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Exacerbation of autoimmune uveitis by obesity occurs through the melanocortin 5 receptor

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Abstract

Autoimmune uveitis is a leading cause of blindness with a complex etiology. Obesity is considered a chronic disease with a connection with autoimmune diseases through systemic inflammation. However, an obesity and autoimmune disease connection is not consistently true in rodent models of autoimmune disease. A mouse model of human autoimmune uveitis, experimental autoimmune uveitis (EAU) has been used to better understand the immunobiology of uveitis. In this study, we assessed EAU in a high-fat diet (HFD) obesity model and found that the EAU severity is significantly higher in wild-type mice, but not in HFD melanocortin 5 receptor deficient mice. We find a decrease in $CD11b^{+}F4/80^{+}Ly\text{-}6C^{lo}Ly\text{-}6G^{+}$ Mϕs, previously shown to be suppressive, and an enhancement of a Th1 response at the onset of EAU in obese mice. We further demonstrate that at recovery of EAU, obese mice lack regulatory immunity that provides protection from EAU. This report demonstrates that obesity exacerbates autoimmune uveitis and inhibits the promotion of post-EAU regulatory immunity through the melanocortin 5 receptor. The implication of this work is that obesity may contribute to the prevalence of autoimmune uveitis.

Keywords

autoimmune uveitis; melanocortin 5 receptor; obesity; ocular inflammation

1 | INTRODUCTION

Obesity is a growing epidemic in the United States that is now considered a chronic disease.[1–4] The health consequences of this epidemic are dire, with type 2 diabetes among the list of diseases that result from obesity.[1,5] With an increase in white adipose tissue is a corresponding increase in the production of inflammatory cytokines and an overall

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AUTHORSHIP

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DISCLOSURE

All other authors have no relevant competing financial interests.

SUPPORTING INFORMATION

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

subclinical systemic state of inflammation.[5] Therefore, it is of interest to determine if an autoimmune disease, such as autoimmune uveitis, is influenced by obesity.

Autoimmune diseases are thought to occur because of a loss of tolerance to the target tissue and/or an overactive immune response toward a particular tissue. Autoimmune uveitis is the third leading cause of blindness in Western countries,[6–8] and the incidence is increasing. This increase mirrors the increased incidence of other autoimmune diseases such as multiple sclerosis, Crohn's disease, and asthma. Further, the increased incidence of autoimmune diseases also mirrors the increase in obesity. This suggests a potential link between obesity and autoimmune disease. Further support of this concept is present in HFD animal studies with an exacerbation of experimental autoimmune encephalomyelitis (EAE),[9] the multiple sclerosis rodent model, and collagen-induced arthritis (CIA),[10] the rodent arthritis model.

The melanocortin system is involved in metabolism and immunology. While the melancortin 4 receptor is the most studied with respect to metabolism, it has been demonstrated that the melanocortin 5 receptor (MC5r) has a role in lipid metabolism and is expressed on adipose tissue.[11,12] The ligand for MC5r, MC1r, MC3r, and MC4r is α-MSH,[13] a potent immunosuppressive neuropeptide constitutively expressed in the eye.[14] In addition to adipose, MC5r is also expressed on Mϕs, T cells, and within the retina.[15] As such, MC5r has been studied using the mouse model for autoimmune uveitis, experimental autoimmune uveitis (EAU). $MC5r^{(-/-)}$ mice recover from EAU normally, but do not have post-EAU regulatory immunity in the spleen.[16–18] This lack of post-EAU regulatory immunity in the spleen is because of a loss of suppressor APC that are $CD11b^{+}F4/80^{+}Ly\text{-}6G^{+}Ly\text{-}6C^{lo}(Ly\text{-}6G^{+})$.[16,18]

In this report, we investigate the effect of a diet-induced obesity model on EAU and the role that MC5r has on obesity and EAU. We assay for the effect of a high-fat diet (HFD) on the suppressor APC population and the dominant T cell type at the onset of EAU. We also investigate if HFD abrogates the emergence of post-EAU regulatory immunity in the spleen. To our knowledge, this is the first report of an exacerbation of EAU by HFD that occurs through promoting a systemic inflammatory response, and a loss of post-EAU regulatory immunity because of HFD.

2 | RESULTS

2.1 | Autoimmune uveitis is exacerbated by obesity

We first asked if a moderate HFD exacerbates EAU by starting mice on a chow diet in which 45% of the calories are obtained through fat (45% HFD). These mice were started on the 45% HFD 2 weeks before immunization for EAU and were weighed weekly for body weight changes and food consumption. A cohort of control mice received chow containing 13% calories from fat (NFD) were used for comparison with the HFD mice. Mice on the 45% HFD showed a significant increase in weight at week 9 after HFD was started (Supplementary Fig. 1A), and weight gain was similar in mice with and without EAU (Supplementary Fig. 1B and C). Moderately elevated blood glucose that corresponds to moderate but not overt diabetes was observed in HFD mice at resolution of EAU, but it was significantly lower in EAU mice and the blood glucose in 45% HFD EAU mice was not

significantly different (Supplementary Fig. 1D). The tempo and course of EAU was not significantly different in 45% HFD mice compared to NFD mice (Supplementary Fig. 1E). Because the tempo can vary with groups of EAU mice, we then compared the highest EAU score for each mouse over the entire course of disease to determine severity of disease (Supplementary Fig. 1F). Despite the similar overall tempo of disease the 45% HFD mice did show a significantly increased severity of disease. These observations indicate that a moderate 45% HFD increases the severity of EAU, but obesity is not affected by EAU.

We next asked if mice on an extreme HFD that consisted of 60% calories from fat showed exacerbated EAU. Mice on 60% HFD showed a more rapid weight gain compared to mice on the 45% HFD with a significant increase in the body weight at 5 weeks after 60% HFD was started Supplementary Fig 1A, (Fig. 1A). EAU did not affect the weight gain in mice on the 60% HFD (Fig. 1B and C), and blood glucose showed a similar moderate but significant increase (Fig. 1D). However, in contrast to the 45% HFD fed EAU mice, the blood glucose in EAU mice on 60% HFD was significantly elevated compared to NFD mice. Resolution of EAU was significantly delayed in the 60% HFD mice compared with NFD mice and the severity was significantly elevated (Fig. 1E and F). Taken together these observations demonstrate an extreme HFD exacerbates EAU and delays the resolution of EAU and in the extreme HFD model EAU does not protect from weight gain or elevated blood glucose.

2.2 | The melanocortin 5 receptor has a role in HFD-induced obesity

The melanocortin 5 receptor (MC5r) has an immunological role on APCs in EAU,[16–18] and a metabolic role in adipose tissue.[11] Since the extreme HFD showed a more consistent effect in the WT mice, we asked if this was the case with MC5r$^{(-/-)}$ mice as well. MC5r$^{(-/-)}$ mice on 60% HFD showed a significant increase in weight gain at week five compared to MC5r$^{(-/-)}$ mice on NFD (Fig. 2A). EAU did not change the weight gain in MC5r$^{(-/-)}$ mice on NFD (Fig. 2B), but did allow for mild protection from weight gain on the HFD (Fig. 2C). Fasting blood glucose in 60% HFD fed MC5r$^{(-/-)}$ mice had elevated blood glucose that is considered moderate diabetes, but was significantly elevated compared to NFD mice (Fig. 2D). Unexpectedly, the MC5r$^{(-/-)}$ mice showed similar EAU scores when on HFD compared to NFD (Fig. 2E) with a similar onset and duration of disease with no significant increase in the severity of disease (Fig. 2F). These observations suggest that there may be a compensatory mechanism in MC5r$^{(-/-)}$ mice that protects from exacerbated EAU when fed HFD.

2.3 | Obesity suppresses the emergence of CD11b^{+}F4/80^{+}Ly-6C^{lo}Ly-6G^{+} APC in the spleen during EAU

We next sought to address how the HFD exacerbates EAU. Because there is systemic inflammation associated with obesity, we asked if the suppressor APC, previously identified as CD11b^{+}F4/80^{+}Ly-6C^{lo}Ly-6G^{+} (Ly-6G^{+} cells), that emerges in the spleen during the resolution of EAU[18] is diminished in HFD mice at the onset of EAU. Because the 60% HFD has a much more consistent phenotype than the 45% HFD, we focused on the response to the 60% HFD. At the onset of EAU 4 weeks after immunization, we determined if there was a change in the number of suppressor APC in the spleen based on CD11b, F4/80, Ly-6G, and Ly-6C expression as we have previously shown to be CD11b^{+}F4/80^{+}Ly-6C^{lo}Ly-6G^{+}.[18]

We found that the Ly-6G$^+$ population was significantly decreased with HFD (Fig. 3A, B, and E). In contrast, there was no difference in the number of Ly-6G$^+$ cells between NFD and HFD MC5r$^{(-/-)}$ mice (Fig. 3C, D, and E). As we previously observed, the Ly-6G$^+$ population in MC5r$^{(-/-)}$ mice was significantly lower than wild-type mice (Fig. 3E). These observations demonstrate that HFD reduces the Ly-6G$^+$ APC population that emerges in the spleen at the onset of EAU.

2.4 | Obesity increases the Th1 response during EAU

We next asked if the T cell response is altered because of HFD. At the onset of EAU, we examined the T cell population in the spleen following reactivation with IRBP residues 1-20 (IRBP). The reactivated T cells were analyzed by flow cytometry for expression of Th1 and Th17 specific transcription factors, T-bet and ROR-γt, and gating placement was based on the fluorescence minus one (FMO) controls (Supplementary Fig. 2). We found that T-bet expression was significantly greater in HFD mice compared to NFD mice (Fig. 4A, B, and I). In contrast, the Th17 specific transcription factor, ROR-γt, was not significantly different depending on the diet (Fig. 4C, D, and I). In addition, we observed significantly greater T-bet expression in MC5r$^{(-/-)}$ mice compared to wild-type mice, but HFD did not further increase expression in MC5r$^{(-/-)}$ HFD mice compared with MC5r$^{(-/-)}$ NFD mice (Fig. 4E–J). When we measured the IFN-γ production, we found an increase in HFD compared with NFD in both WT and MC5r$^{(-/-)}$ mice (Fig. 5A). In contrast and in agreement with the ROR-γt staining, secreted IL-17 was not significantly different (Fig. 5B). We also compared the double positive Tbet$^+$ROR-γt$^+$ subset and did not observe a significant difference between HFD and NFD, but there was a significant increase in MC5r$^{(-/-)}$ compared to WT (Supplementary Fig. 3). These observations demonstrate that obesity promotes an inflammatory T cell response, even when a significant change in weight is first observed, and this T cell response is also significantly greater in MC5r$^{(-/-)}$ mice.

2.5 | Obesity blocks induction of post-EAU regulatory immunity

The recovery of EAU is marked with the emergence of regulatory immunity in the spleen that protects from a memory response to ocular autoantigen.[16–21] We asked if diet induced obesity abrogates this response. The spleen from NFD and HFD mice that have recovered from EAU were collected and re-activated in vitro with IRBP and the functional regulatory capacity was assessed. We assessed the functional regulatory capacity by transferring the re-activated spleen cells into recipient mice that were immunized for EAU. The mice that received spleen cells from NFD post-EAU mice showed a significantly decreased duration and severity of disease compared to control EAU mice (Fig. 6A and B), as expected.[16–18,20,21] In contrast, mice that received spleen cells from post-EAU mice on 45% HFD showed a normal course of EAU (Fig. 6). When post-EAU spleen cells were transferred from 60% HFD mice, the recipient mice also showed a normal course of EAU (Fig. 7A and B). Since post-EAU regulatory immunity does not emerge in the spleen of MC5r$^{(-/-)}$ mice,[16–18,21] it was not unexpected to observe mice that received spleen cells from post-EAU MC5r$^{(-/-)}$ mice on 60% HFD did not show any suppression of EAU (Fig. 7C and D). These observations demonstrate that diet-induced obesity prevents the emergence of post-EAU regulatory immunity in the spleen.

3 | DISCUSSION

These observations demonstrate for the first time to our knowledge that EAU is exacerbated by diet-induced obesity. We further show at the onset of EAU, HFD decreased the number of suppressor APC in wild-type mice and promoted an inflammatory T cell response to ocular autoantigen. Importantly, HFD prevented the emergence of post-EAU regulatory immunity in the spleen. The implications of this study are that a diet rich in fat or overt obesity may contribute to the incidence and prevalence of autoimmune disease, specifically autoimmune uveitis.

Exacerbation of autoimmune disease in a diet-induced obesity model has been previously demonstrated in mouse models of arthritis and multiple sclerosis.[9,10] Systemic inflammation associated with obesity has been demonstrated in animal models and in human studies with an increased production of circulating inflammatory cytokines and greater T cell differentiation toward Th1 and Th17 with an increase in the M1 Mϕ population as well.[22,23] Therefore, it is not unexpected that autoimmune disease would be aggravated in an obese model. Our observations confirm that another Ag-specific autoimmune disease can be added to the list of autoimmune diseases that are exacerbated by obesity, likely due to the systemic inflammatory response that coincides with obesity and an HFD.

There have been reports that diabetic retinopathy can exacerbate uveitis and uveitis can accelerate diabetic retinopathy.[24–32] While the HFD model we used only achieved moderate diabetes, it did exacerbate the uveitis. A small case study reported an increased incidence of uveitis in Behcet's Disease patients with metabolic syndrome.[32] Because obesity is one of several criteria of metabolic syndrome, this suggests a clinical correlation between obesity and uveitis, but requires a much larger epidemiological study to confirm. Therefore, an additional larger case study is necessary to confirm our mouse findings in human patients, and these findings are exciting because they could provide the basis for a novel research area.

It has not been previously investigated how obesity affects different aspects of immune privilege in tissues such as the brain and eye. One aspect of immune privilege that has been demonstrated in both the eye and brain is the induction of regulatory immunity that functions to prevent a memory response to that tissue.[16–18,20] Because when EAU-recovered mice that have this regulatory immunity are re-immunized for EAU, the uveitic response is that of a naïve response, the purpose of this systemic regulatory immunity is to prevent relapse. Since it is thought that post-EAU systemic regulatory immunity provides protection from relapse, if the increased white fat in obese individuals contributes to systemic inflammation that inhibits induction of this regulatory immunity, then obesity may contribute to chronic autoimmune disease, specifically of immune-privileged tissues such as the eye. As such, obesity and/or an imbalanced HFD could be a contributing factor in the failure of immunosuppressive therapies.

Another interesting observation is that we see an increased severity of symptoms in the extreme HFD compared with the moderate HFD. This suggests there may be a resistance threshold that is broken with a much higher fat diet and illustrates the potency of ocular

immune privilege. We also observed the transfer of post-EAU splenocytes from HFD mice on 45% and 60% HFD had no regulatory immunity that provided resistance to EAU in recipient mice, but the mice that received 60% HFD post-EAU splenocytes did not have significantly more severe disease. This is likely because there are additional mechanisms of ocular immune privilege that help to minimize the amount of EAU. The implications of this observation is that if there is a dietary contribution to autoimmune disease, then a moderate reduction of fat consumption may be effective rather than making drastic dietary changes.

This report demonstrates a role for the melanocortin 5 receptor (MC5r) in obesity and exacerbation of autoimmune uveitis by HFD. Albeit, the role is unexpected because it has been previously demonstrated to be anti-inflammatory,[16–18] but our observations show that it is necessary for the exacerbation of EAU by HFD. The majority of previous work with MC5r has focused on the immunological role. Whereas, the majority of the focus with respect to metabolism has been on the melanocortin 4 receptor[33]. However, recent studies have demonstrated MC5r is expressed on adipose tissue, it has a role in lipid metabolism, and polymorphisms are associated with obesity.[11,34,35] These previous reports and our observations indicate a role for MC5r in the exacerbation of EAU by HFD. Despite this observation, it still remains to be shown if the effect of HFD on EAU through MC5r is due to its immunological role, metabolic role, or both.

A skewed Th1 profile with a reduction in Ly-6G^{+} Mϕs was observed in HFD EAU mice at the onset of EAU and requires further investigation to determine if this is due to the course of EAU or if it is caused by the HFD. The observation that Th1 cells are increased at the onset of EAU in MC5r$^{(-/-)}$ mice suggests a greater inflammatory state compared to WT mice that may explain the compensatory weight gain and glucose control that we observed. It may be the case that the regulatory effect exerted through MC5r may suppress vascularization of the adipose tissue. Therefore, adipose tissue expansion in MC5r$^{(-/-)}$ mice may be less likely to experience hypoxia and produce additional inflammatory adipokines. Further studies are necessary to explore this hypothesis.

In summary, this is the first report that an HFD aggravates autoimmune uveitis, prevents the induction of systemic regulatory immunity, and the melanocortin 5 receptor has a role in the aggravation of autoimmune uveitis. As such, these observations support the need for a large epidemiological study to investigate a role for obesity with autoimmune uveitis that may be due in part with the melanocortin 5 receptor.

4 | METHODS

4.1 | Mice

All mouse procedures described in this study were approved by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee (OUHSC IACUC) and all mouse study methods were carried out in accordance with the relevant guidelines approved by the OUHSC IACUC. C57BL/6J mice were purchased from Jackson Laboratories. MC5r$^{(-/-)}$ were provided by Dr. Andrew Taylor (Boston University School of Medicine) who obtained them from Dr. Robert Cone (Oregon Health Sciences Center), these mice were bred at OUHSC.

4.2 | Experimental Autoimmune Uveoretinitis

Mice were immunized for EAU as previously described.[20] Briefly, an emulsion of complete Freund's adjuvant (CFA) with 5 mg/ml desiccated *M. tuberculosis* (Difco Laboratories, Detroit, MI) and 2 mg/ml interphotoreceptor retinoid binding protein (peptides 1-20) (IRBP) (Genscript, Piscataway, NJ) was used to immunize mice for EAU. A volume of 100 μl of the emulsion was injected subcutaneously at 2 sites in the lower back followed by an intraperitoneal injection of 0.3 μg pertussis toxin. The severity of 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, the cornea was numbed with 0.5% proparacaine, and the cornea was flattened with a glass coverslip in order 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.[36] Both eyes were scored and the higher score was used to represent that mouse for that day, the average score for the group of mice was then calculated.

4.3 | HFD administration

Mice were placed on the indicated HFD (Research Diets, New Brunswick, NJ) that contained either 45% or 60% calories from fat. The normal fat diet had a comparable nutritional content with the HFDs but consisted of 13% calories from fat (Lab Diet, St. Louis, MO). All diets are based on the AIN-76A semi-purified diet.[37] Mice were fed ad libitum from 5 weeks of age as indicated. Food was replaced weekly at which time the remaining food from the previous week was weighed to determine the amount consumed. Mice were weighed each week, and the percentage increase compared to the initial weight was calculated. Blood glucose was measured following a 16 h fast.

4.4 | In vitro stimulation

Spleens were collected into 5% FBS in RPMI supplemented with 10 μg/ml Gentamycin (Sigma), 10 mM HEPES, 1 mM sodium pyruvate (BioWhittaker), nonessential amino acids 0.2% (BioWhittaker), and made into a single cell suspension that was depleted of red blood cells using RBC lysis buffer (Sigma, St Louis, MO). The spleen cells were resuspended in serum free media (SFM) and IRBP residues 1-20 (IRBP) was added at 50 μg/ml for 48 h at 37°C and 5% CO_2 to reactivate Ag-specific T cells. SFM consisted of RPMI-1640 with 1% ITS+1 solution (Sigma) and 0.1% BSA (Sigma). Following the reactivation, supernatants were collected and analyzed and/or cells were collected for adoptive transfer into recipient mice.

4.5 | Flow cytometry

Mouse spleen cells were washed with PBS with 1% BSA (staining buffer), blocked with mouse IgG in staining buffer, then stained with conjugated Abs. Abs used were anti-CD11b (clone M1/70, Biolegend, San Diego, CA), anti-Ly-6C (clone HK1.4, Biolegend), anti-Ly-6G (clone 1A8, Biolegend), F4/80 (clone BM8, eBioscience, San Diego, CA), anti-T-Bet (clone 4B10, Biolegend), anti-RORγt (clone AFDKS-9, eBioscience), anti-CD4 (clone RM4-5, Biolegend), and anti-CD25 (clone PC61, Biolegend). Intracellular Ab staining was done with eBioscience Intracellular Fixation and Permeabilization Buffer set (eBioscience).

Stained cells were analyzed in the Oklahoma Medical Research Facility (OMRF) Flow Cytometry Core Facility on a BD LSRII (BD Biosciences) and data were analyzed using FlowJo Software (Tree Star, Inc., Ashland, OR).

4.6 | Statistics

Statistical significance between max EAU scores was determined using nonparametric Mann-Whitney U test between groups of mice. Two-way ANOVA was used to assess significant changes in the tempo of disease between the groups of EAU mice with Bonferroni posttest. Normally distributed groups were assessed by Student's unpaired t-test to determine statistical significance. GraphPad Prism software was used for all statistical analyses. Statistical significance was determined when $P \leq 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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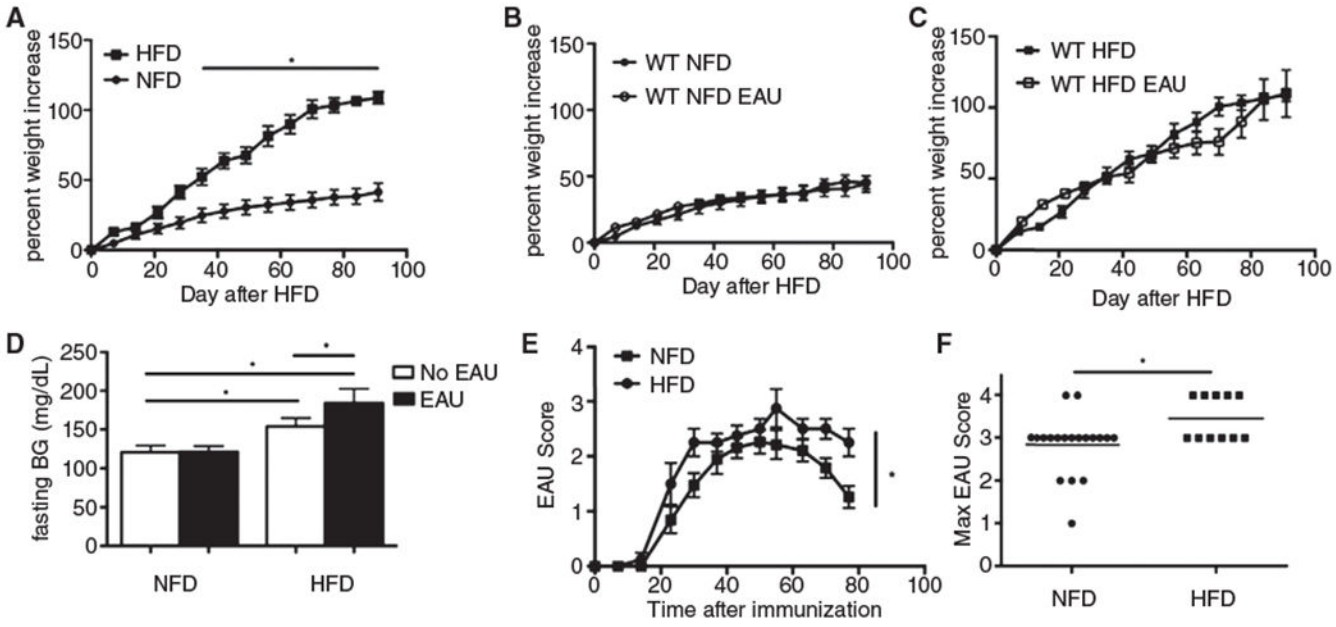

FIGURE 1. EAU is exacerbated by 60% HFD.

HFD mice received food containing 60% of the calories from fat at 5 weeks of age. The mice were weighed every 5–7 days and the average and SEM percent of weight increase is shown. Weight gain of WT mice on NFD (filled circle, solid line, $n = 14$) and HFD (filled square, dashed line, $n = 8$; **A**). On day 15 after the 60% HFD was started, a group of NFD or HFD mice were immunized for EAU. Weight gain of NFD EAU mice (filled circle, solid line, $n = 15$; **B**) or HFD EAU mice (filled square, solid line, $n = 11$; **C**). In order to facilitate better comparison the WT mice on NFD or HFD from panel (A) are shown (filled circle, solid line) and the HFD mice are shown (filled square, solid line). Mice were fasted for 16 h and blood glucose (BG) was measured, the mean and SEM is shown for fasting blood glucose at week 14 after HFD was started for NFD mice (No EAU $n = 8$, EAU $n = 10$) and HFD mice (No EAU $n = 8$, EAU $n = 10$; **D**). The clinical EAU score was assessed by slit lamp examination of the retina every 3-4 days for NFD ($n = 19$) and HFD mice ($n = 11$). The highest score for each mouse on the indicated day was averaged with all the mice on that day and graphed with the SEM (**E**). The highest EAU score for each mouse over the course of EAU was also determined (**F**). Each experiment was repeated three times with 2-5 mice in each experiment, so the indicated n is the total mice pooled from all the experiments. The "*" indicates $P \leq 0.05$ determined by 2-way ANOVA with Bonferroni posttest to determine significance of EAU course of disease or Mann-Whitney nonparametric 2-tailed test for comparison of maximum EAU scores or unpaired Student's *t*-test for comparison of BG

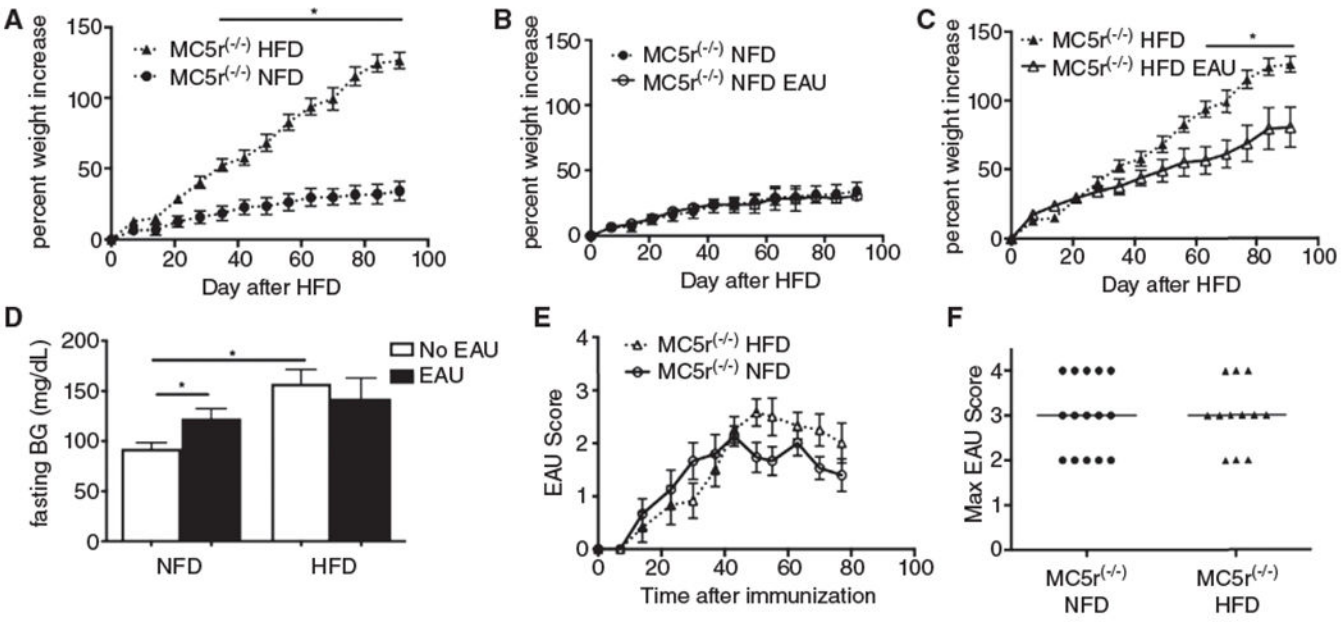

FIGURE 2. MC5r$^{(-/-)}$ mice have normal EAU during 60% HFD.

Percent weight gain of MC5r$^{(-/-)}$ mice on an NFD (n = 10) or 60% HFD (n = 8; **A**). MC5r$^{(-/-)}$ mice were immunized for EAU at 7 weeks of age, 2 weeks after the 60% HFD group were switched to the HFD. Percent weight gain of NFD EAU mice (open circles, solid line, n = 15; **B**) or HFD EAU mice (open triangle, solid line, n = 12) on HFD (**C**). In order to facilitate better comparison the MC5r$^{(-/-)}$ mice on NFD or HFD from panel (**A**) are shown. Mice were fasted for 16 h and glucose was measured, the mean and SEM is shown for fasting blood glucose (BG) at week 12 after EAU was induced in NFD mice (No EAU n = 10, EAU n = 8) and HFD mice (No EAU n = 15, EAU n = 12) (**D**). The clinical EAU score was assessed by slit lamp examination of the retina every 3-4 days. The highest score for each mouse on NFD (n = 15) or HFD (n = 12) on the indicated day was averaged with all the mice on that day and graphed with the SEM (**E**). The highest EAU score for each mouse over the course of EAU was also determined (**F**). Each experiment was repeated three times with 2-5 mice in each experiment, so the indicated n is the total mice pooled from all the experiments. The "*" indicates $P \leq 0.05$ determined by 2-way ANOVA with Bonferroni posttest to determine significance of EAU course of disease or Mann-Whitney nonparametric 2-tailed test for comparison of maximum EAU scores or unpaired Student's t-test for comparison of BG

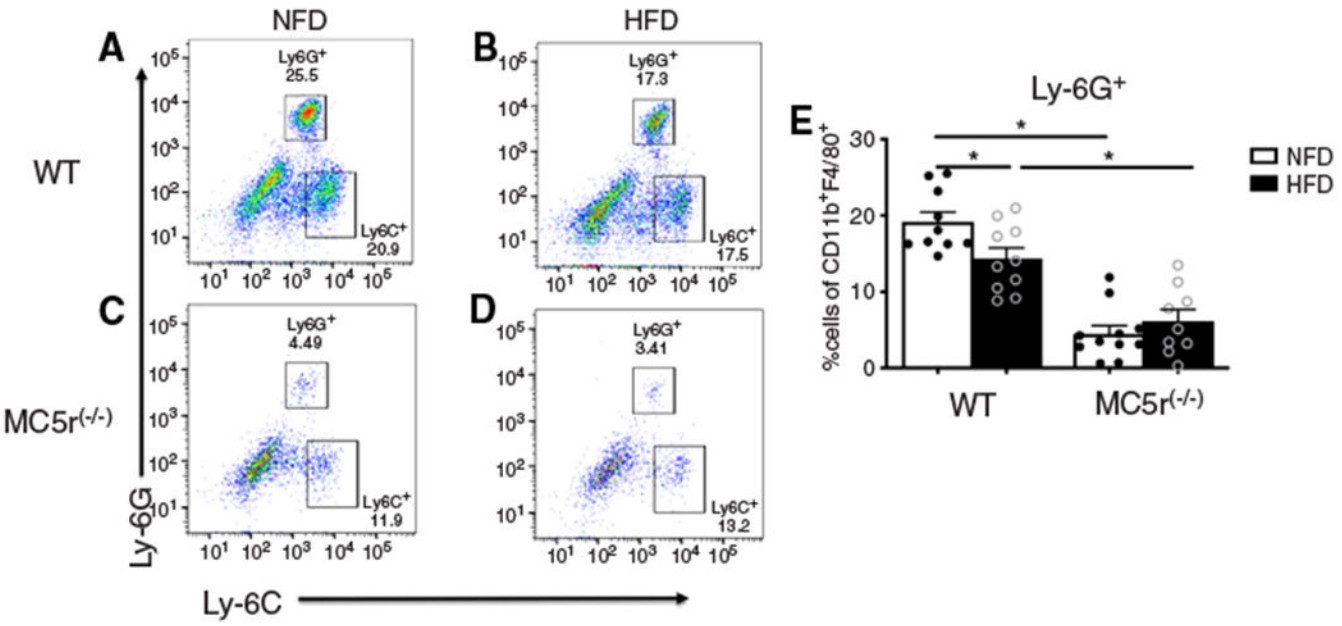

FIGURE 3. Ly-6G$^+$ APC are suppressed during 60% HFD at the onset of EAU.

WT (**A** and **B**) and MC5r$^{(-/-)}$ (**C** and **D**) mice on a NFD or 60% HFD were immunized for EAU at 7 weeks of age, 2 weeks after the 60% HFD group were switched to the HFD. Four weeks after EAU was induced splenocytes were stained for CD11b+ F4/80, Ly-6C, and Ly-6G. Dot plots shown are gated on CD11b$^+$ F4/80$^+$ cells with Ly-6C and Ly-6G expression, and are representative of 4-5 mice per experiment from 2 to 3 experiments. The mean and SEM of Ly-6G$^+$ cells for all mice is shown (**E**). The "*" indicates $P \leq 0.05$ determined by unpaired Student's *t*-test

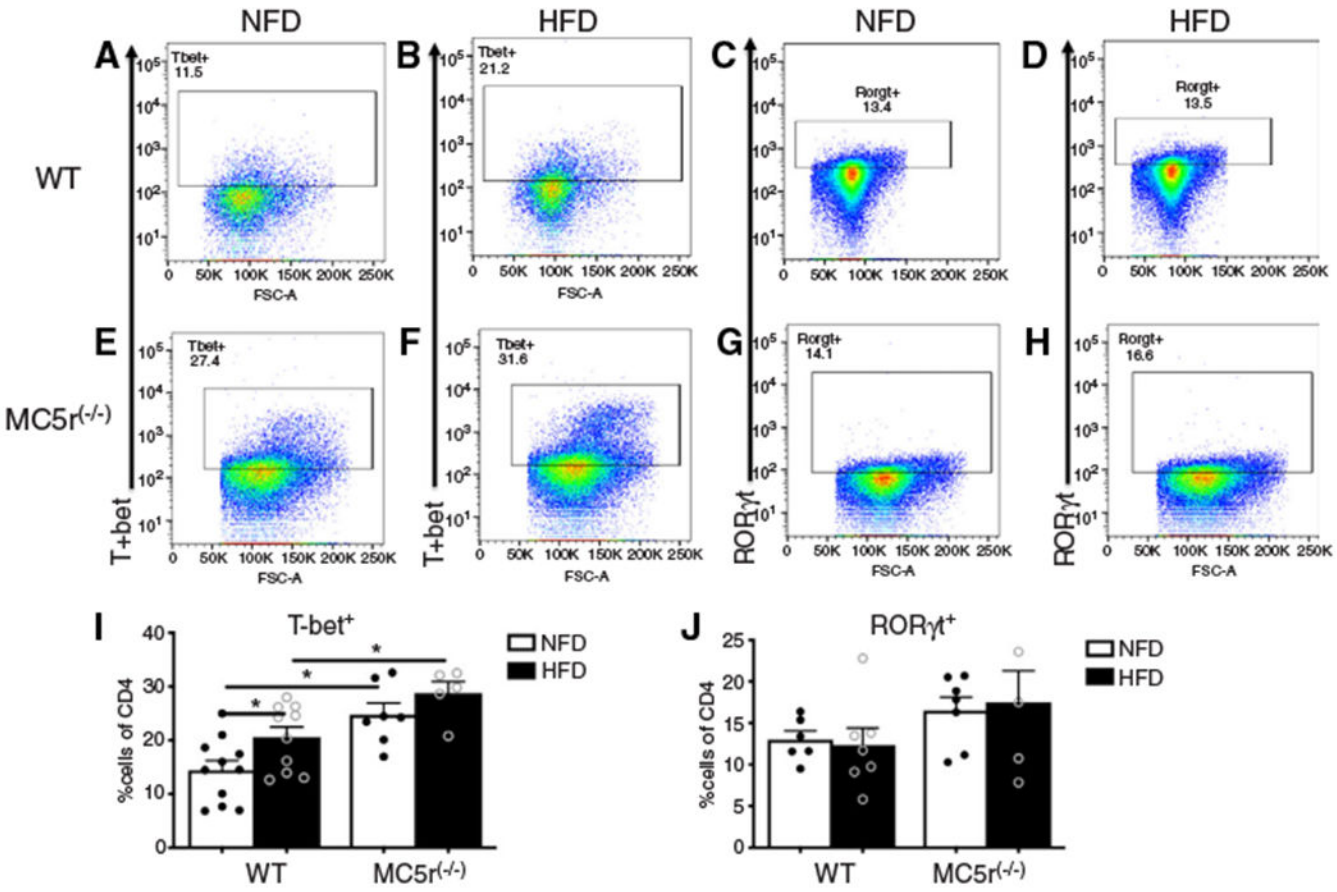

FIGURE 4. Th1 cells are increased during 60% HFD at the onset of EAU.

WT (**A–D**) and MC5r$^{(-/-)}$ (**E–H**) mice on a NFD or 60% HFD were immunized for EAU at 7 weeks of age, 2 weeks after the 60% HFD group were switched to the HFD. Four weeks after EAU was induced splenocytes were stained for CD4, CD25, T-bet, and RORgt. Dot plots shown are gated on CD4$^+$ CD25$^+$ cells with T-bet or RORgt expression and FSC, and are representative of 2-5 mice per experiment from 2 to 3 experiments. The mean and SEM of T-bet$^+$ or RORgt$^+$ cells for all mice is shown (**I** and **J**). The "*" indicates $P \leq 0.05$ determined by unpaired Student's *t*-test

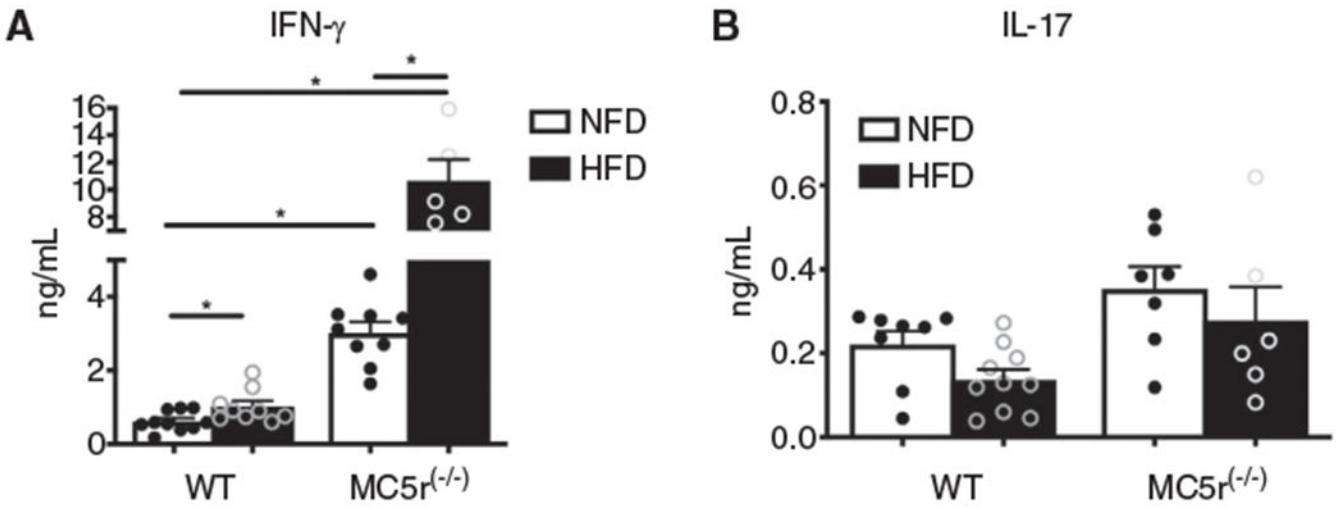

FIGURE 5. IFN-γ but not IL-17 production Is increased during 60% HFD at the onset of EAU.
WT and MC5r$^{(-/-)}$ mice on a NFD or 60% HFD were immunized for EAU at 7 weeks of age, 2 weeks after the 60% HFD group were switched to the HFD. Four weeks after EAU was induced, splenocytes were cultured with IRBP for 2 days. Culture supernatants were collected and IFN-γ (**A**) and IL-17 (**B**) was measured. The mean and SEM is shown for 2-5 mice per experiment from 2 to 3 experiments. The "*" indicates $P \leq 0.05$ determined by unpaired Student's *t*-test

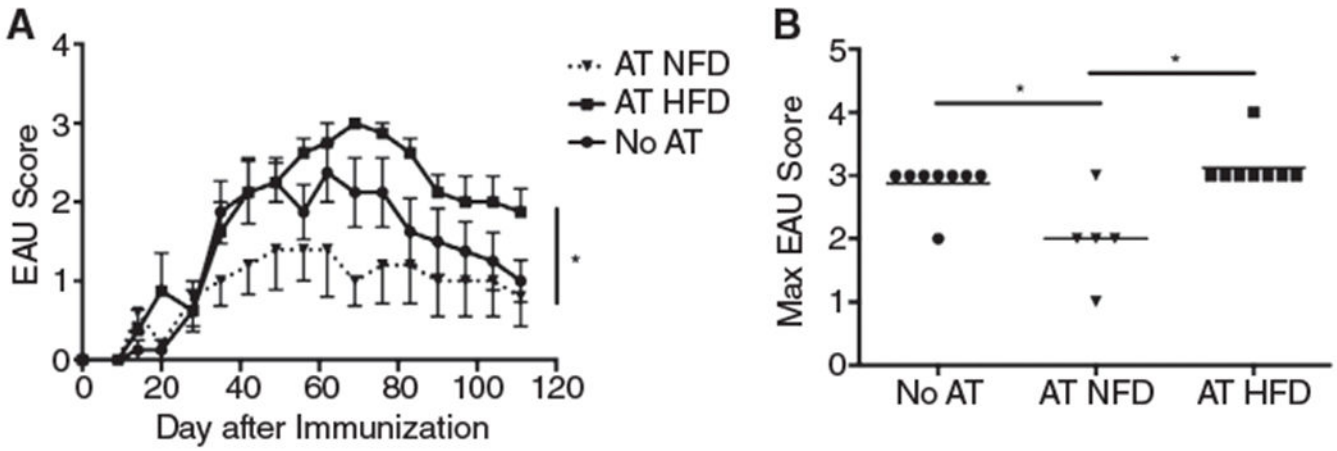

FIGURE 6. Post-EAU regulatory Immunity Is absent In the spleen of mice on 45% HFD.

At recovery of EAU the spleens from NFD or 45% HFD mice were collected and reactivated in vitro with IRBP for 48 h. Following the reactivation, 1×10^6 spleen cells were transferred IV to recipient WT mice immunized for EAU. The course of disease with mean and SEM is shown (**A**). The closed circle is the average for mice that were immunized for EAU but did not receive an adoptive transfer (AT) of spleen cells (No AT, $n = 8$), the triangle with the dashed line are mice that received spleen cells from NFD post-EAU mice (AT NFD, $n = 5$), and the square are mice that received spleen cells from post-EAU 45% HFD mice ($n = 8$). The highest EAU score for each mouse over the course of EAU was also determined (**B**). Each experiment was repeated 2 times with 2-5 mice in each experiment, so the indicated n is the total mice pooled from all the experiments. The "*" indicates $P \leq 0.05$ determined by 2-way ANOVA with Bonferroni posttest to determine significance of EAU course of disease between the AT NFD group and AT HFD group or Mann-Whitney nonparametric 2-tailed test to determine max EAU score significance

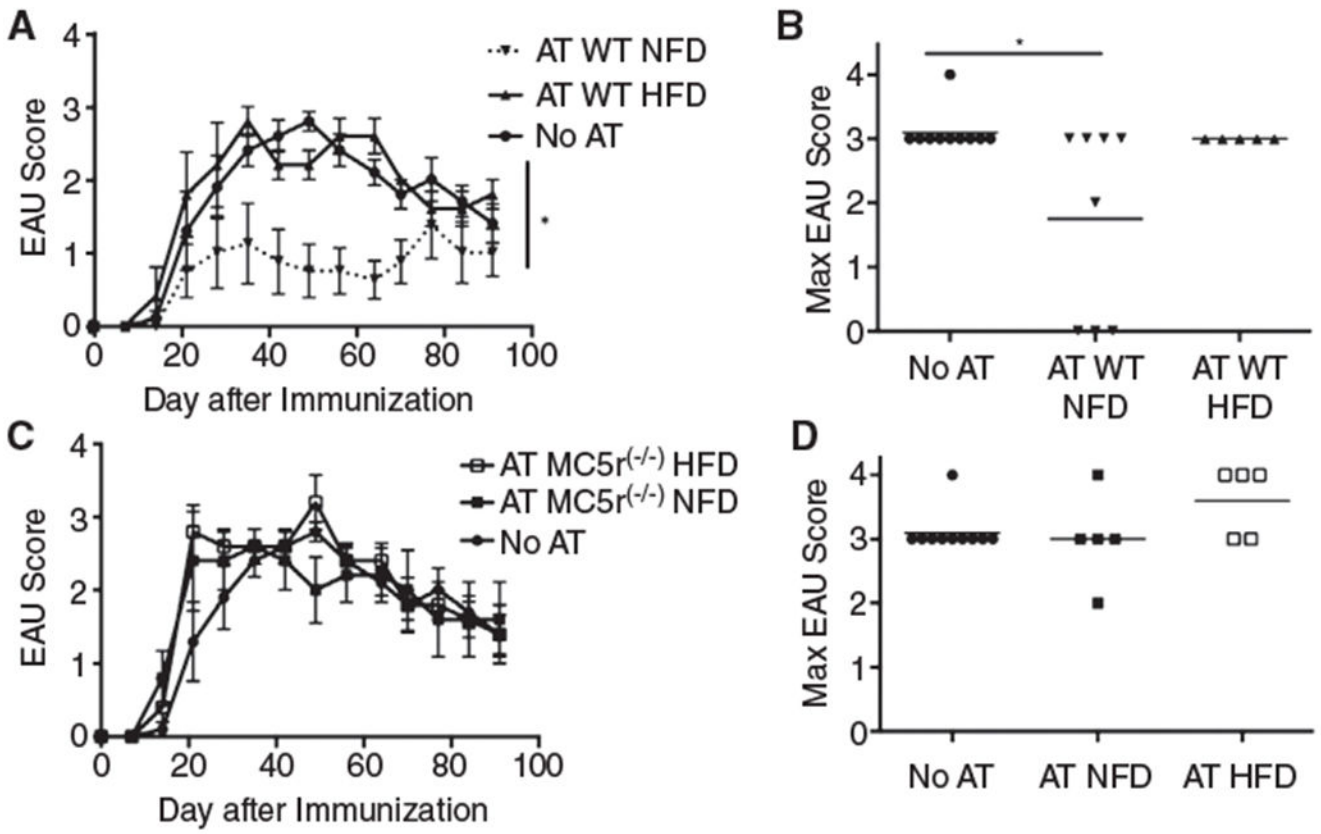

FIGURE 7. Post-EAU regulatory immunity is absent in the spleen of mice on 60% HFD.

At recovery of EAU, the spleens from WT or MC5r$^{(-/-)}$ on NFD or 60% HFD diet were collected and reactivated in vitro with IRBP for 48 h. Following the reactivation, 1×10^6 spleen cells were transferred to recipient mice immunized for EAU. The course of disease with mean and SEM is shown for mice that received cells from WT mice (**A**). The closed circle is the average for mice that were immunized for EAU but did not receive an adoptive transfer (AT) of spleen cells (No AT, $n = 10$), the inverted triangle with the dashed line are mice that received spleen cells from NFD post-EAU mice (AT NFD, $n = 9$), and the triangle with the solid line are mice that received spleen cells from post-EAU 60% HFD mice ($n = 5$). The highest EAU score for each mouse over the course of EAU was also determined (**B**). EAU scores of mice that received cells from MC5r$^{(-/-)}$ mice are shown (**C**). The filled square are mice that received cells from NFD mice ($n = 5$) and open squares are mice that received cells from HFD mice. The maximum EAU scores are shown (**D**). Each group was repeated two times with 2-5 mice in each experiment, so the indicated n is the total mice pooled from all the experiments. The "*" indicates $P \leq 0.05$ determined by 2-way ANOVA with Bonferroni posttest to determine significance of EAU course of disease between the AT NFD group and AT HFD group or Mann-Whitney nonparametric 2-tailed test to determine Max EAU Score significance