Published in final edited form as:

Ann Neurol. 2014 January ; 75(1): 50–66. doi:10.1002/ana.24020.

Sex-specific control of CNS autoimmunity by p38 MAPK signaling in myeloid cells

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Abstract

Objective—Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), characterized by a global increasing incidence driven by relapsing-remitting disease in females. p38 MAP kinase (MAPK) has been described as a key regulator of inflammatory responses in autoimmunity, but its role in the sexual dimorphism in MS or MS models remains unexplored.

Methods—Toward this end, we used experimental autoimmune encephalomyelitis (EAE), the principal animal model of MS, combined with pharmacologic and genetic inhibition of p38 MAPK activity and transcriptomic analyses.

Results—Pharmacologic inhibition of p38 MAPK selectively ameliorated EAE in female mice. Conditional deletion studies demonstrated that p38 α signaling in macrophages/myeloid cells, but not T cells or dendritic cells, recapitulated this sexual dimorphism. Analysis of CNS inflammatory infiltrates showed that female, but not male mice lacking p38 α in myeloid cells exhibited reduced immune cell activation compared with controls, while peripheral T cell priming was unaffected in both sexes. Transcriptomic analyses of myeloid cells revealed differences in p38 α -controlled transcripts comprising female- and male-specific gene modules, with greater p38 α dependence of pro-inflammatory gene expression in females.

Interpretation—Our findings demonstrate a key role for p38 α in myeloid cells in CNS autoimmunity and uncover important molecular mechanisms underlying sex differences in disease pathogenesis. Taken together, our results suggest that the p38 MAPK signaling pathway represents a novel target for much needed disease modifying therapies for MS.

Introduction

Multiple sclerosis (MS), the most common disabling neurologic disease of young adults, is considered a classical T cell-mediated disease and is characterized by demyelination, axonal

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Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

damage, and progressive neurological dysfunction^{1, 2}. Recent genetic studies further confirmed the role of cell-mediated immunity in MS, with an emphasis on T helper cell function³. Despite these insights, the etiopathogenesis of this devastating disease is poorly understood and current disease-modifying therapies (DMTs) have limited efficacy. Importantly, like many other autoimmune diseases, MS is characterized by a female bias. Epidemiological studies have demonstrated a significant increase in the incidence of relapsing-remitting MS in females over the last 50 years⁴. This rate of change is suggestive of environmental factors acting specifically in females at the population level. Despite the fact that such sexual dimorphisms in autoimmunity are well-documented, the mechanistic knowledge for the development of sex-specific DMTs is lacking.

The p38 mitogen-activated kinase (MAPK) pathway plays a prominent role in innate and adaptive immunity⁵. p38 MAPK was identified as the target of a series of small molecules that inhibited toll-like receptor (TLR)-induced inflammatory cytokine production by macrophages⁶. As a key regulator of pro-inflammatory cytokine production, this molecule was expected to be a promising drug target in autoimmune inflammatory disorders where these cytokines were overproduced. Indeed, animal studies have shown efficacy of p38 MAPK inhibitors in models of rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and type 1 diabetes (T1D)⁷⁻⁹, although these compounds have not yet had success in the clinic^{10, 11}. Until recently, this pathway has not been evaluated in MS or its models, despite the fact that MS shares many etiopathogenic features with these autoimmune diseases, such as activation of self-reactive T cells and augmented production of proinflammatory cytokines by innate cells¹².

Early evidence for the involvement of p38 MAPK in autoimmune neuroinflammation came from studies showing increased phosphorylation of this kinase in inflammatory cells and glia in the central nervous system (CNS) during the course of experimental autoimmune encephalomyelitis (EAE), the principal model of MS¹³. Moreover, mRNA for *MAPK14* (encoding p38 α) was found to be overexpressed in CNS lesions of MS patients¹⁴. Subsequently, several recent studies have documented a functional requirement for p38 MAPK signaling in EAE progression. Treatment with pharmacological inhibitors of p38 MAPK inhibited clinical signs of EAE, which correlated with inhibition of pathogenic IL-17 producing T helper cell (Th17) responses¹⁵⁻¹⁷. Genetic inhibition of p38 α , the predominant p38 MAPK isoform in immune cells, also potently ameliorated EAE, suggesting that p38 α is the primary target underlying pharmacologic inhibition of disease^{17, 18}. EAE severity was also reduced by inhibition of p38 MAPK signaling specifically in T cells, either by expression of dominant negative p38 transgene in T cells, or by the mutation of a residue required for T cell-specific activation of p38 α / β ^{16, 19}. Accordingly, augmentation of p38 MAPK signaling by expression of a constitutively active MKK6 transgene in T cells enhanced EAE severity¹⁶. In contrast, Huang *et al* showed that genetic ablation of p38 α in dendritic cells (DCs), but not in T cells or macrophages, inhibited EAE and led to impaired Th17 responses¹⁸. Moreover, deletion of apoptosis signal-regulating kinase 1 (ASK1), a TLR-controlled kinase that is known to activate p38 MAPK, attenuated p38 MAPK activation, EAE severity, and production of pro-inflammatory cytokines by astrocytes and microglia, without affecting peripheral T cell responses, suggesting that ASK1-dependent activation of p38 MAPK in glial cells may promote EAE pathogenesis²⁰. Here, we show sex-specific effects of p38 MAPK signaling in myeloid cells in EAE pathogenesis, suggesting that this signaling pathway may yield novel sex- and cell type-specific targets for treatment of MS.

Materials and Methods

Mice

Lysm-Cre mice (B6.129P2-Lyz2tm1(cre)Ifo/J)²¹, Cd11c-Cre mice (B6.Cg-Tg(Itgax-cre)1-1Reiz/J)²², p38 α floxed mice (Mapk14^{tm1.2Otsu})²³ have been described previously and were obtained from Jackson Laboratories (USA) or RIKEN BioResource Center (Japan). Lck-Cre mice (B6.Cg-Tg(Lck-cre)1Cwi N9)²⁴ were obtained from Taconic (USA). Wild type C57BL/6J mice were purchased from Jackson Laboratories (USA) and were rested at the animal facility at UVM for at least 2 weeks prior to any experimentation. The experimental procedures used in this study were approved by the Animal Care and Use Committee of the University of Vermont.

EAE induction and scoring

EAE was induced essentially as described previously²⁵. Briefly, mice were injected s.c. with an emulsion containing 100 μ g of MOG₃₅₋₅₅ peptide (MEVGWYRSPFSRVVHLYRNGK) (Anaspec, USA) and complete Freund's adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO) supplemented with 200 μ g of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) in the posterior right and left flanks. One week later all mice were similarly injected at two sites on the right and left flank anterior of the initial injection sites (2 $\times$ MOG₃₅₋₅₅/CFA). Alternatively, mice were immunized with 200 μ g of MOG₃₅₋₅₅ in CFA, followed by i.v. administration of 200 ng pertussis toxin (List Biological) (1 $\times$ MOG₃₅₋₅₅/CFA/PTX). In some experiments (as indicated), mice received 5 mg/kg/day of SB203580 dihydrochloride (Tocris, Ellisville, MO) by i.p. injection in a total volume of 200 μ l or an equal volume of carrier daily starting on the day of immunization. Mice were scored daily starting at day 10 post-injection as previously described²⁵. Passive EAE was induced as follows. WT male and female mice were immunized with 2 $\times$ MOG₃₅₋₅₅/CFA. On day 10, effector cells from LN and spleen were harvested and restimulated *ex vivo* with 10 μ g/ml MOG₃₅₋₅₅ and 0.5 ng/ml IL-12 for 72 hr. 20 $\times$ 10⁶ sex-matched effector cells were transferred to female and male recipients.

Cytokine quantification

For the detection of cytokines in the cell culture supernatants, ELISAs were performed as described previously²⁵, using the primary capture mAbs: anti-IFN γ , anti-IL-17A, anti-TNF α , and anti-IL-6 and their corresponding biotinylated detection mAbs (BD Pharmingen, San Diego, CA). Other ELISA reagents included: HRP-conjugated avidin D (Vector Laboratories, Burlingame, CA), TMB microwell peroxidase substrate and stop solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD). rIFN γ , rIL-17A, rGM-CSF (Biolegend, USA), and rTNF α and rIL-6 (BD Pharmingen, San Diego, CA) were used as standards.

For analysis of antigen-specific cytokine production by lymphocytes from mice immunized with 2 $\times$ MOG₃₅₋₅₅/CFA, spleen and draining lymph nodes (DLN) were harvested on day 10 post-immunization, single cell suspensions were prepared at 1 $\times$ 10⁶ cells/ml in RPMI medium with 5% FBS, and stimulated with 50 μ g/ml of MOG₃₅₋₅₅. Cell culture supernatants were collected at 72 hours and cytokine levels were measured by ELISA as described above.

Isolation and stimulation of thioglycolate-elicited macrophages

Mice were immunized using the 2 $\times$ MOG₃₅₋₅₅/CFA protocol. On day 6 post-immunization, mice were injected with 1ml of a 4% solution of thioglycolate broth (Sigma-Aldrich, USA) i.p. 96 hours later mice were sacrificed and the peritoneal cavity was flushed with 15 ml of cold PBS. Cells were washed and cultured overnight in RPMI + 5% FBS, then washed to

remove non-adherent cells. The remaining adherent cells were stimulated with purified LPS (Sigma) or heat-killed *Mycobacterium tuberculosis* H37Ra (Difco, USA).

Flow cytometry

For intracellular cytokine staining *ex vivo*, mice were immunized with 2×MOG₃₅₋₅₅/CFA, spleen and DLN were harvested on day 10 post-immunization, and cells were stimulated with 5 ng/ml of PMA, 250 ng/ml of ionomycin (Sigma-Aldrich) and Golgi Plug reagent (BD Biosciences) for 4 hours. Cells were then stained with the LIVE/DEAD fixable stain (Invitrogen) and then surface stained for the following markers: CD11b, CD4, CD8, TCRγδ, and TCRβ. Cells were then fixed with 1% paraformaldehyde (Sigma-Aldrich), permeabilized with buffer containing 0.2% saponin and stained with anti-IL-17A, anti-IFNγ, and anti-GM-CSF (Biolegend).

For surface marker analysis, unstimulated isolated cells were stained directly *ex vivo* with the LIVE/DEAD fixable stain (Invitrogen) and then surface labeled for different combinations of following markers: CD11b, CD11c, MHCII, CD80, CD86, Ly6C, Ly6G, MHCII, CD4, CD8, TCRγδ, and TCRβ (Biolegend, USA) and fixed with 1% paraformaldehyde. All antibodies used for flow cytometry were directly conjugated to fluorophores.

Cells were analyzed using an LSR II cytometer (BD Biosciences). Compensation was calculated using appropriate single color controls. Data were analyzed using FlowJo software (Tree Star Inc, Ashland, OR).

CNS-infiltrating mononuclear cell isolation

Animals were perfused with saline and brains and spinal cords were removed. A single cell suspension was obtained and passed through a 70 μm strainer. Mononuclear cells were obtained by Percoll gradient (37%/70%) centrifugation and collected from the interphase. For mRNA analysis, cells were lysed and total RNA was isolated using the RNEasy kit (Qiagen). For intracellular cytokine analysis, cells were washed and stimulated with 50 μg/ml of MOG₃₅₋₅₅ for 4 hours in the presence of Golgi Plug reagent (BD Bioscience). Cells were labeled with LIVE/DEAD UV-Blue dye (Invitrogen) followed by surface staining (anti-CD45 from Invitrogen and anti- CD11b, CD11c, Ly6C, Ly6G, MHCII, CD4, CD8, TCRγδ, and TCRβ from Biolegend). Afterwards, cells were fixed, permeabilized and stained for intracellular IL-17A, IFNγ, and GM-CSF (Biolegend) as described above. For surface marker analysis, unstimulated isolated cells were stained directly *ex vivo* for the following markers: CD45, CD11b, CD11c, MHCII, CD80, CD86, Ly6C, Ly6G, MHCII, CD4, CD8, TCRγδ, and TCRβ.

Cell lysates and immunoblot analysis

Whole-cell lysates were prepared by lysing adherent macrophages directly in Triton lysis buffer, separated by SDS-PAGE, and transferred to PVDF membranes as described previously²⁵. Primary antibodies used for western blot analysis included anti-phospho-p38, anti-p38α, and anti-GAPDH (Cell Signaling Technologies, Danvers, MA). Anti-mouse and anti-rabbit secondary antibodies were conjugated to DyLight680 and DyLight800, respectively (Jackson ImmunoResearch Laboratories, West Grove, PA). Membranes were imaged using fluorescent detection on the Odyssey CLx instrument (Li-Cor Biosciences, USA), and images were processed using the Image Studio program (Li-Cor Biosciences, USA).

RNA isolation and quantitative real-time PCR (qRT-PCR)

RNA was extracted using the RNEasy kit (Invitrogen) according to manufacturer's instructions. cDNA was reverse transcribed using the Taqman Gold RT-PCR kit using the oligo-dT method (Applied Biosciences, USA). qRT-PCR was performed using the DyNAmo Colorflash SYBR green qPCR kit (Thermofisher) and the following primer sets: *Il10*, GAAGCTGAAGACCCTCAGGA and TTTTCACAGGGGAGAAATCG; *Tnfa*, GAACTGGCAGAAGAGGCACT and AGGGTCTGGGCCATAGAACT; *Il6*, CCGGAGAGGAGACTTCACAG and GAGCATTGGAAATTGGGGTA; *Il1b*, AGGCCACAGGTATTTTGTCTG and GCCCATCCTCTGTGACTCAT; *B2m*, CATGGCTCGCTCGGTGACC and AATGTGAGGCGGGTGGAAGT. *B2m* was used as a reference gene and relative mRNA levels were calculated using the comparative delta-delta C_T method, normalizing first by the expression of the reference gene, then normalizing to the mean WT female expression level for each gene of interest.

Microarray sample preparation and hybridization

RNA was isolated using the RNEasy kit (Qiagen) according to manufacturer's instructions. Microarray was performed on 3 biological replicates for each condition (e.g. female KO). To create each replicate, equal amounts of RNA from 2–3 different mice were pooled.

An RNA input of 25ng was used to generate cDNA through the First Strand and Second Strand synthesis reactions of the Ovation[®] Pico WTA System V2 from NuGEN. The cDNA samples were then purified using an Agencourt[®] RNAClean[®] XP magnetic bead protocol. Following purification, samples were amplified using SPIA reagents from the Ovation[®] Pico WTA System V2 from NuGEN. A final cDNA purification is performed using an Agencourt[®] RNAClean[®] XP magnetic bead protocol. Sample concentrations were determined using a 33ug/mL/A260 constant on a Nanodrop 1000 Spectrophotometer. Approximately 4ug of cDNA generated using the Ovation[®] Pico WTA System V2 was fragmented and labeled using the Encore[®] Biotin Module from NuGEN. Efficiency of the biotin labeling reaction was verified using NeutrAvidin (10mg/mL) with a gel-shift assay. Samples were injected into arrays and placed in the Affymetrix Genechip[®] Hybridization Oven 640 at 45° C and 60 RPM for 16–18 hours overnight. Arrays were stained using the Affymetrix Genechip[®] Fluidics Station 450 and scanned with the Affymetrix Genechip[®] Scanner 3000. Mouse Gene 2.0 ST arrays were used (Affymetrix).

Microarray analysis - calculation of probe set statistics

Raw GeneChip data (one DAT file for each chip) includes a collection of images, one for each probe and chip. Each image was summarized by Affymetrix GCOS software using one probe intensity (in CEL files, one per chip). Information from multiple probes can be combined to obtain a single measure of expression for each probe set and sample. Probe-level intensities were calculated using the Robust Multichip Average (RMA) algorithm, including background-correction, normalization (quantile), and summarization (median polish), for each probe set and sample, as is implemented in Partek Genomic Suites[®], version 6.6 (Copyright © 2009, Partek Inc., St. Louis, MO, USA). Sample quality was assessed based on the 3':5' ratio (3' arrays only), relative log expression (RLE), and normalized unscaled standard error (NUSE).

Microarray analysis - identification of differential expression and alternative splicing

Univariate linear modeling of sample groups is performed using ANOVA as implemented in Partek Genomic Suites. The magnitude of the response (fold change calculated using the least square mean) and the p-value associated with each probe set and binary comparison are calculated, as well a "step-up," adjusted p-value for the purpose of controlling the false

discovery rate (FDR) ²⁶. For identification of differentially expressed genes between WT and KO in females and males, a binary filter of $|FC| > 1.5$ and $p < 0.05$ was used. ANOVA was also performed to detect alternative splicing, using exon-specific probe sets (at least one probe per exon). A filter of $FDR < 0.05$ was used as a cut off for alternative splicing analysis.

Bioinformatic identification of biological processes associated with differentially expressed genes

Data were analyzed through the use of Ingenuity Pathways Analysis (IPA; Ingenuity® Systems, www.ingenuity.com). Lists of differentially expressed genes in females and males were uploaded and analyzed using IPA Core Analysis. The Upstream Analysis function was used to identify predicted upstream regulators. The overlap p value generated by Upstream Analysis indicates the significance of the number of genes in the data set regulated by a given upstream regulator. The Z-score indicates the predicted direction of change for a given upstream regulator, with the sign indicating repression (negative) or upregulation (positive). Estrogen (beta-estradiol) and testosterone (dihydrotestosterone) were both in the top ten upstream regulators with the lowest Z-score (indicative of inactivation in p38 α -deficient cells) for female and male data sets, respectively.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc, San Diego, CA). The significance of differences in cytokine production and flow cytometry data were determined using 2-way ANOVA. The significance of differences observed in clinical course of EAE was determined by 2-way ANOVA and post-hoc analysis using Fisher's LSD test for individual time points (significance indicated by “*” above each time point). Nonlinear regression analyses of the mean daily clinical disease scores indicated that the disease course amongst all strain and treatment combinations was best fit by a variable slope dose response curve, which together with daily mean score was used to represent the change in clinical disease over time. EAE data from replicate experiments were analyzed by heterogeneity testing. No significant experiment-to-experiment variation was observed, and therefore the data were pooled accordingly.

Results

Pharmacologic inhibition of p38 MAPK signaling ameliorates EAE in a sex-specific manner

We have previously shown that pharmacologic inhibition of p38 MAPK prevented EAE in female C57BL/6J (B6) mice ¹⁶. Since EAE, like MS, can often exhibit sexual dimorphisms²⁷, we tested whether male mice showed a similar therapeutic response. EAE was induced in female and male B6 mice using the 2 $\times$ MOG₃₅₋₅₅/CFA protocol, followed by daily treatment with SB203580, a small molecule inhibitor of p38 α and β . Surprisingly, while disease was robustly ameliorated in female mice as previously described (Fig. 1A), SB203580 treatment showed no therapeutic efficacy in male mice; in fact, EAE onset was accelerated (Fig. 1B). Thus, the therapeutic response to p38 MAPK inhibition is sexually dimorphic.

p38 α signaling in myeloid cells underlies the sexually dimorphic therapeutic efficacy of p38 MAPK inhibition in EAE

To delineate the contribution of p38 MAPK signaling in different cell types to the sexually dimorphic response to SB203580 treatment, as well as to rule out any sex-specific pharmacokinetic differences, we used cell type-specific genetic ablation of p38 α , the predominant isoform expressed in immune cells. Autoreactive Th1 and Th17 cells are

thought to initiate disease in both MS and EAE²⁸, and p38 MAPK signaling in Th cells plays an important role in the generation and function of both Th1 and Th17 cells^{15–17, 29}. Moreover, conventional dendritic cells (DCs) are important in the activation of pathogenic Th cells not only in the lymphoid organs, but also in the CNS during disease progression³⁰, and p38 α signaling in DCs has been recently shown to promote the generation of encephalitogenic Th17 cells¹⁸. Lastly, myeloid cells such as macrophages, microglia, and neutrophils are important mediators of tissue destruction and inflammation in the CNS during EAE and MS³¹, and p38 α is thought to control the release of many pro-inflammatory mediators from these cells⁵. Therefore, we hypothesized that the sex-specific therapeutic efficacy of SB203580 (Fig. 1) is mediated by inhibition of p38 MAPK signaling in one or more of these cell types. To address this hypothesis, B6 mice expressing a floxed allele of p38 α (p38 $\alpha^{fl/fl}$)²³ were crossed to mice expressing Cre recombinase under the control of the *Lck*, *Cd11c*, or *Lysm/Lyz2* promoters^{21, 22, 24}. This selectively ablates p38 α in T cells (p38CKO^{*Lck*}), conventional DCs (p38CKO^{*Cd11c*}), or myeloid cells (p38CKO^{*Lysm*}), respectively.

EAE was induced in the resulting mice using the 2 $\times$ MOG_{35–55}/CFA protocol. Surprisingly, p38CKO^{*Lck*} mice exhibited a disease course similar to littermate p38 $\alpha^{fl/fl}$ Cre-negative controls (these are designated as WT throughout), suggesting that p38 α in T cells is not essential for EAE in B6 mice (Fig. 2A and B). Deletion of p38 α in DCs ameliorated disease, as recently reported¹⁸, but in a non sex-specific manner (Fig. 2C and D). However, deletion of p38 α in myeloid cells resulted in diminished disease in females, but not males (Fig. 2E and F). EAE in males was in fact augmented by deletion of p38 α in myeloid cells (Fig. 2F), as seen with SB203580 treatment (Fig. 1B). Expression of any of the above Cre alleles in p38 $\alpha^{wt/fl}$ or p38 $\alpha^{wt/wt}$ mice did not affect EAE (data not shown), thereby excluding any non-specific effects of Cre expression. Furthermore, p38 α was deleted with equal efficiency in myeloid cells from female and male p38CKO^{*Lysm*} mice (Supplementary Fig. 1). Taken together, these results suggest that inhibition of p38 α in myeloid cells underlies the sexual dimorphism observed with pharmacologic inhibition of p38 MAPK (Fig. 1A).

p38 α signaling in myeloid cells promotes CNS inflammation

Based on the results above, we hypothesized that deletion of p38 α in myeloid cells affected their proinflammatory functions selectively in females. Myeloid cells such as macrophages are potent antigen-presenting cells (APCs) that can influence T cell priming and effector responses by presenting antigen and regulating the cytokine milieu³⁰. Thus, we first determined whether deletion of p38 α in myeloid cells affected the priming of myelin-specific Th1 and Th17 cells in secondary lymphoid organs. p38CKO^{*Lysm*} mice and WT control littermates were immunized with 2 $\times$ MOG_{35–55}/CFA, and 10 days later Th1 and Th17 responses in lymph nodes (LN) and spleen were assessed using *ex vivo* cytokine staining or by measuring MOG_{35–55}-stimulated cytokine production by ELISA. No significant effect of p38 α deletion on the production of IFN γ , IL-17, or GM-CSF was found in female or male mice (Fig. 3). Moreover, normal numbers and percentages of myeloid cells were found in lymphoid tissues of p38CKO^{*Lysm*} mice, and the expression of MHC Class II and co-stimulatory molecules CD80 and CD86 on these cells was not affected (Supplementary Fig. 2). Similar results were obtained in thioglycolate-elicited peritoneal macrophages (data not shown). Taken together, these results suggest that p38 α in myeloid cells is dispensable for normal myeloid cell homeostasis and for efficient priming of peripheral Th1 and Th17 responses.

We next assessed whether deletion of p38 α in myeloid cells affected the inflammatory response in the CNS, since peripheral immune responses may not fully represent what occurs in the target organ. p38CKO^{*Lysm*} mice and WT control littermates were immunized

with 2×MOG₃₅₋₅₅/CFA, and infiltrating mononuclear cells were isolated from the CNS at the peak clinical disease (day 19). Total mononuclear cell numbers were significantly reduced in p38CKO^{Lysm} female mice compared to WT females (Fig. 4A), suggesting reduced infiltration of immune cells into the CNS. CD11b expression on macrophages and microglia is upregulated by activation of these cells during neuroinflammation³². There was a reduction in the percentage of activated CD11b^{hi} myeloid cells, with corresponding increase in the CD11b^{int} population in female p38CKO^{Lysm} mice (Fig. 4B–E). Furthermore, production of IFN γ , IL-17, and GM-CSF by CD4 T cells was reduced in p38CKO^{Lysm} female mice compared to WT females (Fig. 4F–L). None of these changes were seen in male p38CKO^{Lysm} mice compared to WT males; in fact, GM-CSF production was significantly increased (Fig. 4H). Taken together, these results indicate that in females p38 α in myeloid cells promotes CNS inflammation and indirectly promotes CNS T cell responses, whereas in males it may play an opposing role. To further verify that myeloid cell-specific deletion of p38 α impacted the effector phase of EAE, we induced passive EAE in WT or p38CKO^{Lysm} mice by adoptive transfer of effector T cells harvested from WT sex-matched donors. EAE severity in p38CKO^{Lysm} female mice was significantly reduced compared to WT females (Supplementary Fig. 3A), while in males no significant difference was observed (Supplementary Fig. 3B).

p38 α signaling in myeloid cells is not required for TLR-induced TNF α and IL-6 production

Based on the results above, we hypothesized that p38 α controls a subset of pro-inflammatory mediators in female myeloid cells. p38 MAPK has long been known to control the production of proinflammatory cytokines by macrophages in response to TLR stimulation. Therefore we tested the effect of p38 α ablation on macrophage responses to TLR agonists. Thioglycolate-elicited macrophages were isolated from WT and p38CKO^{Lysm} mice immunized with 2×MOG₃₅₋₅₅/CFA in order to more closely mimic the *in vivo* environment to which myeloid cells are exposed during EAE. Macrophages were stimulated *ex vivo* by LPS, a TLR4 agonist, or heat-killed *Mycobacterium tuberculosis* H37Ra (MTB), the primary adjuvant used to induce EAE which contains several TLR ligands³³. These stimuli resulted in increased phosphorylation of p38 MAPK, which was strongly reduced in p38CKO^{Lysm} mice (Fig. 5A and B). However, the production of TNF α and IL-6, two TLR-induced cytokines that have been previously shown to be controlled by p38 MAPK⁵, was not reduced in macrophages from female or male p38CKO^{Lysm} mice (Fig. 5C–F). Production of these two cytokines was in fact modestly enhanced in p38CKO^{Lysm} macrophages relative to WT at several of the time points assayed, but this was not sex-specific. Similarly, no sex-specific differences in the production of these cytokines were observed using bone marrow-derived macrophages (data not shown). These results suggest that production of cytokines classically associated with p38 MAPK signaling is not reduced in p38CKO^{Lysm} macrophages, and hence does not explain the sex-specific effects of p38 α deletion on EAE susceptibility.

p38 α signaling in myeloid cells controls unique sex-specific gene expression modules

Since cytokines classically associated with p38 MAPK signaling in macrophages were not altered by p38 α deletion in a sex-specific manner, we undertook a genome-wide transcriptomic approach to identify sex-specific p38 α -regulated transcripts in macrophages. Thioglycolate-elicited macrophages were isolated from WT and p38CKO^{Lysm} female and male mice immunized using the 2×MOG₃₅₋₅₅/CFA protocol, and restimulated *ex vivo* with MTB for 4 hours, at which point RNA was extracted and subjected to microarray analysis. Differential expression analysis revealed three unique modules of p38 α -dependent genes in macrophages: non-sex-specific (i.e. controlled by p38 α in both sexes), female-specific, and male-specific (Tables 1–3, Fig. 6A and B). The number p38 α -dependent transcripts was

higher in females than in males, with limited overlap (70 vs. 44 genes; 8 genes in common), suggesting a greater dependence on p38 α in females.

p38 α differentially regulates pro- and anti-inflammatory genes in females and males

MS and EAE are polygenic diseases, where small effects of multiple loci contribute to overall disease susceptibility^{34, 35}. By analogy, we hypothesize that the effect of p38 α deletion in EAE is mediated by the combined effects of multiple genes within the p38 α -dependent modules, rather than a single key p38 α -dependent gene. Within the non-sex-specific module, several genes of interest were downregulated in the absence of p38 α (Table 1). *Serpinb2* (plasminogen activator inhibitor 2, PAI-2) and *Mmp13* (matrix metalloproteinase 13, MMP-13) have both been previously shown to be controlled by p38 α in macrophages, where PAI-2 can inhibit apoptotic responses and IL-1 β production^{36–38}. Although the role of MMP-13 in EAE/MS is so far unexplored, MMPs are well-known to be involved in the pathogenesis of these diseases³⁹. *Il1f9* encodes IL-36 γ , a novel cytokine with a potential role in psoriasis^{40–42}, a Th17-driven autoimmune disease. *Mirlet7e* encodes a microRNA that was recently shown to promote EAE pathogenesis⁴³. In contrast, *Ccr5* (chemokine (C-C motif) receptor 5), which was also downregulated in the absence of p38 α , is not required for EAE⁴⁴. It is important to note that while these genes did not exhibit sex-specific p38 α -dependence, their altered expression may nevertheless contribute to the observed sex-specific phenotypes in EAE by interacting with genes within the female- or male-specific p38 α -controlled modules.

In females, deletion of p38 α downregulated several genes that are known to promote EAE or MS pathogenesis (Table 2). The product of *Maoa*, monoamine oxidase A, has been successfully targeted to treat EAE⁴⁵. *Oas1g* (2'-5' oligoadenylate synthetase 1G) is an ortholog of human *OAS1*, which was recently found to be associated with MS susceptibility and severity^{46–48}. *Fcgr1* is a mouse ortholog of human *FCGR1a* (encoding the high affinity IgG Fc receptor), which was upregulated in chronic CNS lesions in MS patients, and targeting the Fc gamma receptor pathway ameliorated EAE in mice¹⁴. Lastly, *Ccr1* (chemokine (C-C motif) receptor 1) has been shown to be critical for EAE pathogenesis⁴⁴. In contrast, in males, deletion of p38 α resulted in downregulation of *Il10*, an immunosuppressive cytokine well-known to inhibit EAE⁴⁹. Meanwhile, *Il12b*, the p40 subunit of IL-12 and IL-23, and known to be required for EAE⁵⁰, was upregulated.

As an additional filter to determine the *in vivo* relevance of genes differentially regulated by p38 α in macrophages stimulated *in vitro*, we analyzed the expression of multiple transcripts in mononuclear cells isolated from the inflamed CNS at peak EAE (day 21). Consistent with microarray results, we found that *Il10* mRNA was downregulated in cells from male but not female p38CKO^{Lysm} mice (Fig. 7A), while *Oas1g* mRNA was downregulated specifically in females in the absence of p38 α (Fig. 7B). In contrast to the microarray results, *Fcgr1*, *Ccr1* and *Ccr5* mRNAs were unchanged in either sex (Fig. 7C–E), suggesting differential regulation *in vivo*. Several other transcripts of interest were either undetectable, or their expression was highly variable (data not shown), owing likely to the heterogeneity in CNS-infiltrating cells and/or EAE timing/onset, thus precluding their analysis. We also examined the expression of several pro-inflammatory cytokines thought to be controlled by p38 MAPK. *Il1b*, was upregulated in males in an inverse relationship with *Il10* (Fig. 7F). *Tnfa* expression was not affected by p38 α deletion, while *Il6* was upregulated in both females and males in the absence of p38 α (Fig. 7G and H), similar to the results observed by ELISA *in vitro* (Fig. 5). Taken together, these results suggest that differential sex-specific regulation of pro- and anti-inflammatory gene expression by p38 α underlies the opposing protective and pathogenic effects of p38 MAPK inhibition in EAE in females and males, respectively, and

identify *Il10* and *Oas1g* as key p38 α -controlled sex-specific genes in the CNS during peak neuroinflammation.

Sex hormones contribute to the sexual dimorphism in EAE in p38CKO^{Lysm} mice

Bioinformatic analysis of differentially expressed transcript modules in macrophages from female and male p38CKO^{Lysm} mice identified estrogen as a highly significant positive upstream regulator of genes within the female-specific module (activation Z score = -2.21; p value of overlap = 2.29E-04), while testosterone was a positive regulator of genes within the male-specific module (activation Z score = -1.47; p value of overlap = 1.32E-06). This suggested that sex hormones may be responsible for differential EAE outcomes in female and male p38CKO^{Lysm} mice. To test this hypothesis, we performed gonadectomies (or sham control surgeries) on adult WT and p38CKO^{Lysm} mice, followed by EAE induction. Sham surgeries did not affect the sexual dimorphism, as female p38CKO^{Lysm} mice were highly resistant to EAE induction compared to WT (Fig. 8A), while EAE in p38CKO^{Lysm} males was not significantly different from WT males (Fig. 8B). However, removal of adult sex hormones by gonadectomy completely reversed the sexual dimorphism, as ovariectomized p38CKO^{Lysm} females lost EAE resistance (Fig. 8C), and orchiectomized p38CKO^{Lysm} males gained it (Fig. 8D). These results demonstrate the role of adult sex hormones in determining the sexual dimorphism in EAE pathogenesis mediated by p38 α signaling in myeloid cells.

Discussion

In this study, we showed that deletion of p38 α in T cells did not affect EAE in B6 mice (Fig. 2A and B). This is in contrast to our previous results showing that augmentation of p38 MAPK activity by a constitutively active MKK6 transgene, or its inhibition by a dominant negative p38 transgene, expressed specifically in T cells in B10.BR mice, enhanced or diminished EAE severity, respectively¹⁶. This discrepancy may be due to genetic differences between B6 and B10.BR mice, or the fact the transgenic approaches affect all four p38 MAPK isoforms. Furthermore, we found that in transgenic B10.BR mice EAE was modulated in a non-sex specific fashion (data not shown), suggesting a different mechanism from the one described herein.

The finding that SB203580 treatment did not reduce EAE in males (Fig. 1B) is somewhat counter-intuitive given reduced EAE in p38CKO^{Cd11c} males (Fig. 2D). However, based on the findings that p38CKO^{Lysm} males exhibited augmented EAE (Fig. 2F), we predict that pharmacological inhibition of p38 concurrently in myeloid cells and DCs has opposing effects on EAE in males which effectively cancel each other out. An alternative explanation is the involvement of other cell types targeted by SB203580 besides myeloid cells and DCs. Lastly, pharmacological experiments are difficult to compare to p38 α genetic deletion studies due to effects of SB203580 on p38 α or potential off-target effects⁵¹.

Chi and colleagues recently demonstrated that deletion of p38 α in myeloid cells did not affect EAE, although the sex of the animals was not reported¹⁸. Another important difference is that Chi and colleagues used pertussis toxin (PTX) as an ancillary adjuvant in the EAE induction protocol, which is absent in the 2 $\times$ MOG₃₅₋₅₅/CFA protocol used in our study (Fig. 2). Because PTX overrides many genetic checkpoints (e.g., see ⁵²⁻⁵⁴), we hypothesized that it can override disease protection provided by deletion of p38 α in myeloid cells. In agreement with this, we found that WT and p38CKO^{Lysm} female and male mice exhibited no difference in EAE disease course when the 1 $\times$ MOG₃₅₋₅₅/CFA/PTX protocol was used (Supplementary Fig. 4). MS exhibits remarkable heterogeneity in disease course and severity, similar to what is seen across in different EAE models elicited with or without PTX.⁵³ It is likely that different adjuvants used in EAE model different infectious/

environmental risk factors in MS. Consequently, inhibitors of p38 MAPK-related pathways may have differential therapeutic efficacy depending on disease etiopathogenesis.

Our findings that deletion of p38 α in macrophages altered a relatively small subset of transcripts are somewhat surprising, since p38 inhibitors were reported to inhibit wide range of proinflammatory cytokines and mediators. However, our findings are in agreement with previous reports using conditional deletion of p38 α , which demonstrated that this kinase controls a limited spectrum of pro-inflammatory genes^{37, 55}. Interestingly, IL-10 production was shown to be dependent on p38 α in these reports and in our study. The differences in the results observed between pharmacologic and genetic studies may be due to the poor specificity of inhibitors⁵¹. Furthermore, p38 MAPK controls many of its targets, at the post-translational level^{56, 57}, including IL-17 in T cells¹⁶, while our current analysis focused mainly on transcriptional control. Nonetheless, while it appears that effects of p38 α inhibition in macrophages may be far more limited than previously suggested, it is clear that these subtle effects are sufficient to modulate EAE severity. As such, more specific and/or cell type-targeted inhibitors of p38 α could represent an attractive therapeutic approach in MS.

In humans, gender influences immunity, although controversial results regarding gender and/or sex hormone treatment in *ex vivo* studies are frequently reported⁵⁸. While p38 MAPK is well-known to control pro-inflammatory functions in human monocytes/macrophages⁶, to the best of our knowledge, little has been reported on gender differences in this regard. One study examined LPS-induced phosphorylation of p38 in PBMCs from males and females, and found that it was somewhat lower in females, correlating with lower proinflammatory cytokine production⁵⁹. Hence, it is possible that the p38-dependent sex bias in macrophages observed in our mouse model will translate to humans, but further studies are needed to address this possibility.

The incidence of MS has approximately tripled in the last 50 years, driven by an increase in relapsing-remitting disease in women⁴. This rate of change strongly suggests the existence of environmental risk factors acting at the population level in females. p38 MAPK is a well-known evolutionarily conserved component of the response to environmental stress stimuli, such as hypertonicity and UV radiation^{60–62}. Recent epidemiological data indicate that the prevalence of MS correlates with decreased UV-radiation exposure more highly in females than it does in males⁶³. The immunomodulatory effects of UV-radiation⁶⁴ are in part mediated by p38 MAPK signaling⁶² and UV-irradiation has been shown to influence EAE susceptibility⁶⁵ independent of vitamin D⁶⁶. With respect to hypertonicity, two recent reports suggested that dietary sodium may also represent an environmental risk factor for MS, as increased dietary sodium exacerbated EAE, in association with enhanced generation of pathogenic Th17 cells^{67, 68}. p38 MAPK appears to transduce this sodium stress signal, as inhibition of p38 MAPK abrogated sodium-induced upregulation of IL-17 production⁶⁷. However, the effect of p38 MAPK inhibition on sodium-exacerbated EAE was not reported⁶⁷. Since our data show that the p38 MAPK signaling pathway is central to EAE pathogenesis in females, it is tempting to speculate that environmental stress signals acting through the p38 MAPK pathway may contribute to the increasing MS risk in females. Consequently, understanding this pathway may reveal mechanistic insight into gene-by-environment interactions in MS etiopathogenesis. Moreover, our results suggest that targeting the p38 MAPK signaling pathway in MS could represent a novel and much needed female-specific DMT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institute of Health Grants AI041747, NS036526, and NS060901 to CT. This work was also supported a postdoctoral fellowship from the National Multiple Sclerosis Society to DNK.

Matt Poynter, John Boyson, Sean Diehl, Laure Case, Roxana del Rio, Naresha Saligrama, Emma Wall and Elizabeth P. Blankenhorn are acknowledged for helpful discussions and advice. Kristiaan Finstaad and Erin Osmanski are acknowledged for technical assistance. Meghann Palermo and Tim Hunter at the VCC facility at UVM are acknowledged for the help with microarrays. Additional thanks to Jeff Bond and the Molecular Bioinformatics Shared Resource of the UVM College of Medicine.

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Figure 1. Sex-specific modulation of EAE susceptibility by p38 MAPK

Female (A) and male (B) B6 mice were immunized using the 2×MOG₃₅₋₅₅/CFA protocol, followed by daily injections of SB203580 or carrier. Data represent 2 independent experiments, pooled. Data were analyzed as indicated in Materials and Methods. A significant difference in EAE course was observed in females [treatment, $p < 0.0001$; time, $p < 0.0001$; time-by-treatment interaction, $p < 0.0001$], and in males [treatment, $p = 0.71$; time, $p < 0.0001$; time-by-treatment interaction, $p < 0.0001$]. For individual time points, * 0.05; ** 0.01, *** 0.001, **** 0.0001. Numbers in parentheses indicate the total number of animals studied.

Figure 2. Differential control of EAE by p38 α signaling in T cells, DCs, and myeloid cells Female (left panels) and male (right panels) WT and p38CKO^{Lck} (A and B), WT and p38CKO^{Cd11c} (C and D), and WT and p38CKO^{Lysm} (E and F) mice were immunized with 2 $\times$ MOG₃₅₋₅₅/CFA. Data represent two pooled independent experiments for each strain combination, and were analyzed as in Fig. 1. No significant effect of KO on EAE course was found in (A and B), females [strain, $p=1.0$; time, $p<0.0001$; time-by-treatment interaction, $p=0.4$], males [strain, $p=0.3$; time, $p<0.0001$; time-by-treatment interaction, $p=0.6$]. In (C and D), a significant effect of KO on EAE course was found in both females [strain, $p=0.006$; time, $p<0.0001$; time-by-treatment interaction, $p<0.0001$], and males [strain, $p=0.3$; time, $p<0.0001$; time-by-treatment interaction, $p<0.0001$]. In (E and F), a significant effect of KO on EAE course was found in both females [strain, $p=0.008$; time, $p<0.0001$; time-by-treatment interaction, $p<0.0001$], and males [strain, $p=0.2$; time, $p<0.0001$; time-by-treatment interaction, $p=0.02$]. For individual time points, * 0.05; ** 0.01, *** 0.001, **** 0.0001. Numbers in parentheses indicate the total number of animals studied.

Figure 3. Ex vivo peripheral recall responses in p38CKO^{LysM} mice

Female and male littermate WT and p38CKO^{LysM} mice were immunized with 2×MOG₃₅₋₅₅/CFA. On D10, LN and spleen cells were isolated, restimulated with 50 μg/ml MOG₃₅₋₅₅, and production of the following cytokines was determined by ELISA: IFNγ (A), IL-17 (B), and GM-CSF (C). Alternatively, LN and spleen cells were stimulated with PMA/Ionomycin in the presence of brefeldin A for 4 hours, then stained and analyzed by intracellular cytokine staining and flow cytometry (D–G). Percentage of TCRβ+CD4+ cells positive for IFNγ (D) or IL-17 (E) is shown. Representative flow cytometry plots are shown for female WT (F) and p38CKO^{LysM} (G) mice. Data are representative of two independent experiments. WT female (n=7), p38CKO^{LysM} female (n=10), WT male (n=10), p38CKO^{LysM} male (n=10). No significant effect of KO was detected.

Figure 4. Dampened CNS inflammatory response in female p38CKO^{LysM} mice

Female and male WT and p38CKO^{LysM} mice were immunized using the 2 $\times$ MOG₃₅₋₅₅/CFA protocol. On day 19, mononuclear cells were isolated from the CNS using a Percoll gradient, counted (A), and surface stained for the indicated markers. Cells were gated on the CD45⁺TCR β ⁻ population and expression of CD11b (CD11b^{hi} vs. CD11b^{int}) was analyzed (B and C; representative flow cytometry plots shown in D and E). Alternatively, mononuclear cells were stimulated with MOG₃₅₋₅₅ for 4 hr in the presence of brefeldin A, then analyzed by intracellular cytokine staining and flow cytometry (F–L). Percentage of TCR β ⁺CD4⁺ cells positive for IFN γ (F), IL-17 (G), or GM-CSF (H) is shown. Representative flow cytometry plots are shown in (I–L). WT female (n=6), p38CKO^{LysM} female (n=10), WT male (n=6), p38CKO^{LysM} male (n=6).

Figure 5. Macrophage responses to TLR stimulation in p38CKO^{Lysm} mice

Female and male WT and p38CKO^{Lysm} mice were immunized using the 2×MOG₃₅₋₅₅/CFA protocol. On day 6 thioglycolate was injected i.p., and elicited peritoneal macrophages were isolated on day 10 post-immunization. Adherent macrophages were stimulated for 30 min with 100 ng/ml LPS (A) or 50 µg/ml heat killed MTB (B), lysed, and analyzed by immunoblot for phosphorylation of p38 MAPK (p-p38). GAPDH is shown as a loading control. Panel (B) is a composite image of two different parts of the same membrane image, as indicated by the lines, treated identically and shown at the same exposure. Alternatively, adherent macrophages were stimulated for 4 or 24 hrs (as indicated) with LPS (C and D) or MTB (E and F), supernatants were collected and analyzed by ELISA for the presence of TNFα or IL-6. Data are representative of 3 independent experiments. * 0.05; ** 0.01. WT female (n=6), p38CKO^{Lysm} female (n=9), WT male (n=8), p38CKO^{Lysm} male (n=8).

Figure 6. Sex-specific p38α-dependent transcript modules in macrophages

Female and male WT and p38CKO^{LysM} macrophages were isolated as described for Fig. 5, stimulated for 4 hrs with 50 μg/ml heat killed MTB, RNA was extracted, reverse transcribed, and cDNA subjected to microarray analysis. **(A)** A heat map of gene expression across triplicate samples of WT and p38CKO^{LysM} (KO) macrophages from female and male mice. Each triplicate represents a pool of 2–3 different biological replicates. Expression is shown relative to the centered mean of all samples for a given gene (see Materials and Methods). Genes passing the binary filter of $p < 0.05$ and $|FC| > 1.5$ (of KO relative to WT for either sex) are shown, ordered by FC in descending order. “Up” or “down” refers to the direction of change in KO relative to WT. **(B)** A Venn diagram indicating the overlap in p38α-dependent transcripts between females and males. Both annotated and non-annotated genes were included.

Figure 7. Sex-specific regulation of inflammatory gene expression in the CNS of p38CKO^{LysM} mice

Female and male WT and p38CKO^{LysM} mice were immunized using the 2×MOG₃₅₋₅₅/CFA protocol. On day 21, mononuclear cells were isolated from the CNS using a Percoll gradient and RNA was extracted. Relative mRNA abundance of *Il10* (A), *Oas1g* (B), *Fcgr1* (C), *Ccr1* (D), *Ccr5* (E), *Il1b* (F), *Tnfa* (G), and *Il6* (H) was quantified by qRT-PCR using the delta-delta CT method with *B2m* as an endogenous control. * 0.05. WT female (n=8), p38CKO^{LysM} female (n=6), WT male (n=5), p38CKO^{LysM} male (n=4).

Figure 8. Sex hormones contribute to the sexual dimorphism in EAE in p38CKO^{Lysm} mice
 Female (left panels) and male (right panels) WT and p38CKO^{Lysm} mice underwent sham surgeries (Sham) (A and B) or gonadectomies (GndX) (C and D). 3 weeks later, mice were immunized using the 2×MOG₃₅₋₅₅/CFA protocol. Data represent one independent experiment, and were analyzed as in Fig. 1. A significant effect of KO on EAE course was found in (A) [strain, $p=0.05$; time, $p<0.0001$; time-by-treatment interaction, $p<0.0001$] and in (D) [strain, $p<0.0001$; time, $p<0.0001$; time-by-treatment interaction, $p=0.03$]. No significant effect of KO on EAE course was found in (B) [strain, $p=0.4$; time, $p<0.0001$; time-by-treatment interaction, $p=0.3$] and in (C) [strain, $p=0.7$; time, $p<0.0001$; time-by-treatment interaction, $p=0.7$]. For individual time points, * 0.05; ** 0.01, *** 0.001, **** 0.0001. Numbers in parentheses indicate the total number of animals studied.

Table 1
Non-sex-specific p38 α -dependent transcripts in macrophages

Thioglycolate-elicited macrophages were isolated from female and male p38CKO^{Lysm} and WT mice, and stimulated with 50 μ g/ml heat-killed MTB *in vitro* for 4hrs. mRNA was isolated and subjected to microarray analysis to identify differentially expressed genes between p38CKO^{Lysm} and WT in both females and males. The criteria for differential expression was set at $p < 0.05$ and signed fold change ($|FC|$) > 1.5 . Fold change indicates the change in expression in female p38CKO^{Lysm} relative to WT. Non-annotated genes are not shown.

gene	female		male	
	p val	FC	p val	FC
U90926	0.00	-2.51	0.02	-1.69
Mmp13	0.00	-2.18	0.00	-1.78
Il1f9	0.01	-2.02	0.01	-1.91
Serpib2	0.00	-1.88	0.00	-2.30
Ccr5	0.01	-1.80	0.00	-1.87
Lox	0.01	-1.57	0.02	-1.51
Mirlet7e	0.04	-1.57	0.05	-1.54
Ch25h	0.00	1.52	0.00	1.51

Table 2
Female-specific p38 α -dependent transcripts in macrophages

Microarray analysis was performed on samples collected as described for Table 1 to identify differentially expressed genes between p38CKO^{Lysm} and WT that were unique to females. The criteria for differential expression was set at $p < 0.05$ and $|FC| > 1.5$. Fold change indicates the change in expression in female p38CKO^{Lysm} relative to female WT. Non-annotated genes are not shown. Genes known to play a role in EAE/MS pathogenesis (discussed in the text) are italicized and bolded.

gene	p val	FC
Retnlg	0.021	-2.17
Snord82	0.050	-2.12
Mir20a	0.005	-1.93
Scd1	0.048	-1.91
Mir1b	0.035	-1.90
Gpr141	0.006	-1.86
Isg15	0.028	-1.80
Trim30c	0.002	-1.79
Snord15a	0.003	-1.77
Cox7b	0.038	-1.76
Trim30d	0.006	-1.74
Syne1	0.011	-1.71
Mpz13	0.027	-1.69
Hist1h2bn	0.000	-1.65
Hist2h3c2	0.004	-1.64
Vsig4	0.047	-1.64
Car2	0.038	-1.64
Mid1	0.036	-1.63
<i>Maoa</i>	0.004	-1.62
Adm	0.016	-1.61
Snord43	0.049	-1.61
Tfrc	0.008	-1.61
Flrt3	0.001	-1.61
Scarna17	0.011	-1.59
<i>Oas1g</i>	0.017	-1.59
Mir187	0.040	-1.58
Tpsb2	0.013	-1.58
Ctla2a	0.005	-1.57
Snord118	0.021	-1.57
Rn5s20	0.049	-1.55
<i>Ccr1</i>	0.009	-1.54
Scg2	0.015	-1.53
<i>Fcgr1</i>	0.003	-1.52
Dio2	0.021	-1.52
Gorab	0.005	-1.52

gene	p val	FC
Zfp68	0.008	-1.51
Mreg	0.014	-1.51
Pyhin1	0.007	-1.51
Tut1	0.021	-1.51
Syne1	0.040	-1.50
Hdhd3	0.015	1.52
Zfp862	0.006	1.53
Snora30	0.024	1.56
Mir23a	0.034	1.57
Kcnj13	0.032	1.58
Ptges3l	0.000	1.61
Pdgfb	0.005	1.62
Kprp	0.002	1.71
Clca2	0.044	1.78
Mir5123	0.020	2.32

Table 3
Male-specific p38α-dependent transcripts in macrophages

Microarray analysis was performed on samples collected as described for Table 1 to identify differentially expressed genes between p38CKO^{Lysm} and WT that were unique to males. The criteria for differential expression was set at $p < 0.05$ and $|FC| > 1.5$. Fold change indicates the change in expression in male p38CKO^{Lysm} relative to male WT. Non-annotated genes are not shown. Genes known to play a role in EAE/MS pathogenesis (discussed in the text) are italicized and bolded.

gene	p val	FC
Mir669a-3	0.05	-1.73
Mup12	0.02	-1.71
Fpr1	0.03	-1.69
Fabp7	0.00	-1.69
Hp	0.00	-1.64
Cd5l	0.04	-1.63
Rrs1	0.02	-1.61
<i>Il10</i>	0.04	-1.56
Mir7-2	0.01	-1.53
Cav1	0.00	-1.52
Vmn2r26	0.02	-1.52
Olf1269	0.02	-1.51
Acpp	0.01	-1.51
Vmn2r113	0.03	-1.51
Olf1566	0.05	-1.51
Cdc14a	0.00	1.52
Mir505	0.04	1.53
Atp5s	0.03	1.53
Kansl2	0.01	1.54
Pgcp	0.01	1.54
Calca	0.01	1.55
Zfp945	0.02	1.56
<i>Il12b</i>	0.00	1.57
Hgf	0.00	1.60
Mir215	0.05	1.73
Cdr1	0.02	2.45