3^+$ Treg cells. Therefore, in the recovery from autoimmune disease the ocular microenvironment induces tolerance through a melanocortin mediated expansion of $Ly\text{-}6G^+$ regulatory APC in the spleen that utilize the adenosinergic pathway to promote activation of autoantigen-specific Treg cells.

Introduction

Experimental autoimmune uveitis (EAU) is a mouse model of endogenous uveitis in humans (1). In order to better understand the mechanisms contributing to this relapsing and remitting nature of chronic autoimmune uveitis, EAU has been studied. C57BL/6J mice immunized with human interphotoreceptor retinoid binding protein peptide amino acids 1–20 (IRBP) display uveoretinitis within 2–3 weeks of immunization. This inflammation resolves on its own 70–90 days after immunization. The resolution of EAU establishes regulatory immunity specific to IRBP in the spleen (2). This post-EAU regulatory immunity suppresses memory/recall immune responses to IRBP, and suppresses induction and reactivation of EAU. The post-EAU protective regulatory immunity involves an undefined interaction between regulatory APC, and their antigen-stimulated IRBP-specific $CD25^+$ $CD4^+$ Treg cells (2). The regulatory activity is not seen with IRBP-immunization only, but requires a uveitic response. This has suggested that during uveitis the ocular mechanisms of immune privilege induce a distinctive regulatory APC to activate IRBP-specific Treg cells in the spleen.

The immunomodulating neuropeptide alpha melanocyte stimulating hormone (α-MSH) is a central mediator of immunosuppression within the ocular microenvironment (3). Therapeutic injections of α-MSH are effective in suppressing EAU (4, 5), experimental autoimmune encephalomyelitis (6), and corneal allograft rejection (7). The melanocortin 5

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receptor (MC5r) is one of four melanocortin receptors that α-MSH is the ligand (8, 9). The presence of the post-EAU regulatory APC requires expression of MC5r (10). When reimmunized after recovery from the initial episode of EAU, the MC5r knockout ($MC5r^{(-/-)}$) mice have a second episode of EAU with a rapid onset and enhanced severity that is characteristic of a memory/recall response (2). This is in contrast to reimmunized wild-type mice that display an immunologically naive-like response for a second episode of EAU. The severity of the second episode in reimmunized $MC5r^{(-/-)}$ mice is suppressed by the adoptive transfer of spleen cells from post-EAU wild-type mice. This failure of the $MC5r^{(-/-)}$ mice to generate post-EAU regulatory immunity is not due to an inability of $MC5r^{(-/-)}$ mice to generate Treg cells, but is a failure of $MC5r^{(-/-)}$ mice to generate a post-EAU regulatory APC (10). Therefore the dependence on MC5r expression is associated with the induction of regulatory APC.

Myeloid derived suppressor cells (MDSCs) are antigen presenting cells capable of suppressing inflammation and promoting regulatory immunity (11–13). MDSCs are identified by CD11b, and can be further subdivided into Ly-$6C^{low}$ Ly-$6G^{+}$ and Ly-$6C^{high}$ Ly-$6G^{-}$ cell populations (14, 15). They can emerge as inflammatory diseases progress to resolution (16), and it has been suggested that they are more characteristic of alternatively activated macrophages (12, 14). The melanocortin pathways that include the melanocortin receptors and their ligand α-MSH, have been shown to induce alternative activation of macrophages and dendritic cells, making them promote activation of regulatory immunity (17, 18). Recently it has been found that α-MSH with Neuropeptide Y mediates induction of myeloid suppressor cell-like activity in resting macrophages, and contributes to regulating similar activity in retinal microglial cells (19). The dependence on MC5r expression for the induction of a post-EAU regulatory APC suggests that the post-EAU APC is potentially similar to myeloid derived suppressor cells.

In this report we further identified the MC5r-dependent post-EAU APC to be $CD11b^{+}$ $F4/80^{+}$ Ly-$6C^{low}$ Ly-$6G^{+}$ cells with adenosine-generating ecto-nucleotidases CD39 and CD73 that mediate activation of regulatory T cells through the adenosine receptor A2Ar expressed on the T cells. These findings demonstrate a link between two highly conserved pathways of immune regulation through MC5r expression on APC, and A2Ar on T cells in establishing protective post-EAU regulatory immunity to ocular autoantigen.

Materials and Methods

Mice

The Boston University Institutional Animal Care and Use Committee approved all procedures used on mice in this study. C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). The $MC5r^{(-/-)}$ mice on a C57BL/6J background were obtained from Roger D. Cone (Oregon Health Sciences, Portland, Oregon), and the $A2Ar^{(-/-)}$ mice also on a C57BL/6J background, were obtained from Dr. Jiang-Fen Chen (Boston University School of Medicine, Boston, MA). Both strains were housed and bred in the Boston University Laboratory Animal Science Center.

Induction of Experimental Autoimmune Uveitis

EAU was induced by immunizing the mice with an emulsion of complete Freund's adjuvant (CFA) with added 5 mg/mL desiccated *M. tuberculosis* (Difco Laboratories, Detroit, MI), and 2 mg/ml IRBP peptide amino acids 1–20 (Genscript, Piscataway, NJ). Mice received a subcutaneous injection of the emulsion (100 μl) into two sites on the lower back followed by an intraperitoneal injection of 0.3 μg pertussis toxin. Every 3–4 days the course of EAU was evaluated by fundus examination. The ocular fundus was examined using a slit lamp

microscope. The corneas were flattened with a glass coverslip, the cornea was numbed with 0.5% proparacaine, and the iris dilated with 1% tropicamide. The clinical signs of observable infiltration and vasculitis in the retina were scored on a 5 point scale as previously described (20). The maximum score from either eye per mouse was recorded and mean score for each group per day was calculated. The mean maximum score was calculated by averaging the maximum scores of each mouse in each group over the entire course of disease.

Flow Cytometry Analysis

The spleens from post-EAU mice (day 70–80 after immunization for EAU) were collected, and placed in RPMI-1640 supplemented with 5% FBS, 10 μg/ml Gentamycin (Sigma, St Louis, MO), 10 mM HEPES (BioWhittaker, Walkersville, MD), 1 mM Sodium Pyruvate (BioWhittaker), and Nonessential Amino Acids (BioWhittaker). Cells were made into a single cell suspension, depleted of red blood cells using RBC lysis buffer (Sigma, St Louis, MO), washed, and resuspended in serum free media (SFM), SFM consisted of RPMI-1640 supplemented with 10 μg/ml Gentamycin (Sigma), 10 mM HEPES, 1 mM Sodium Pyruvate (BioWhittaker), Nonessential Amino Acids 0.2% (BioWhittaker), 1% ITS+1 solution (Sigma), and 0.1% BSA (Sigma). Splenocytes were incubated in SFM at 37°C and 5% CO_2 for 90 minutes in tissue culture plates, washed twice, and adherent cells were scraped off of the plastic in ice cold SFM. The cells were washed with staining buffer, PBS with 1% BSA, blocked with mouse IgG in staining buffer, then stained with the conjugated antibodies. Antibodies used in this study were CD11b-FITC (clone M1/70, Biolegend, San Diego, CA), Ly-6C (clone HK1.4, Biolegend), Ly-6G (clone 1A8, Biolegend), CD73 (clone 496406, R&D Systems, Minneapolis, MN), CD39 (clone 495826, R&D Systems) and F4/80 (clone BM8, eBiosciences, San Diego, CA). Stained cells were analyzed in the Boston University Flow Cytometry Core Facility on a BD LSRII (BD Biosciences) and data was analyzed using FlowJo Software (Tree Star, Inc., Ashland, OR).

Cell Sorting and Adoptive Transfer

Adherent spleen cells were collected as described above and pooled from post-EAU mice (day 70–80 after immunization for EAU). Staining was performed as described above. Stained cells were sorted in the Boston University Flow Cytometry Core Facility on a MoFlo Cell Sorter (Beckman Coulter, Inc., Brea, CA) or a FACSAria III (BD Biosciences). Cells were sorted into tubes containing 10% FBS, immediately after sorting an aliquot of cells was analyzed on the FACSAria III and found to be ≥96% pure (data not shown). Sorted cells were washed with SFM, and plated at 5×10^4 cells per well. CD3 enriched T cells were obtained from post-EAU spleens using a CD3 enrichment column (R&D Systems), added to the sorted APC at 8×10^5 cells per well with 50 μg IRBP peptide, and cultured at 37°C 5% CO_2 for 48 hours. After 48 hours T cells and APC were collected, and washed in PBS. Mice were injected with 1×10^6 activated post-EAU cells in PBS into the tail vein. Following the adoptive transfer, the mice were immunized for EAU as described above. Each group of adoptive transfer experiments were repeated at least two times, the EAU scores shown were pooled from all repeated experiments.

Cytokine Analysis

Cytokine production was measured in the supernatant of cells cultured for 48 hours. After incubation, the supernatants were assayed for IFN–γ and TGF–β. The concentration of IFN–γ was measured using a sandwich ELISA (IFN-γ detection and biotinylated IFN-γ antibodies from BD Pharmingen). The concentration of TGF–β was measured using the standard Mv1Lu bioassay (21) or TGF-β ELISA (R&D systems, Minneapolis, MN). The concentration of IL-17 in the culture media was measured with an IL-17 ELISA kit (R&D systems).

α–MSH Plasmid Treatment

Treatment of mice immunized for EAU with α–MSH expression plasmid was performed as previously described (4). Briefly, the expression vector was pCMV-Script with the portion of the POMC gene that encodes the ACTH residues 1–17 that results in production of a properly structured α–MSH peptide (22). Mice received 6 μL subconjunctival injections of 1 mg/mL in PBS on days 17 and 19 after EAU immunization. The mice were then evaluated for EAU as described above.

CGS21680 Treatment

The CGS21680 (CGS) (Tocris, Bristol, UK) was reconstituted in DMSO at 27.7 mg/mL, and then diluted in PBS to 0.05 mg/mL. Mice were immunized for EAU, as described above, and followed until maximum EAU score was obtained (Day 23 after immunization). Then the mice were given one *ip* injection of CGS at 0.5 mg/kg once a day for three consecutive days, and evaluated for EAU.

In Vitro α-MSH Treatment and RT-PCR Analysis

Spleen APC were collected as described above from naive mice. APC were plated at 6×10^6 cells per well in a 24-well plate in SFM, with or without 1 ng/mL α– MSH (19). Cells were incubated at 37°C 5% CO_2 for 48 hours, then collected, sorted, and processed for RT-PCR. The RNA was extracted from the sorted APC subsets with a RNeasy kit (Qiagen, Germantown, MD). Reverse transcription of extracted RNA into cDNA was done with the QuantiTect Reverse Transcription kit, (Qiagen), and PCR reactions were run with the QuantiTech Probe PCR kit (Qiagen). The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1, CD39), and 5-nucleotidase (NT5E, CD73) primers and probes were purchased from Integrated DNA Technologies, Inc. (Skokie, IL). PCR reactions were run in the Boston University School of Medicine RT-PCR core facility on an Applied Biosystems 7300 (Applied Biosystems, Foster City, CA). Data were analyzed by the $2 \wedge -\Delta\Delta C_T$ method (23).

Statistical Analysis

The experimental results for the EAU scoring used nonparametric Mann-Whitney U test for statistical differences in EAU scores between groups of mice. In addition, changes in the tempo of disease between the groups of treated EAU mice were analyzed by two-way ANOVA. Comparisons of flow cytometry results and cytokine concentrations were statistically analyzed by one-way ANOVA with post-test Bonferroni comparison analysis. Statistical significance was determined when $P \leq 0.05$.

Results

There is a MC5r Dependent Increase in post-EAU CD11b$^+$ F4/80$^+$ Ly-6C^{low} Ly-6G$^+$ Spleen Cells

It has been previously shown that MC5r is required for emergence of post-EAU protective regulatory immunity in the spleen, and that it is the APC that must express MC5r to have the post-EAU regulatory immunity (10). The post-EAU APC from wild-type mice were compared with post-EAU APC from MC5r$^{(-/-)}$ mice for their expression of CD11b, F4/80, Gr-1 (Ly-6G), and Ly-6C. Flow cytometry analysis of stained cells showed a significant expansion of CD11b$^+$ F4/80$^+$ post-EAU APC compared to the APC found in the spleen of unimmunized mice (Fig. 1A). In contrast, post-EAU MC5r$^{(-/-)}$ APC showed no significant change in CD11b or F4/80 expression compared to unimmunized MC5r$^{(-/-)}$ mice. Also, the post-EAU wild-type spleen cells showed a significant increase in CD11b$^+$ F4/80$^+$ cells compared to post-EAU MC5r$^{(-/-)}$ spleen cells (Fig. 1B). The post-EAU wild-type and

MC5r$^{(-/-)}$ expression of Ly-6G and Ly-6C was observed for cells gated on CD11b^{+} F4/80^{+} APC (Fig. 1C). The cells from the wild type post-EAU spleen, but not the cells from the post-EAU MC5r$^{(-/-)}$ mice, had a significant increase in the proportion of cells expressing CD11b^{+} F4/80^{+} Ly-6C^{low} Ly-6G^{+} compared to spleen cells from unimmunized mice (Fig. 1D). In contrast, there is only a moderate increase in CD11b^{+} F4/80^{+} Ly-6C^{high} Ly-6G^{-} cells in both post-EAU wild-type and MC5r$^{(-/-)}$ CD11b^{+} F4/80^{+} spleen cells. Overall there was a significant increase in both populations in post-EAU spleens of wild-type mice over the cells from post-EAU MC5r$^{(-/-)}$ mice (Fig. 1D). From this point on the CD11b^{+} F4/80^{+} Ly-6C^{low} Ly-6G^{+} population of APC will be labeled Ly-6C^{low} Ly-6G^{+}; and the CD11b^{+} F4/80^{+} Ly-6C^{high} Ly-6G^{-} APC will be labeled Ly-6C^{high} Ly-6G^{-}. These results show that in the post-EAU spleen there was a MC5r expression-dependent expansion of Ly-6C^{low} Ly-6G^{+} cells.

Post-EAU Ly-6C^{low} Ly-6G^{+} APC Mediate Regulatory Immunity

The regulatory activity of the post-EAU Ly-6C^{low}Ly-6G^{+} and Ly-6C^{high}Ly-6G^{-} cells was determined by pulsing sorted Ly-6C^{low}Ly-6G^{+} and Ly-6C^{high}Ly-6G^{-} cells with IRBP, and using these cells as APC to activate post-EAU CD3^{+} T cells in culture for 48 hours. The T cells and APC were then adoptively transferred into wild-type mice immunized for EAU. Mice that received the post-EAU T cells activated with Ly-6C^{high}Ly-6G^{-} APC showed no significant change in the tempo and severity of EAU compared to mice that were not injected with cells (Fig. 2A). In contrast, mice that received post-EAU T cells activated by the post-EAU Ly-6C^{low}Ly-6G^{+} APC showed a significant change in the tempo of EAU with an early resolution of EAU (Fig. 2A). This demonstrates that the post-EAU regulatory immunity was with the Ly-6C^{low}Ly-6G^{+} APC.

As with the post-EAU wild-type spleen cells, the cells from post-EAU MC5r$^{(-/-)}$ mice spleens were sorted and used to activate post-EAU wild-type T cells to see if these cells can suppress EAU. Neither the post-EAU Ly-6C^{high}Ly-6G^{-} APC nor the post-EAU Ly-6C^{low}Ly-6G^{+} APC from MC5r$^{(-/-)}$ mice activated post-EAU T cells to suppress EAU (Fig. 2B). Therefore, while there are potential Ly-6C^{low}Ly-6G^{+} APC present in the post-EAU spleen of MC5r$^{(-/-)}$ mice, they do not have the ability to activate regulatory immunity to suppress EAU. This corresponds with previously published observations on post-EAU MC5r$^{(-/-)}$ spleen cells (10). However, it has previously been reported that APC from post-EAU wild type spleens stimulate regulatory activity in T cells from post-EAU spleens of MC5r$^{(-/-)}$ mice. To see whether the post-EAU Ly-6C^{high}Ly-6G^{-} or Ly-6C^{low}Ly-6G^{+} APC of wild type mice stimulate regulatory activity in post-EAU MC5r$^{(-/-)}$ spleen T cells, MC5r$^{(-/-)}$ post-EAU spleen T cells were antigen stimulated with sorted Ly-6C^{high}Ly-6G^{-} or Ly-6C^{low}Ly-6G^{+} APC from post-EAU wild type mice. The post-EAU Ly-6C^{low}Ly-6G^{+} APC, but not the Ly-6C^{high}Ly-6G^{-} APC, with MC5r$^{(-/-)}$ post-EAU T cells significantly changed the tempo and severity of EAU in the recipient mice (Fig. 2C). These results demonstrate that it is the spleen Ly-6C^{low}Ly-6G^{+} APC that mediates the post-EAU regulatory immunity.

α-MSH Treatment of EAU Significantly Increases Regulatory APC in the Spleen

Since the results show a dependency on MC5r expression for the emergence of a post-EAU regulatory APC in the spleen of mice (10). This suggests that if MC5r is stimulated with its ligand, α-MSH, it should mediate expansion of the regulatory APC in the spleens of EAU mice. It is known that a local application of α-MSH expression plasmid at the onset of EAU is effective in promoting an early resolution of EAU and preserving retinal structure (4). EAU mice treated with the α-MSH plasmid showed suppressed EAU as seen before (Fig. 2D). Analysis of culture supernatants of spleen cells stimulated for 48 hours with IRBP from α-MSH-plasmid treated and untreated mice showed no change in the low levels of IL-17

with a decrease in IFN-γ production; however, there was a significant increase in TGF-β production (Fig. 2E). This shows that in the spleens of α-MSH treated EAU mice the antigen-specific response is tilted toward Treg cell activation. To see if this is because α-MSH treatment induced regulatory APC in the spleen, the APC from treated EAU mice were used to activate spleen T cells from the untreated EAU mice. Significantly higher levels of TGF–β with significantly suppressed production of IFN-γ and IL-17 was seen when the T cells were stimulated by the APC from the α-MSH treated EAU mice (Fig. 2E). To determine whether the α-MSH treatment promoted expansion of post-EAU Ly-6C^{low}Ly-6G^{+} APC in the spleen, the spleen cells were collected on day 42 from the EAU treated mice and untreated EAU mice and stained for CD11b, F4/80, Ly-6G, and Ly-6C. There was an overall expansion of CD11b^{+} F4/80^{+} cells in the spleens of α-MSH treated EAU mice (data not shown) with a two-fold expansion of both Ly-6C^{high}Ly-6G^{-} and Ly-6C^{low} Ly-6G^{+} cells (Fig. 2F). Therefore, α-MSH plasmid treatment promotes the expansion of the Ly-6C^{low}Ly-6G^{+} cells, and regulatory activity in the post-EAU mice.

Post-EAU Protective Regulatory Immunity Also Requires A2Ar Expression

Since the adenosinergic pathway has the potential to govern the intensity of an immune response (24, 25), and that there is some hint in the literature of a potential link between the melanocortin and adenosinergic pathways (26), a possible role of A2Ar in post-EAU protective regulatory immunity and a link with MC5r was investigated. Mice with the A2Ar knocked out were immunized to induce EAU and clinically scored for uveoretinitis. The course and severity of EAU in wild-type and A2Ar$^{(-/-)}$ mice were insignificantly different and nearly the same (Fig. 3A). The post-EAU spleen cells were collected and restimulated in vitro with IRBP to assay their regulatory potential as done before with the MC5r$^{(-/-)}$ post-EAU spleen cells (2, 10). The post-EAU spleen cells from A2Ar$^{(-/-)}$ mice were unable to suppress EAU in contrast to the adoptive transfer of post-EAU spleen cells from wild-type mice (Fig. 3B – D). Therefore, like the MC5r$^{(-/-)}$ mice, the A2Ar$^{(-/-)}$ mice do not develop post-EAU regulatory immunity in the spleen.

Induction of post-EAU regulatory immunity requires A2Ar expression on the T cells

Since A2Ar expression has been reported on both APC and T cells (27), the post-EAU A2Ar$^{(-/-)}$ APC were used to IRBP-activate the Treg cells from the post-EAU wild type spleen. Both APC and T cells were transferred into mice immunized for EAU. Mice that received post-EAU Treg cells activated by post-EAU A2Ar$^{(-/-)}$ APC showed significantly suppressed clinical scoring of EAU, and a significantly lower severity of EAU (Fig. 4A). In contrast, mice that received T cells isolated from the spleen of post-EAU A2Ar$^{(-/-)}$ mice restimulated by APC from post-EAU wild type mice showed no significant change in the course of EAU or severity of disease (Fig. 4B). This suggests that the lack of post-EAU regulatory immunity in the spleens of A2Ar$^{(-/-)}$ mice is because of a requirement for the T cells to express A2Ar as there is a requirement for MC5r on the APC. The A2Ar$^{(-/-)}$ post-EAU T cells were stained for FoxP3, and the surface markers, CD4 and CD25. Post-EAU spleen T cells from A2Ar$^{(-/-)}$ mice showed significantly lower proportion of FoxP3^{+} CD25^{+} CD4^{+} T cells compared to post-EAU T cells from the spleens of wild-type mice (Fig. 4C). Therefore, the expression of A2Ar is necessary for there to be Treg cells associated with the regulatory immunity in the spleens of post-EAU mice.

If the stimulation of A2Ar is sufficient to induce the post-EAU Treg cells, then stimulation of A2Ar should establish regulatory immunity in the mice with EAU. Mice with EAU were injected systemically with a selective A2Ar agonist, CGS21680 (CGS), during EAU on days 23, 24, 25, when the mice first reach the height of retinal inflammation. On day 35, treated mice began to show resolution of ocular inflammation and by day 60 all mice were at a score of 1 or below (Fig. 4D). In contrast, PBS injected mice maintained EAU through day

60 (Fig. 4D). To see whether there was an early emergence of regulatory immunity in the spleen, the spleen cells were collected on day 38 and assayed for IRBP stimulated expression of IFN-γ, IL-17 and TGF-β. The spleen cells from CGS treated mice had significantly lower IFN–γ production, and no change in TGF-β production compared to T cells from the untreated mice (Fig. 4E). From both untreated and CGS-treated mice the levels of IL-17 production were very low. These findings showed that the CGS treatment suppressed Th1 cell activity while promoting or maintaining Treg activity. In addition, the results showed that stimulating the A2Ar during EAU promotes early resolution of uveitis.

To see if the α-MSH-plasmid treatment of EAU can induce regulatory immunity and early resolution of EAU in $A2Ar^{(-/-)}$ mice, the $A2Ar^{(-/-)}$ EAU mice were treated with α MSH expression plasmid on day 17 and 19 after immunization to induce EAU. These treated mice showed a normal course of EAU with no early EAU resolution, and no change in disease severity (Fig. 4F). At the resolution of EAU, spleen cells were re-stimulated and cytokine production was measured. Untreated $A2Ar^{(-/-)}$ mice and α MSH expression plasmid treated $A2Ar^{(-/-)}$ mice showed no significant differences in IL-17, IFN–γ, and TGF–β production (Fig. 4G). Therefore, the melanocortin-driven induction of regulatory immunity also required the expression of A2Ar on T cells.

Emergence of Post-EAU Regulatory Immunity is Linked Through MC5r and A2Ar

If adenosine is a mediator in the induction of post-EAU Treg cells, then the post-EAU APC should express the ecto-nucleotidases CD39 and CD73 that convert ATP, ADP, and AMP into adenosine (28). The CD39 and CD73 mRNA in the sorted Ly-$6C^{low}$Ly-$6G^{+}$ and Ly-$6C^{high}$Ly-$6G^{-}$ subsets from unimmunized and post-EAU mice was measured by real-time PCR (Fig. 5A). Both CD39 and CD73 mRNA expression was significantly greater in the Ly-$6C^{low}$Ly-$6G^{+}$ subset compared with the Ly-$6C^{high}$Ly-$6G^{-}$ subset (Fig. 5A). Moreover, the Ly-$6C^{low}$Ly-$6G^{+}$ subset from post-EAU mice had significantly higher levels of CD39 and CD73 expression compared with cells from unimmunized mice (Fig. 5A). In contrast, the CD39 and CD73 mRNA expression was suppressed in the Ly-$6C^{high}$Ly-$6G^{-}$ subset from post-EAU mice (Fig. 5A). This showed that the regulatory Ly-$6C^{low}$Ly-$6G^{+}$ APC subset in the post-EAU mice spleens highly express CD39 and CD73 mRNA.

To see if the increased expression of CD39 and CD73 mRNA was dependent on MC5r, the APC were collected from the spleens of unimmunized mice, treated with α-MSH, sorted into Ly-$6C^{low}$Ly-$6G^{+}$ and Ly-$6C^{high}$Ly-$6G^{-}$ subsets, and analyzed for CD39 and CD73 expression by RT-PCR. The Ly-$6C^{low}$Ly-$6G^{+}$ APC showed no change in CD39 and CD73 expression with α-MSH treatment (Fig. 5B). The Ly-$6C^{high}$Ly-$6G^{-}$ APC showed suppression of CD39 and increased CD73 expression with α-MSH treatment (Fig. 5B). This demonstrated that the MC5r-dependent induction of post-EAU regulatory immunity was not associated with up regulating CD39 and CD73 in the regulatory APC. Therefore, the induction of post-EAU protective immunity in the spleen is a MC5r-driven expansion of ocular-autoantigen presenting Ly-$6C^{low}$Ly-$6G^{+}$ APC in the spleen that activate Treg cells through the adenosinergic pathway (Fig 6).

Discussion

Our results demonstrated that the emergence of post-EAU protective regulatory immunity in the spleen is dependent on a sequential activity of the melanocortin and adenosinergic pathways of regulatory APC and T cells. At resolution of EAU there was a melanocortin-driven expansion of $CD11b^{+}$ $F4/80^{+}$ Ly-$6C^{low}$Ly-$6G^{+}$ APC that mediate the induction of regulatory T cells in the spleen. These APC and the induction of regulatory immunity were not seen in the spleens of post-EAU $MC5r^{(-/-)}$ mice, but the APC were seen in the spleens of post-EAU $A2Ar^{(-/-)}$ mice. This difference was caused by the different roles that the

melanocortin and adenosinergic pathways have in the induction of post-EAU regulatory immunity. The melanocortin pathway through MC5r on APC was associated with the induction and expansion of the regulatory APC, and the adenosinergic pathway through A2Ar on T cells was associated with the APC induction and activation of Treg cells.

How the expression of MC5r is associated with the expansion or localization of suppressor cells in the spleen is unknown. This is a new finding. There are at least two other melanocortin receptors on macrophages and dendritic cells, MC1r and MC3r. The α-MSH suppression of macrophages and dendritic cell production and response to proinflammatory cytokines is through MC1r and MC3r (29–31). Through these receptors α-MSH blocks TLR-signaling pathways, and inhibits activation of NF-κB and p38MAPK (31–33). The macrophages treated with α-MSH produce TGF-β and IL-10, which also is seen with α-MSH treated Langerhans cells that prevent contact hypersensitivity (17). While these reports suggest that it is a cytokine-mediated suppression of immunity caused by α-MSH treatment, our results demonstrated that a mediator of melanocortin-induced regulatory activity in the post-EAU mouse is through an APC with CD39 and CD73 expression that has localized to the spleen and subsequently activates Treg cells through the immunoregulating adenosinergic pathway.

Ly-6C^{low}Ly-6G^{+} suppressor cells have been found in the retina of mice recovering from EAU, and are considered important in mediating local resolution of the inflammation (34). Recently it has been found that the microglia in healthy retina express characteristics of myeloid suppressor cells that are mediated by the presence of α MSH (19). Since we have shown that this regulatory immunity requires an intact eye, and recovery from uveitis, it is possible that the source of the splenic post-EAU regulatory APC is the eye itself. It is known that foreign antigen placed into the anterior chamber of the healthy eye ends up being presented in the spleen by regulatory/tolerogenic F4/80^{+} APC that activate CD8^{+} Treg cells as part of the anterior chamber associated immune deviation (ACAID) phenomena (35). This suggests that the ocular microenvironment both healthy and inflamed is uniquely capable of driving APC toward suppressive functions where induction of immune tolerance is found in the spleen. The post-EAU induced tolerance is dependent on a functional MC5r melanocortin pathway and a significantly different mechanism of tolerance induction than the ACAID phenomena.

Through MC5r, α-MSH induces regulatory activity in effector T cells (21, 36, 37), and from our results regulatory APC. While most of the intracellular signaling pathways associated with the melanocortin receptors are unclear, α-MSH stimulation of MC5r activates the JAK2 and STAT1 pathway (38). The activation of STAT1 is necessary in the activity of tumor derived myeloid suppressor cells, suggesting that α-MSH uses STAT1 to induce regulatory activity in APC (13, 39, 40). However there are other receptors on APC through which α-MSH suppresses inflammatory cytokine production and certain innate immune responses, and it is not possible to rule out their role in α-MSH-mediated induction of regulatory APC. Recently it has been found that α-MSH with another constitutive neuropeptide in the eye, Neuropeptide Y (NPY), together promote myeloid suppressor cell characteristics in macrophages (19). It is not known which melanocortin receptor is involved in this interaction; however, the findings suggest that it may most likely be MC5r. While MC5r is required for the expansion or localization of the Ly-6C^{low}Ly-6G^{+} APC seen in the post-EAU spleen, the effects of α-MSH cannot account for the expression of the ecto-nucleotidases, nor is it understood how through MC5r cell migration to the spleen is regulated. It is known that α-MSH does regulate the migratory activity of neutrophils, and melanocyte adhesion and migration, but this is through MC1r (41, 42). Therefore, while the presence of regulatory Ly-6C^{low}Ly-6G^{+} APC in the spleen is dependent on MC5r expression, the induction of the regulatory activity must be more than α-MSH alone.

Adenosine-stimulation of A2Ar on T cells has been demonstrated to be effective and possibly required for Treg cell induction (24, 43, 44). A2Ar stimulation protects against inflammatory bowel disease (45), neuroinflammatory injury (46), reperfusion injury in the lung and liver (47, 48), and allograft rejection (49). Stimulating another adenosine receptor, A3Ar, suppresses EAU by suppressing the activation of effector T cells and up regulating IL-10 production in cultured splenocytes from treated mice (50). Our results demonstrated that the post-EAU protective immunity is mediated by a peripheral mechanism of Treg cell activation through A2Ar.

Our results demonstrated that there is a pathway of inducing regulatory immunity that is through MC5r-dependent expansion in the spleen of ecto-nucleotidase-expressing $CD11b^{+}$ $F4/80^{+}$ $Ly\text{-}6C^{low}Ly\text{-}6G^{+}$ regulatory APC that through A2Ar on T cells activate $FoxP3^{+}$ $CD4^{+}$ Treg cells (Fig. 6). The results of this pathway is the activation of antigen-specific inducible $CD4^{+}$ Treg cells that when specific to ocular autoantigens protect the mice from the recrudescence of uveitis. Therefore, the highly conserved melanocortin and adenosinergic signaling pathways of immune regulation and homeostasis function together to induce a protective regulatory immune response that can be exploited to (re)establish tolerance to autoantigens.

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Abbreviations

A2Ar	adenosine 2A receptor
α-MSH	alpha-Melanocyte Stimulating Hormone
AT	adoptive transfer
CGS	CGS21680, A2Ar agonist
EAU	experimental autoimmune uveoretinitis
IRBP	interphotoreceptor retinoid binding protein
MC5r	melanocortin 5 receptor

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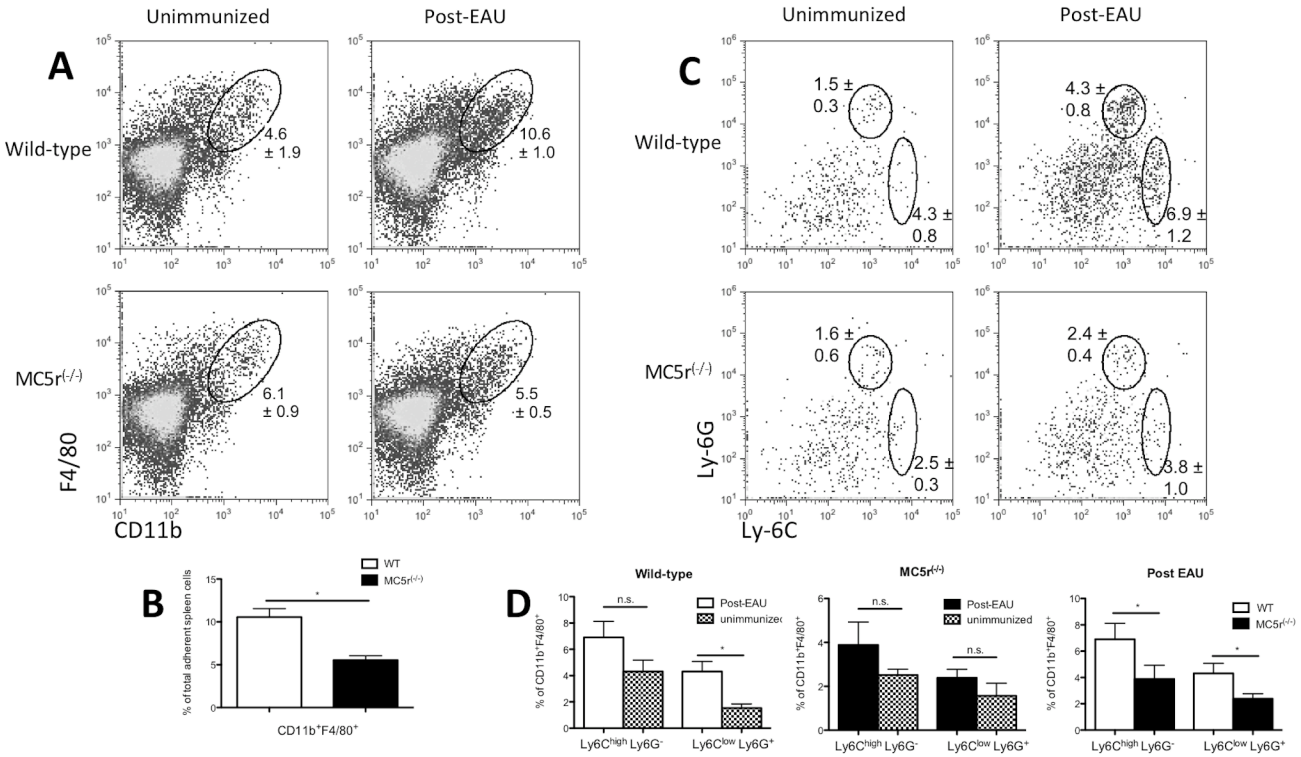

Figure 1. MC5r expression is required for expansion of CD11b$^+$ F4/80$^+$ Ly-6C^{low}Ly-6G$^+$ APC

Wild-type and MC5r$^{(-/-)}$ mice were immunized for EAU, and APC were collected from the spleens when the mice recovered from EAU (day 70–80). (**A**) The spleen post-EAU APC were stained for CD11b and F4/80 and analyzed by flow cytometry. (**B**) The bar graph shows the quantified CD11b$^+$ F4/80$^+$ APC from all wild type and MC5r$^{(-/-)}$ mice given as mean percentage ± SEM, P = 0.007. (**C**) Graphs show the expression of Ly-6G and Ly-6C on gated CD11b$^+$ F4/80$^+$ APC from post-EAU spleens was assayed by flow cytometry. (**D**) The bar graphs show the quantified Ly-6C^{low} Ly-6G$^+$ and Ly-6C^{high} Ly-6G$^-$ APC populations from all mice, given as mean percentage ± SEM. The bar graphs compare post-EAU wild-type and unimmunized wild-type (left panel, P = 0.0037), post-EAU MC5r$^{(-/-)}$ and unimmunized MC5r$^{(-/-)}$ (center panel), and post-EAU wild-type and post-EAU MC5r$^{(-/-)}$ (right panel). n.s. No significant differences were seen between the groups. Data are representative of five mice individually assayed (**A** – **D**).

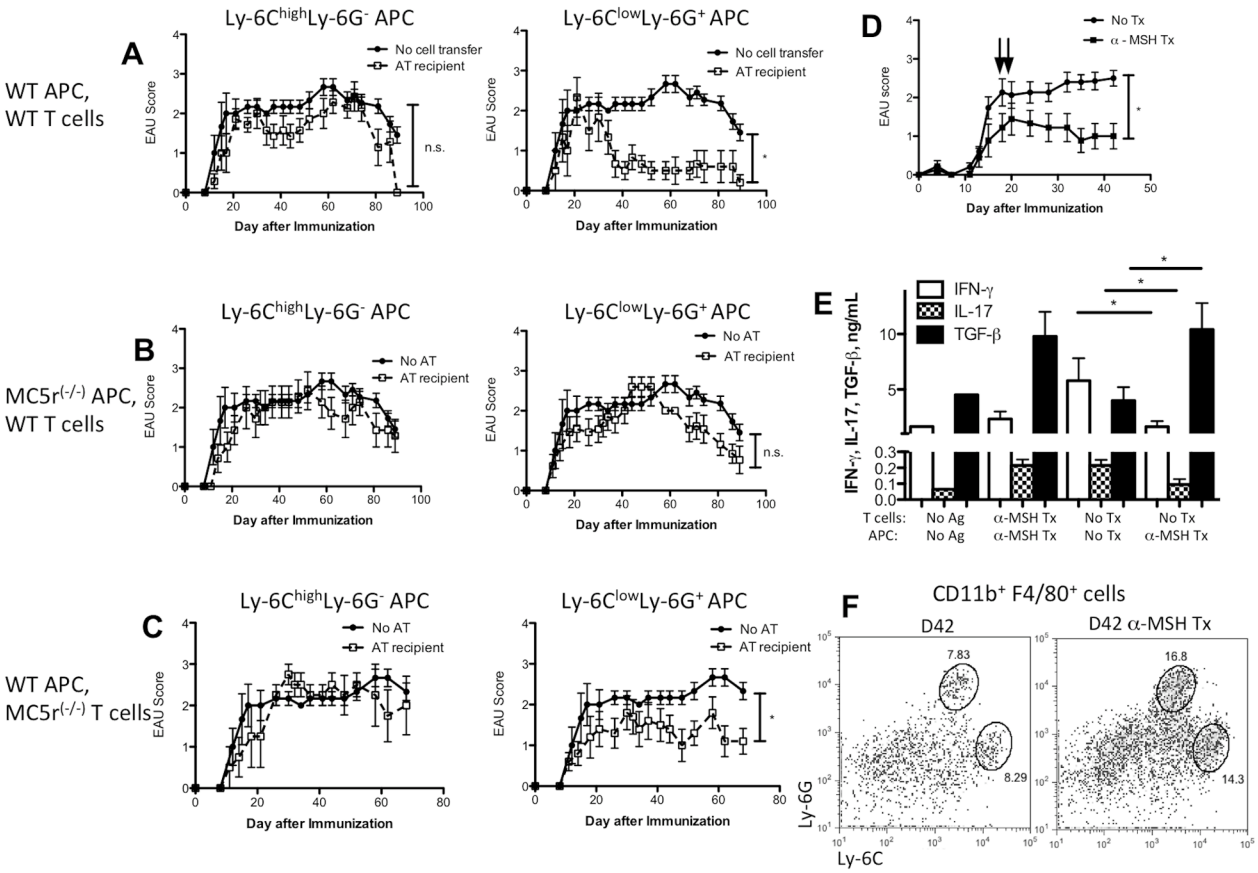

Figure 2. Ly-6C^{low}Ly-6G^{+} post-EAU APC require MC5r to activate post-EAU Treg cells

APC and T cells from the spleens of EAU recovered (day 70–80) wild-type and MC5r$^{(-/-)}$ mice were collected. CD11b^{+} F4/80^{+} APC were sorted based on Ly-6C and Ly-6G expression and the sorted APC were used to activate the T cells. (**A** – **C**) Shown are EAU scores (mean ± SEM) for each group of EAU mice that received an adoptive transfer (AT) of both APC and T cells. Mice that received no cells are shown as a control in each graph (n = 11). * Statistical significant P ≤ 0.05 difference was determined. (**A**) Cells transferred were T cells activated with Ly-6C^{high}Ly-6G^{-} APC (7 recipient mice) or Ly-6C^{low}Ly-6G^{+} APC (6 recipient mice), P ≤ 0.0001. (**B**) Mice received T cells activated by MC5r$^{(-/-)}$ Ly-6C^{high}Ly-6G^{-} APC (9 recipient mice) or MC5r$^{(-/-)}$ Ly-6C^{low}Ly-6G^{+} APC (13 recipient mice). (**C**) Transferred cells were MC5r$^{(-/-)}$ T cells activated by Ly-6C^{high}Ly-6G^{-} APC (4 recipient mice) or Ly-6C^{low}Ly-6G^{+} APC (10 recipient mice), P = 0.02. (**D**) EAU scores (mean ± SEM) of mice treated with α MSH expression plasmid (n = 10, α-MSH Tx) on day 17 and 19 (arrows) or untreated EAU mice (n = 11, No Tx), P = 0.002. (**E**) IFN-γ, IL-17, and TGF-β were measured from spleen APC and T cells from treated or untreated mice on day 42. The ng/ml (mean ± SEM) are shown for each combination of APC and T cells of 5 individual mice, * P 0.05. (**F**) The expression of Ly-6C and Ly-6G on CD11b^{+} F4/80^{+} APC from the spleens of treated (α MSH Tx) or untreated (No Tx) EAU mice on day 42, scatter plot shown is representative of 7 different mice.

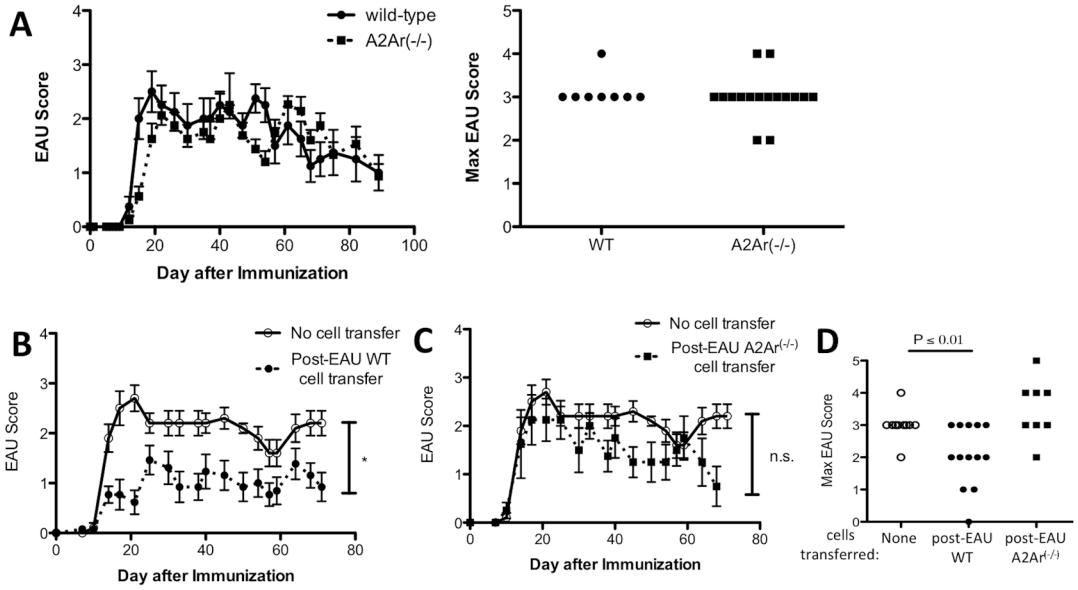

Figure 3. A2Ar$^{(-/-)}$mice do not develop protective post-EAU regulatory immunity

Wild-type and A2Ar$^{(-/-)}$ mice were immunized for EAU, and the fundus was examined until resolution. * Statistical significant P ≤ 0.05 differences are identified. (**A**) EAU scores of wild-type (n = 10), and of A2Ar$^{(-/-)}$ (n = 15) mice (left panel), and maximum EAU scores of each mouse over the entire course of disease (right panel). Spleen cells from post-EAU mice (day 70–80) were collected and stimulated with IRBP for 48 hours, then transferred to mice immunized for EAU (**B** – **D**). Each graph shows the EAU scores (mean ± SEM) for each group over time. Cells transferred to recipient mice were post-EAU spleen cells from wild-type mice, n = 14, P = 0.0001 (**B**); or post-EAU spleen cells from A2Ar$^{(-/-)}$ mice, n = 8 (**C**). (**D**) Maximum EAU scores of each mouse over the course of disease.

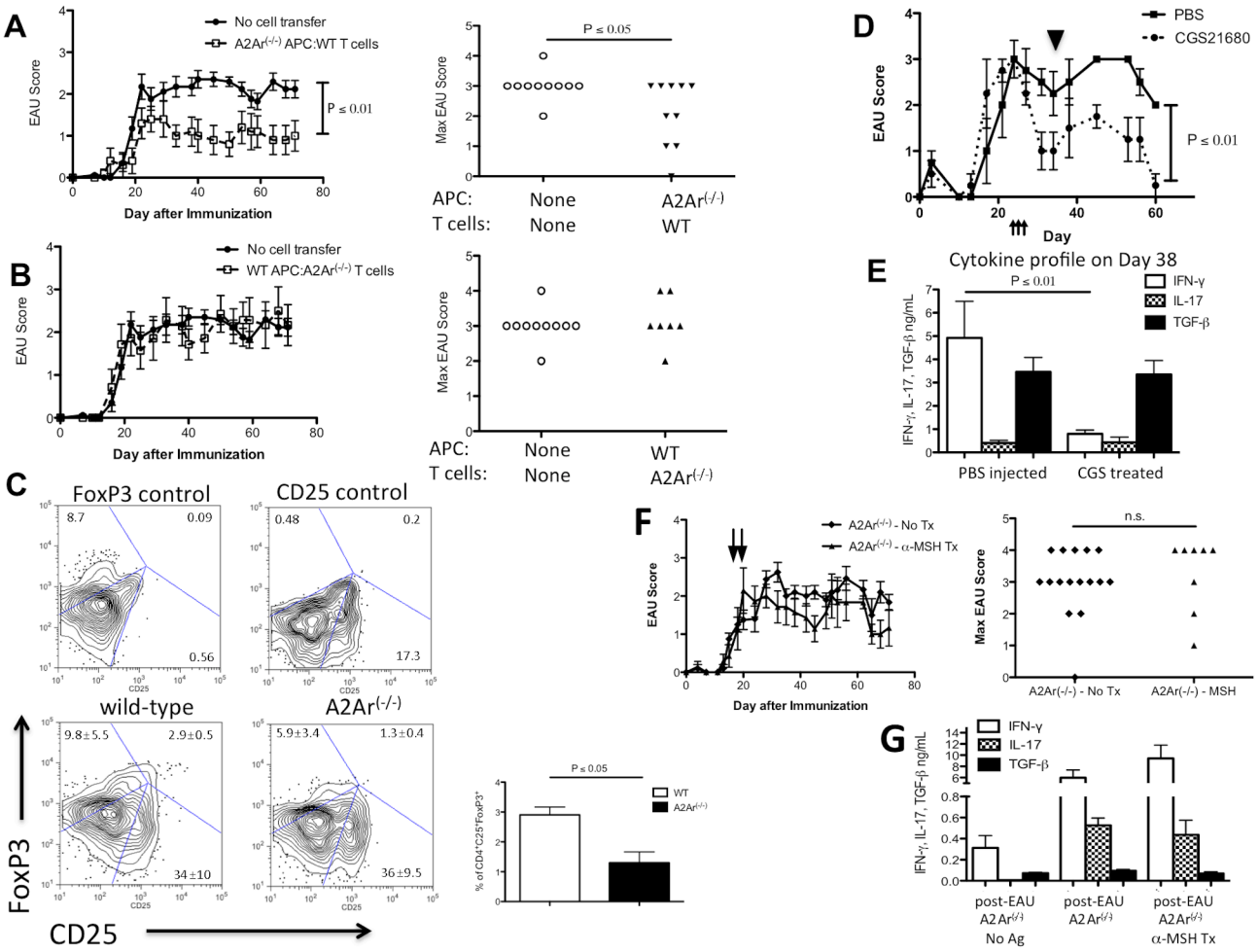

Figure 4. A2Ar on the post-EAU Treg cell is necessary for activation by post-EAU regulatory APC

(**A** – **B**) The EAU scores (mean ± SEM) over the course of disease (left panels) and maximum scores (right panels) of mice immunized for EAU that received an adoptive transfer of both post-EAU (day 70–80) APC and T cells. The cells transferred to recipient mice were wild type (WT) post-EAU T cells activated by post-EAU A2Ar$^{(-/-)}$ APC, n = 10 (**A**), or post-EAU A2Ar$^{(-/-)}$ T cells activated by WT post-EAU APC, n = 7 (**B**). (**C**) FoxP3 and CD25 expression of IRBP restimulated post-EAU CD4^{+} T cells from wild-type or A2Ar$^{(-/-)}$ mice; the scatter plot shown is representative of three different mice. Cells were gated on CD4^{+} T cells and stained for FoxP3 and CD25. The scatter plot labeled FoxP3 control is the CD25 isotype control of cells stained for CD4 and FoxP3. The scatter plot labeled CD25 control is the FoxP3 isotype control of cells stained for CD4 and CD25. The bar graph is the percentage (mean ± SEM) of CD4^{+} CD25^{+} FoxP3^{+} T cells of three different post-EAU mice. (**D**) EAU scores of mice that received CGS21680 (n = 9) on days 23, 24, 25 (black arrows) or PBS injections (n = 9), P = 0.0017. The inverted triangle indicates day 38 when the spleen cells were collected, IRBP restimulated, and assayed for IFN–γ, IL-17, and TGF–β production. (**E**) The bar graphs are the mean ± SEM of the concentration of IFN-γ, IL-17, and TGF-β in the cultures of spleen cells from post-EAU mice (day 70–80) of CGS treated or untreated mice, n = 4, P = 0.007. (**F**) EAU scores over time (left panel) and maximum EAU scores (right panel) of A2Ar$^{(-/-)}$ mice treated with α MSH expression plasmid on day 17 and 19 (arrows), n = 7. (**G**) The concentration of IFN–γ, IL-17, and

TGF–β production (mean ± SEM) by IRBP-restimulated spleen cells from treated (Tx, n = 6) or untreated A2Ar$^{(-/-)}$ mice (n = 14) on day 71. Spleen cells incubated without IRBP is used as a no Ag control. n.s. No significant differences were seen between the groups.

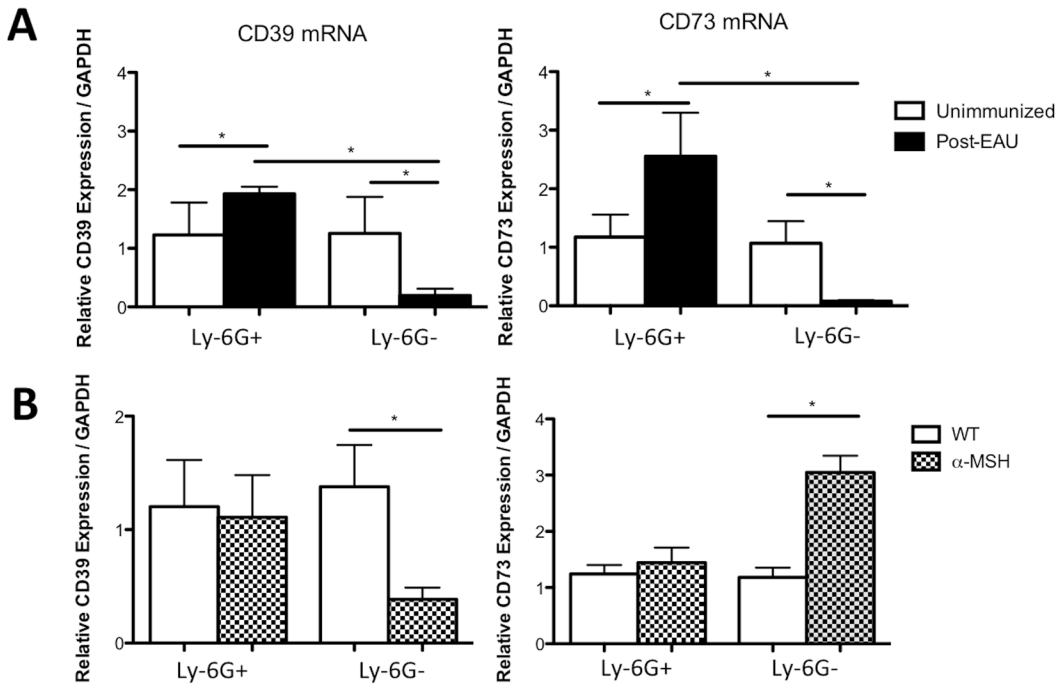

Figure 5. Expression of CD39 and CD73 by Ly-6C^{high}Ly-6G^{-} and Ly-6C^{low}Ly-6G^{+} APC

(**A**) CD39 and CD73 mRNA in Ly-6C^{low}Ly-6G^{+} APC and Ly-6C^{high}Ly-6G^{-} APC sorted from the spleen of unimmunized or post-EAU mice were assayed by RT-PCR. The 2 ^ - $\Delta\Delta C_T$ for CD39 and CD73 mRNA were normalized to GAPDH and their relative expressions is presented. The data shown is the mean ± SEM where two mice were pooled per experiment and the experiment was done twice. *Significance $P \leq 0.05$ was measured between the results. (**B**) The mRNA for CD39 and CD73 mRNA was measured in Ly-6C^{low}Ly-6G^{+} APC and Ly-6C^{high}Ly-6G^{-} APC that were sorted from α-MSH treated and untreated APC of unimmunized mice spleens. Presented are the relative expressions (mean ± SEM) of CD39 and CD73 mRNA normalized to GAPDH mRNA expression of 5 mice pooled per experiment and the experiment was done twice, *Significance $P \leq 0.05$ was measured between the results.

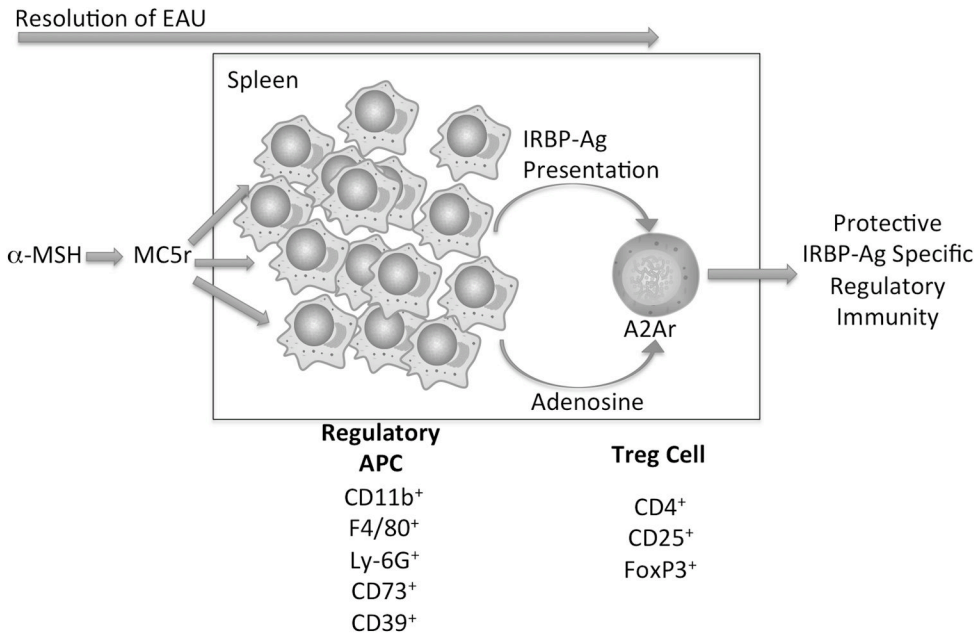

Figure 6. The induction of post-EAU protective regulatory immunity

At resolution of EAU, through MC5r, presumably stimulated by the neuropeptide alpha-melanocyte stimulating hormone (α-MSH), regulatory Ly-6C^{low}Ly-6G^{+} APC expressing CD39 (ecto-nucleoside triphosphate diphosphohydrolase) and CD73 (ecto-5′-nucleotidase) localize or accumulate in the spleen. The Ly-6C^{low}Ly-6G^{+} APC present ocular-autoantigens, and through their expression of CD39 and CD73 generate adenosine from ATP to induce in an A2Ar-dependent manner autoantigen-specific Treg cells, which mediate a post-EAU protective regulatory immune response to ocular-autoantigens.