HHS Public Access

Author manuscript

J Neuroimmunol. Author manuscript; available in PMC 2022 April 04.

Published in final edited form as:

J Neuroimmunol. ; 342: 577209. doi:10.1016/j.jneuroim.2020.577209.

The sex-specific role of p38 MAP kinase in CNS autoimmunity is regulated by estrogen receptor alpha

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Abstract

Biological sex is a critical factor in regulating immune function. A striking example of this is the higher prevalence of autoimmune diseases such as multiple sclerosis (MS) and lupus in females compared to males. While many studies have implicated the role of sex hormones such as estrogens and androgens in these sex differences, surprisingly little is known about other molecular pathways that underlie sex differences or interact with sex hormones. We have previously shown that conditional ablation of p38α MAP kinase signaling in myeloid cells (p38αCKO) was protective in a mouse model of MS, experimental autoimmune encephalomyelitis (EAE), in female but not male mice. This sex difference was dependent on the presence of sex hormones, leading us to hypothesize that the pathogenic function of p38α in EAE depends on estrogen signaling via one of the two nuclear estrogen receptors, encoded by *Esr1* and *Esr2*. To test this hypothesis, we performed experiments with p38αCKO macrophages, which demonstrated that the effects of estradiol and p38α were independent of one another *in vitro*. Since many sex hormone effects are lost *in vitro,* we generated p38αCKO mice lacking either *Esr1* or *Esr2*, and evaluated their EAE susceptibility *in vivo*. Myeloid-specific deletion of *Esr1* abrogated protection in p38αCKO females, although global deletion of *Esr1* and *Esr2* did not. Moreover, global or myeloid-specific disruption of *Esr1* unexpectedly promoted protection from EAE in p38αCKO males. Mechanistically, *Esr1* deletion resulted in partial reprogramming of p38α-dependent transcriptional modules in male macrophages, in particular those regulated by TGFβ, BRD4, and SMARCA4. These results demonstrate that estrogen signaling in myeloid cells plays an important sex-specific role in programming their dependence on specific intracellular signaling pathways in the context of autoimmune disease pathogenesis, suggesting potential avenues for sex-specific therapeutics or combinatorial approaches for the treatment of such diseases.

Graphical Abstract

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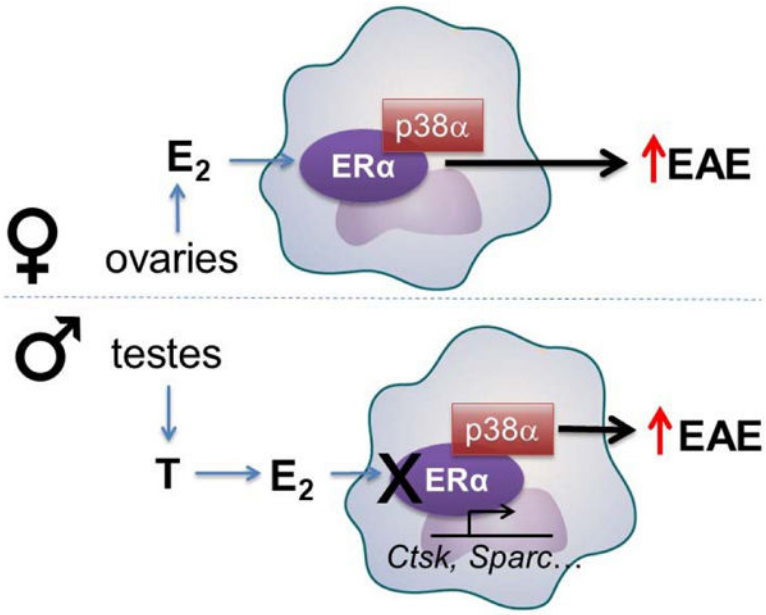

1. Introduction

Multiple sclerosis (MS) is a multifactorial inflammatory disease of the central nervous system (CNS) characterized by demyelination, gliosis, axonal loss, and progressive neurological dysfunction. The etiology of MS is incompletely understood, but current evidence suggests that peripheral activation of myelin-reactive CD4 T cells triggers an inflammatory cascade in the CNS, recruiting other immune cells that mediate the subsequent tissue destruction and pathology [1, 2]. While CD4 T cells initiate the inflammatory cascade in CNS, other immune and CNS resident cells, such as macrophages, B cells, CD8 T cells, microglia, and astrocytes, are thought to mediate tissue destruction and pathology [3]. Several therapies for MS have been developed recently, but their efficacy and success rate are relatively low [4]. While most of these therapies are aimed at modulating lymphocyte function, there is increasing interest in targeting myeloid cells, which serve as the secondary effector cells in MS [5].

The principal animal model of MS, experimental autoimmune encephalomyelitis (EAE), is an autoimmune disease induced in various animal species by active immunization with CNS homogenate or specific myelin proteins/peptides, or by adoptive transfer of CD4 T cells reactive to these antigens. As in MS, myelin-reactive CD4 T cells enter the CNS to initiate inflammation and pathology, recruiting other immune cells, and eventually resulting in neurologic disability and clinical signs [6]. The EAE model has been instrumental in improving our understanding of MS pathogenesis and the development of disease-modifying therapies [7].

Most autoimmune diseases, such as MS or systemic lupus erythematosus (SLE), are more prevalent in females, while the disease course tends to be more severe in males [8]. In spite of these sex differences being well-established, their underlying molecular

mechanisms remain poorly understood. The prevailing hypothesis suggests that these sex differences result from the influence of sex hormones (estrogens and androgens) and/or sex chromosomes [8, 9]. Sex hormones are known to influence MS disease course, supported by the observation that MS disease activity is reduced during pregnancy and flares post-partum [8, 10]. Increasing estrogens during pregnancy are thought to be primarily responsible for these disease suppressive effects, and EAE can also be robustly suppressed by administration of relatively high doses of *exogenous* estrogens, although the role of *endogenous* non-pregnancy levels of estrogens is less clear (reviewed in [11] and [12]). In line with this, a recent clinical trial in MS patients demonstrated modest efficacy of exogenous estriol treatment that did not reach significance compared with placebo [13].

At the molecular level, the vast majority of physiological actions of estrogens are thought to be mediated by the binding of estrogen to the classical nuclear estrogen receptors ERα and β, (encoded by *Esr1* and *Esr2*, respectively), which can directly or indirectly induce context-specific gene expression programs [14]. The protective effects of *exogenous* estrogens in EAE are thought to be primarily mediated by ERα and β in a variety of different immune and non-immune cell types, and to a lesser extent by the putative membrane ER, GPR30 [8, 12]. However, virtually nothing is known about druggable signaling pathways that exhibit crosstalk (whether synergistic or inhibitory) with *endogenous* estrogen signaling through ERs in EAE/MS. Such knowledge could provide the rationale for sex-specific therapeutic approaches targeting different signaling pathways in men and women, or combinatorial therapies targeting ERs and other signaling pathways simultaneously.

The p38 MAP kinase (MAPK) pathway is activated by inflammatory insults (e.g., toll-like receptor (TLR) ligands, cytokines), and stress stimuli (e.g., UV radiation, osmotic stress, DNA damage), via the upstream kinases MKK3 and MKK6 that are in turn regulated by numerous MKK kinases [15]. Four isoforms of p38 MAPK (p38α, p38β, p38γ and p38δ) have been identified, each encoded by a separate gene. The ubiquitously expressed p38α (*MAPK14/Mapk14*) is the best characterized isoform, which is thought to be responsible for the vast majority of the inflammatory functions of this Ser/Thr kinase family [16]. While well-studied in inflammatory bowel disease and arthritis [17], the p38 MAPK pathway has received little attention in MS or its models until recently. Early evidence for the involvement of p38 MAPK in autoimmune neuroinflammation came from microarray studies showing that the expression of *MAPK14* (encoding p38α) was elevated ~5-fold in MS lesions in the CNS [18]. Additionally, meta-analyses of transcriptional profiling in MS have identified the p38 MAPK pathway as a key predicted regulatory node [19], a finding that was later confirmed in patient samples [20]. Recent studies from our laboratory and others have suggested that p38 MAPK signaling in myeloid cells [21], T cells [22–24], dendritic cells [21, 25], and astrocytes [26, 27] can promote EAE pathogenesis (reviewed in [28]), but many of the underlying mechanisms remain unclear.

With regard to sex differences, in our previous studies in EAE we demonstrated that pharmacologic inhibition of p38 MAPK is protective in female but not male mice [21]. Conditional genetic deletion of p38α using LysM-Cre recapitulated this phenotype, suggesting that p38α signaling in myeloid cells promotes EAE in females but not males [21]. Importantly, we also demonstrated that removal of adult sex hormones by

gonadectomy of males or females reversed this sex difference [21]. Based on these findings, we hypothesized the estrogen signaling via ERα or ERβ promotes the pathogenic role of p38α in EAE in females. To test this hypothesis, we genetically ablated *Esr1* or *Esr2* in p38α-deficient mice. Surprisingly, we found that global ablation of *Esr1* or *Esr2* did not affect the role of p38α in females. However, conditional deletion of *Esr1* in myeloid cells reversed protection in p38α-deficient females. Moreover, global or myeloid cell-intrinsic *Esr1* deletion unexpectedly restored p38α dependence of EAE in males, accompanied by transcriptional reprogramming of myeloid cells. These results demonstrate that estrogen signaling in myeloid cells plays an important sex-specific role in programming their dependence on specific intracellular signaling pathways in the context of autoimmune disease pathogenesis.

2. Materials and Methods

2.1 Animals

p38αCKO$_{LysM}$ mice were generated in our laboratory previously [21], by crossing LysM-Cre mice (B6.129P2-*Lyz2tm1(cre)Ifo/J*) [29], and p38α$^{f/f}$ (floxed) mice (*Mapk14*$^{tm1.2Otsu}$) [30]. p38αCKO^{LysM} mice were crossed with *Esr1*−/− (global KO), *Esr1*$^{f/f}$ (*Esr1*-floxed), and *Esr2*−/− (global KO) mice to generate double knockout mice (see Tables 1 and 2). All estrogen receptor gene-targeted mice were generated and characterized [31, 32], and generously provided by the Korach laboratory (NIEHS). Wild type C57BL/6J mice were purchased from Jackson Laboratories (Maine, 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 Institutional Animal Care and Use Committee of the University of Vermont.

2.2 EAE induction and scoring

EAE was induced in 8–12 week old mice using the 2×MOG$_{35–55}$/CFA protocol, as previously described [33]. Mice were injected subcutaneously with 0.1 mL of emulsion containing 0.1 mg of myelin oligodendrocyte glycoprotein peptide 35–55 (MOG$_{35–55}$) peptide (Anaspec Inc., MA, USA) in PBS and 50% complete Freund's adjuvant (CFA; Sigma, USA) on day 0 on the lower flanks (50 μl per flank), followed by an identical injection on upper flanks on day 7. CFA was supplemented with 4 mg/mL *Mycobacterium tuberculosis* H37Ra (Difco, USA). Pertussis toxin (PTX) was not used in this induction protocol because the molecular and cellular targets and mechanism of PTX in EAE remain poorly defined, and because this protocol (unlike that with PTX) results in moderate, rather than maximal disease severity, allowing us to detect changes in severity in either direction (exacerbation or amelioration). Starting on day 10, mice were scored visually, as follows: 0.5 - partial loss of tail tone, 1 – full loss of tail tone, 2 - loss of tail tone and weakened hind limbs, 3 - hind limb paralysis, 4 - hind limb paralysis and incontinence, 5 - quadriplegia or death. EAE scoring was performed by an observer blinded to the animals' genotypes. Significance of differences in disease course were determined using Friedman's non-parametric two-way ANOVA using ranked data [34, 35], as previously described [33], using the treatment*time interaction term to evaluate differences.

2.3 Cell culture experiments

Bone marrow-derived macrophages (BMDM) were generated from bone marrow of age- and sex-matched 8–12 week old male and female p38αCKO^{LysM} or WT (p38α$^{f/f}$ littermate) mice, essentially as previously described [36], with the following modifications. Bone marrow cells and differentiated macrophages were cultured in hormone free media (phenol red-free DMEM with 2 mM glutamine (Mediatech, USA) supplemented with 10% charcoal-treated fetal calf serum). To isolate peritoneal macrophages, mice were injected i.p with 1mL of a 4% solution of thioglycolate broth (Sigma-Adrich, USA), and 96 hours later mice were sacrificed and the peritoneal cavity was flushed with 15 mL of cold PBS as described previously [36]. Differentiated BMDM and peritoneal macrophages were plated in 96 well plates at 50,000 cells per well (ELISA) or in 12 well plates at 400,000 cells/well (immunoblotting) and stimulated, as indicated, with *E. coli 026:B6* LPS (Sigma, USA) or with the indicated concentrations of estradiol (Sigma, USA). Supernatants and/or cell lysates were collected at the indicated time points.

2.4 Immunoblot analysis

Whole-cell lysates were prepared by lysing adherent macrophages directly in Triton lysis buffer containing a cocktail of protease inhibitors (Roche, USA) and 1μM sodium orthovanadate, separated by SDS-PAGE, and transferred to PVDF membranes as described previously [22]. Primary antibodies used for western blot analysis included anti-phospho-p38, anti-p38α, anti-phospho-ERK and anti-ERK (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).

2.5 Cytokine quantification

For the detection of cytokines in the cell culture supernatants or sera, ELISAs were performed as described previously [37], using the primary capture mAbs anti-TNFα, and anti-IL-6 and their corresponding biotinylated detection mAbs (Biolegend, USA). Recombinant mouse TNFα and IL-6 (Biolegend, USA) were used as standards. IL-10 was measured using a commercial ELISA kit (R&D systems, USA) according to manufacturer's instructions. Other ELISA reagents included: HRP-conjugated avidin D (Vector Laboratories, USA), TMB microwell peroxidase substrate and stop solution (Kirkegaard and Perry Laboratories, USA).

2.6 Transcriptional profiling

BMDM were generated as previously described [36], from bone marrow of individual p38αCKO^{LysM} (n=4) and p38α/Esr1-CKO^{LysM} (n=4) male mice, or control male WT littermates (Cre-negative, p38α$^{f/f}$ and p38α/*Esr1*$^{f/f}$, n=4 and n=2, respectively), and cultured in standard media (DMEM with 10% FBS and 2 mM Glutamine). Cells were stimulated with 100 ng/mL LPS for 4 hrs, followed by RNA extraction using the RNEasy miniprep kit (Qiagen, USA). The cell preparation from each individual mouse was used as a single

biological replicate. The two types of Cre-negative WT mice were treated as a single WT group in the analysis.

For transcriptional profiling, microarray analysis was performed at the UVM Cancer Center Genomics Facility using the Mouse Affymetrix Clariom D Genechip and the GeneChip™ WT Pico Target Preparation reagent kit (ThermoFisher 9026220) as described by the manufactures procedures. Briefly, 5 ng of RNA was used to synthesize cDNA through a First-Strand and Second-Strand reverse transcription reaction followed by conversion to cRNA through an overnight T7 In-vitro Transcription reaction. The resulting cRNA was purified and 5.5 ug was converted to sense, single-strand cDNA using UDG (10U/μL) and APE1 (1000U/μL), provided in the GeneChip® WT PLUS Reagent Kit. cDNA was end labelled with biotin using TdT (30U/μL), and used as input for the hybridization mix for the GeneChip. Mouse Clariom D arrays were incubated in the Affymetrix® GeneChip® Hybridization Oven 645 at 45° C / 60 RPM for 16–18 hours. Arrays were stained using the Affymetrix® GeneChip® staining reagents and scanned with the 7G Affymetrix® GeneChip® Scanner 3000.

RT-qPCR was performed on larger sample groups of each genotype to validate some of the microarray data. RNA was isolated as described above for microarray. cDNA was synthesized using qScript® cDNA Synthesis Kit (Quantabio, USA). Then, SYBR Green-based qRT-PCR was performed as described previously [22]. Data were normalized by the expression of the housekeeping gene, *B2m*.

2.7 Statistical analyses of microarray data

Raw intensity CEL files were imported into Expression Console software (Affymetrix, USA), and CHP files were generated for gene level analysis. CHP files were imported into Transcriptome Analysis Console (TAC) software v4.0.0.25 (Affymetrix, USA), and gene level expression analysis was performed using the default ANOVA settings (e-Bayesian method), comparing WT vs. p38αCKO, WT vs. p38α/Esr1-CKO, and p38αCKO vs. p38α/Esr1-CKO. Genes exhibiting |FC| > 2 and FDR <0.2 (uncorrected ANOVA ~P<0.0002) were considered as differentially expressed. For comparison, data were also analyzed with an alternative cut-off of |FC| > 1.5 and FDR <0.1 (Table S1 and supplementary Figure S1). Gene expression statistics and raw expression values were exported as text files for downstream analysis. In the WT vs. p38α/Esr1-CKO comparison, two probes were removed from the final results presented, for *Hbb-b1* and *Hbb-b2*, since they likely represent a fluctuation in a small number of contaminating blood cells from the bone marrow preparation. All raw microarray data will be deposited into the Gene Expression Omnibus (GEO) database, accession number pending.

2.8 Bioinformatic analyses

Volcano plots were generated using the Galaxy interface (European server) [38] and Volcano plot (ggplot2) tool. Heatmaps were generated using Excel. Pathway analysis was performed using Ingenuity Pathway Analysis™(IPA; Qiagen, Inc, USA) software. The gene expression datasets were exported from TAC software and uploaded into IPA. A relaxed cut-off filter of |FC|>2 and ANOVA p< 0.05 was used to maximize the number of genes in the analysis

(recommended by IPA to enhance the analysis power and accuracy). An alternative more stringent cut-off filter of |FC| >2 and FDR<0.2 was also included for comparison (Figure S2). The IPA Core Analysis function, followed by the Comparison Analysis function was used to compare the effect of p38 and/or *Esr1* deficiency, as follows. The Upstream Regulator function was used to identify the top putative upstream regulators ($p < 0.01$, Z score > |1.5|) affected by genotype (KO). The sign and magnitude of the Z scores are indicative of the predicted strength and direction of the genotype (KO) effect.

2.9 Statistical Analyses

Statistical analyses not pertaining to microarray data were carried out using GraphPad Prism software, version 6. Details of the analyses are provided in the figure legends. All statistical tests were two-sided, and adjustments for multiple comparisons were made as indicated. All center values represent the mean, and error bars represent the standard error of the mean. P-values below 0.05 were considered significant. The significance of differences observed in clinical course of EAE was determined using the Friedman 2-way Analysis of Variance by Ranks test (for overall effect of treatment) [34, 35, 39, 40].

3. Results

3.1 Rapid estrogen signaling does not activate MAPKs in macrophages in vitro

In addition to their classic gene expression regulatory functions in the nucleus, ERs have been shown to rapidly (minutes to hours) activate intracellular signaling cascades, including MAPKs such as p38 MAPK isoforms and extracellular signal regulated kinases (ERK) [41]. Given the link between estrogen and p38α MAPK in our model, we sought to determine whether this signaling cascade was functional in myeloid cells, since the majority of the previous studies on rapid estrogen signaling were done in cancer cell lines [41]. To this end, thioglycolate-elicited peritoneal macrophages and bone marrow-derived macrophages (BMDM) cultured in hormone-free media were treated with various concentrations of estradiol (E_2). E_2 stimulation across a wide range of concentrations and time intervals failed to elicit detectable phosphorylation of p38 MAPK isoforms or ERK (Fig. 1A and B). To ensure that our cells could respond to estrogen stimulation, we measured the expression of 4 select genes whose expression has been previously shown to be regulated by E_2 stimulation in mouse macrophages [42]. E_2 stimulation led to a dose-dependent effect on gene expression of these genes (Fig. 1C), confirming that our cells can respond to estrogens, at least at the transcriptional level. Taken together, these results suggest that rapid activation of p38 MAPK isoforms and ERK by estrogen is not an active signaling mechanism in myeloid cells, leading us to focus on genomic actions of ERs in subsequent experiments.

3.2 Estrogen enhances *in vitro* macrophage pro-inflammatory cytokine production independent of p38α

Estrogen simulation has been shown to modulate macrophage responses *in vivo* and *in vitro* [43–45], as does genetic inhibition of p38α MAPK [36, 46, 47]. Since we have previously demonstrated that ovariectomy, and hence removal of estrogen and other ovarian hormones, abolishes pro-inflammatory effects of p38α *in vivo*, we hypothesized that a similar effects may be recapitulated *in vitro*, by addition or removal of estrogen. To

determine whether estrogen signaling modulates the effects of p38α MAPK inhibition, BMDMs were differentiated from myeloid p38α-deficient mice (p38αCKO^{LysM}) or WT littermate controls (p38α-flox/flox Cre-negative littermates) in the presence or absence of E_2 in hormone-free media, followed by LPS stimulation and measurement of the proinflammatory cytokines IL-6 and TNFα, and the anti-inflammatory IL-10 cytokine. p38α deficiency increased LPS-induced IL-6 production, decreased IL-10 production, and had no significant effect on TNFα production, in line with our published results [36]; however, this occurred in a E_2-independent manner (Fig. 2A–C). E_2 treatment itself increased IL-6 and TNFα production (Fig. 2A and B), and decreased IL-10 production (Fig. 2C), but this occurred in a p38α-independent manner (i.e., to a similar extent in both WT and p38α-CKO cells). These results suggest that estrogen exposure *in vitro* does not modulate p38α-dependent pro- or anti-inflammatory functions in macrophages, and that inhibition of p38α does not affect the pro-inflammatory effects of estrogens on macrophages. Given that many *in vitro* effects of estrogen are not recapitulated *in vivo,* and vice versa [38, 44, 48], we subsequently focused on *in vivo* experiments.

3.3 *Esr1* regulates sex-specific effects of p38α in EAE

We have previously shown that the sex-specific protective effect of myeloid p38α ablation in females is reversed by removal of adult sex hormones by gonadectomy [21]. Given that estrogen is a key sex hormone produced by the ovaries, with known immune-modulatory properties in EAE [11, 12], we hypothesized that endogenous estrogen signaling is required for the protective effects of p38α ablation in females. This hypothesis was tested by genetic ablation of the two canonical nuclear receptors for estrogen, ERα/*Esr1* and ERβ/*Esr2*. Mice globally deficient in *Esr1* (*Esr1−/−*) and *Esr2* (*Esr2−/−*) [31, 32] were crossed to p38αCKO^{LysM} mice to generate mice singly deficient in p38α, *Esr1,* or *Esr2*, or doubly deficient for p38α/*Esr1* or p38α/*Esr2* (see Table 1 for explanation of genotypes). EAE was induced in the resulting mice as previously described (see Materials and Methods). In *Esr1/2*-sufficient control mice (*Esr+/+*), p38α deficiency was protective in female mice, and had no effect in male mice (Fig. 3A and B). This is consistent with our published p38α-dependent sex difference, whereby we saw protection in female, but not male p38αCKO^{LysM} mice [21]. Surprisingly, in *Esr1−/−* and *Esr2−/−* females, p38α deficiency remained protective (Fig. 3C and E), suggesting that signaling by these two receptors is may not be required for the female-specific role of p38α. *Esr2−/−* males behaved similar to control *Esr1/2*-sufficient mice, whereby no protective effect p38α deletion was seen (Fig. 3F). In contrast, p38α deficiency was protective in *Esr1−/−* males (Fig. 3D), thus phenocopying the females, and suggesting that endogenous estrogen signaling through ERα/*Esr1* unexpectedly regulates the role of p38α in EAE in males.

Additionally, we reanalyzed these data to examine the role of endogenous estrogen signaling through ERα/*Esr1* and ERβ/*Esr2* in EAE, independent of p38α (i.e. in p38α-sufficient, p38$^{f/f}$ LysM-Cre-negative mice). This analysis revealed that *Esr1*, but not *Esr2* deficiency in females significantly exacerbated EAE, while deletion of either receptor did not affect EAE in males (Fig. 3G and H). These results suggest that endogenous estrogen signaling via ERα/*Esr1* in females is protective, in line with the published protective effects of endogenous estrogen signaling via *Esr1* in B6 female mice [49]. This also suggests that

estrogen signaling does not modulate EAE in males unless the p38α signaling pathway is also perturbed.

Although global *Esr1* deficiency in males was sufficient to render p38α deletion protective in males, and insufficient to abrogate protection in p38αCKO females (Fig. 3D), one cannot exclude a potentially confounding global developmental effect on sexual differentiation and organismal development, since *Esr1* controls proper development of male and female sex organs [31]. To rule out this possibility, and to determine whether *Esr1* signaling controlling this phenotype is intrinsic to myeloid cells, we conditionally deleted in *Esr1* in myeloid cells using LysM-Cre. Mice carrying a floxed allele of *Esr1* [31, 32] were crossed to p38αCKO^{LysM} mice to generate mice singly conditionally deficient in *Esr1* (Esr1-CKO), as well as conditionally double deficient in both p38α and *Esr1* (p38α/Esr1-CKO) (see Table 2 for detailed description of genotypes). EAE was induced in the resulting mice as above. Myeloid cell-specific deficiency in *Esr1* alone did not significantly affect EAE severity in females or males (Fig. 4A and B), suggesting that the effects of global *Esr1* deletion in p38α-sufficient females (shown in Fig. 3G) cannot be ascribed to myeloid cell *Esr1* alone. However, conditional deletion of *Esr1* in myeloid cells abrogated the protection in p38α-deficient females (Fig. 4C), suggesting that myeloid-intrinsic ERα signaling promotes the pathogenic role of p38α in females, as previously suggested by our ovariectomy experiments [21]. Myeloid-specific deletion of *Esr1* rendered deletion of p38α highly protective in males (Fig. 4B), thus phenocopying the effect of global *Esr1*$^{-/-}$ (Fig. 3D), and orchiectomy [21]. These results suggest that myeloid cell-intrinsic *Esr1* signaling regulates the EAE-promoting role of p38α in males and in females in opposing fashion.

3.4 *Esr1* controls transcriptional programming of p38α-dependent pathways in myeloid cells

In order to understand the molecular mechanisms by which *Esr1* modulates the role of p38α in myeloid cells, we performed transcriptional profiling of macrophages singly deficient in p38α or doubly deficient in p38α and *Esr1*. Since the modulation of p38α effects by *Esr1* deletion was most pronounced in males (Fig. 4D), we chose to restrict this analysis to male macrophages. BMDM were isolated as above, from p38αCKO^{LysM} and p38α/Esr1-CKO^{LysM} male mice, or control male WT littermates (Cre-negative, p38α$^{f/f}$ and p38α$^{f/f}$/Esr1$^{f/f}$). In order to induce the expression of p38α-dependent inflammatory genes, macrophages were stimulated for 4 hours with LPS, a TLR4 stimulus that robustly activates p38α (Fig. 1A), followed by RNA isolation and transcriptional profiling (see Materials and Methods).

We first identified genes whose expression was dependent on p38α in the presence of a functional *Esr1* allele, by comparing WT vs. p38αCKO macrophages. This identified 204 differentially expressed genes, 106 of which were downregulated in the absence of p38α, while 98 genes were upregulated (Fig. 5A and B). Differentially expressed genes (DEGs) included *Il10, Il12b, Ccr1, Ccr3, U90926,* and *Serpinb2,* which were among p38α-regulated genes that we have previously identified in MTB-stimulated peritoneal macrophages [21]. Additionally, we identified a large number of genes not previously known to be regulated by p38α in macrophages, including *Il19, Inpp1, Spic, Icosl, and Ctsk* (Fig. 5A and Table S2).

Next we compared the effect of *Esr1* deficiency superimposed onto p38α deficiency (i.e. WT vs. p38α/Esr1-CKO; double CKO, abbreviated as DKO). This analysis identified 190 DEGs, 71 of which were also differentially expressed in the WT vs p38α comparison (above), and 109 that were unique to DKO (Fig. 5B and Table S3), suggesting that *Esr1* deficiency significantly modulates the p38α-dependent transcriptional landscape.

As a more stringent way to identify p38-dependent genes that were the most significantly modified by *Esr1* deficiency, we directly compared DKO to p38αCKO. This analysis identified 24 DEGs (Fig. 5C and Table S4), with 19 genes that were upregulated in DKO vs. p38αCKO, and 5 that were downregulated (Fig. 5D). We compared the expression of these genes across the three genotypes (WT, p38αCKO, and DKO). Strikingly, the majority of the genes that were upregulated in the double CKO relative to p38αCKO were initially downregulated in the p38αCKO relative to WT, and thus restored to close to or above WT levels by the double CKO, e.g., *Timp3*, *Sparc, Serpinb2, and Ctsk* (Fig. 5D), with the latter gene exhibiting particularly high statistical significance in both comparisons (Fig. 5A and C). The inverse was also true for the some of the genes downregulated in DKO relative to p38αCKO, e.g. *Ch25h* (Fig. 5C). Select DEGs from each comparison were additionally validated by RT-qPCR (Fig. S2A). Taken together, these results suggest that *Esr1* regulates the dependence of specific genes on p38α in macrophages, thereby regulating the outcome of p38α inhibition in EAE (Fig. 3 and 4).

To identify key molecular pathways impacted by p38α/*Esr1* co-dependent transcriptional modules, and their regulation by *Esr1*, we utilized upstream regulator analysis across the two differential expression comparisons above, p38αCKO vs. WT and DKO vs. WT (see Materials and Methods). This approach identified significant enrichment of a number of central inflammatory signaling mediators, such as IL-1A/B, STAT3, TGFB1. Several of these were expected as known pathways regulated by p38α, such as IL10 [36, 46] and DUSP1 [46], and p38 MAPK itself. Interestingly, the p38 MAPK regulator module was downregulated (negative Z-score) by p38αCKO deficiency, as expected, but was in fact upregulated in the DKO (Fig. 5E), suggesting that *Esr1* deficiency abrogates the dependency of multiple gene modules on p38α, consistent with Fig. 5D. Additionally, the estrogen receptor module was a significant positive regulator of p38α-dependent genes (Fig. 5E), in line with our previous bioinformatic analyses of p38α-dependent genes in a different macrophage model [21]. In contrast, in DKO cells, this module was downregulated (Fig. 5E), suggesting that *Esr1* regulates this p38α-dependent transcriptional cluster. Several other upstream regulator modules, including SMARCA4, TGFB1, and BRD4 showed similar opposing effects in DKO vs. p38α-CKO, while other regulator modules, such as IL1A, STAT3, IL10, showed minimal influence of the *Esr1* deletion (Fig. 5E). Taken together, these results demonstrate that *Esr1* modulates the dependence of multiple transcriptional modules on p38α in myeloid cells, in particular those regulated by TGFB1, BRD4, and SMARCA4.

4 Discussion

4.1 Comparison of effects of sex hormone depletion vs. global and conditional depletion of Esr1

Based on our published results with gonadectomy experiments [21], we had hypothesized that gonadal hormones, in particular estrogen and testosterone, respectively, promoted the dependence of EAE on p38α in females, and promoted the lack of dependence of EAE on p38α in males. While global deletion of *Esr1* and *Esr2* did not affect the pathogenic role of p38α in females, myeloid-specific deletion of *Esr1* abrogated it, similar to ovariectomy. The lack of effect seen with the global knockout likely has to do with confounding developmental effects in *Esr1*−/− females, which fail to undergo normal sexual development [14]. Of note, in our previous studies [21], the ovariectomies were performed in adult animals which had undergone normal sexual differentiation, and are thus more comparable to the conditional ablation of *Esr1* herein. Thus, we conclude that signaling by endogenous estrogens via ERα intrinsic to myeloid cells promotes the female-specific EAE-promoting role of p38α. How exactly this is achieved at the molecular level remains to be determined, but we propose that ERα activity results in transcriptional reprogramming of the "wiring" of p38 MAPK-related signaling cascades and/or effector molecules in myeloid cells, as supported by our transcriptional profiling data of male macrophages (discussed below). Additionally, given the previously well documented role of the sex chromosome complement in EAE [9, 50, 51], it would be of interest in future studies to examine sex hormone-independent effects on the p38α pathway in EAE, using, e.g., the four core genotypes model [52].

Contrary to our expectations, global or conditional ablation of *Esr1* in males had the opposite effect compared with females, unexpectedly promoting a pathogenic role for p38α in EAE. While at first glance this effect may be unexpected, estrogen signaling is well documented to play critical roles in various aspects of male physiology [31, 53]. In males, aromatase expression in various tissues allows for conversion of circulating testosterone to estrogen locally, including the CNS and other peripheral tissues [53]. Thus, we believe that *Esr1* deletion in males in this study phenocopies our published results with orchiectomy [21] because testes-derived testosterone is converted to estrogen, which signals via *Esr1* in myeloid cells to transcriptionally reprogram their dependence on p38α signaling in EAE. This conversion could happen locally in the inflamed CNS, where it would regulate the function of infiltrating myeloid cells.

Numerous previous studies, from the Voskuhl, Offner, Guery groups and others have used genetic deletion of *Esr1* and *Esr2* globally and in a cell-type specific manner, to identify the targets of exogenous estrogen-mediated suppression of EAE (reviewed in [11, 12]). The dominant view that has emerged is that most of the anti-inflammatory effects of exogenous estrogens are mediated by *Esr1* signaling on hematopoietic-derived immune cells, most prominently CD4 T cells. Meanwhile, the neuroprotective effects of exogenous estrogens are mediated by mostly by *Esr2* signaling in CNS resident cells, such as microglia or oligodendrocytes. With regard to *endogenous* estrogens, the results are somewhat conflicting, with different groups showing that ovariectomy either exacerbates EAE, while

others reported that this treatment does not affect EAE, possibly dependent on the timing of the surgery and other factors [11, 12]. Intriguingly, Guery and colleagues showed that ovariectomy exacerbates EAE in WT, but not *Esr1*-deficient female B6 mice, a difference that was controlled by non-hematopoetic derived cells [49], suggesting that endogenous estrogen signaling via *Esr1* is protective in females. Our results demonstrating exacerbated EAE in *Esr1*−/− females (in p38α-sufficient mice) are in agreement with these findings. Additionally, our results provide new mechanistic information on a signaling pathway intrinsic to myeloid cells that exhibits cross-talk with endogenous estrogen signaling to regulate EAE in a sex-specific manner.

4.2 Transcriptional regulation of macrophage gene expression by p38α

Our transcriptional profiling experiments in male macrophages identified novel p38α-dependent genes, a subset of which was co-regulated by *Esr1*. With regard to p38α-dependent genes in the context of a wild-type *Esr1* (WT vs. p38αCKO comparison), we provide the first (to our knowledge) genome-wide transcriptional profile of p38α-dependent genes in LPS-stimulated BMDM. Many early studies of p38 MAPK have suggested that this kinase acts as a broad positive regulator of pro-inflammatory mediator production by myeloid cells, akin to NFκB [17]. However, most of these studies have relied on first generation chemical inhibitors of p38, the specificity of which is likely much broader than p38 MAPKs alone [54–56], and we have recently shown that indeed such first generation p38 inhibitors suppress cytokine production in p38α-deficient macrophages [36]. Subsequent studies using genetic targeting of p38α have suggested that this kinase regulates a much smaller subset of pro-inflammatory genes, as well as regulating several key anti-inflammatory genes [21, 46, 57]. Consistent with those findings, in this study we show that the expression of the anti-inflammatory cytokine gene, *Il10*, and the MAPK phosphatase, *Dusp1*, was highly dependent on p38α, as previously shown by us and others [21, 36, 46]. These genes are induced as part of a negative feedback loop that serves to limit inflammation, which has likely contributed to the difficulty of targeting p38α in clinical settings [17, 58, 59]. Additionally, our study has identified the IL-10-family cytokine, *Il19*, as one of the most significantly downregulated genes in p38α-deficient macrophages. The role of this enigmatic cytokine is still being worked out, but like IL-10, it appears to play mainly anti-inflammatory roles [60], suggesting that it serves as a novel part of the p38α-dependent negative feedback loop. We also show that *Il12a* and *Il12b* genes, encoding the two subunits of the proinflammatory cytokine IL-12 are upregulated in p38α-deficient cells. Interestingly, IL-12 appears to be protective rather than pro-inflammatory in the EAE model [61], suggesting that its upregulation may contribute to the suppression of EAE. In contrast, chemokine receptor genes, *Ccr1*, *Ccr3*, and *Ccr5* are downregulated in p38α-deficient macrophages, which may contribute to impaired recruitment of pathogenic monocyte/macrophages into the inflamed CNS, which is supported by our published data in of p38αCKO^{LysM} female mice [21]. Taken together, these results suggest that the net effect of myeloid p38α-deficiency on EAE in *Esr1*-intact males may be zero, due to the loss of both anti- and pro-inflammatory pathways. However, our results demonstrate that deficiency in *Esr1* partially reprograms the p38α-dependent transcriptional landscape, and thus tips the scale towards EAE suppression in Esr1/p38αCKO^{LysM} male mice, as described below.

4.3 Co-regulation of the myeloid cell transcriptional landscape by p38α and Esr1

With regard to the co-regulation of the myeloid cell transcriptional landscape by p38α and *Esr1*, several genes and transcriptional modules are of particular interest. The gene *Ctsk*, encoding the lysosomal cysteine protease cathepsin K, stands out as the most significantly differentially expressed gene in both the WT vs. p38αCKO and p38αCKO vs. p38α/Esr1-CKO comparisons. This gene was downregulated in p38α-deficient macrophages, and its expression was restored by *Esr1* deficiency (Fig. 5D). Interestingly, cathepsin K was previously found to have a pathogenic role in experimental autoimmune arthritis and EAE, as mice globally deficient in this gene were resistant in both models [62]. However, the key cell type or the precise mechanism remain unclear, and it is likely that this gene plays additional roles in CNS-invading monocyte/macrophages in EAE. Several other genes followed a similar expression pattern to *Ctsk*, including *Timp3*, *Sparc*, and *Serpinb2*. Although the role of these genes in neuroinflammation is unclear, they do appear to be involved in regulating macrophage inflammatory functions [63–66]. An inverse pattern was seen for *Ch25h*, which was upregulated in p38α singly-deficient cells, and downregulated to WT levels by *Esr1* deficiency. *Ch25h* encodes the enzyme cholesterol 25 hydroxylase, which generates oxysterols that act as chemoattractants to leukocytes, and the deletion of which is protective in EAE [67]. Lastly, *Esr1* deficiency caused a further downregulation of *Ccr3* (already downregulated in single p38α-deficient cells), which as discussed above, could contribute to EAE resistance. The specific contribution of these differentially regulated genes can be evaluated in future studies.

At the level of transcriptional modules/regulators, we observed that several were differentially affected by p38αCKO vs. *Esr1*/p38αCKO deficiency (Fig. 5C). The observed opposing effects on the p38 MAPK and estrogen receptor regulator modules were expected, and consistent with *Esr1* co-regulating p38α-dependent genes, as discussed above. Three additional regulators with similar opposing effects were identified: TGFB1, BRD4, and SMARCA4, whereby double deficiency caused upregulation of these pathways. The protective role of the TGFβ cytokine in EAE and neuroinflammation is well established [68], and consistent with our findings. The function of the other two regulator genes in this context is less clear, but recent evidence exists for a role of BRD4 in regulating macrophage function [69]. Taken together, these results suggest that *Esr1* co-regulates the p38α-dependent transcriptional landscape in myeloid cells to alter their pathogenicity in EAE.

5. Conclusions

Although great progress in developing disease-modifying therapies for MS has been made in the recent years, the response to treatment varies greatly across individuals, and improved and personalized treatments are desired [70]. Our results suggest that biological sex and sex steroid hormones could play an important role in modifying treatment outcome, which is an important consideration for evaluating the efficacy of existing or future treatments. Additionally, our results suggest the possibility of combinatorial targeting of sex hormone pathways together with other druggable pathways, such as the p38 MAPK signaling pathway. Such synergy could enhance the modest efficacy of sex hormone treatment in

recent clinical trials in MS [13], or the variable response to other disease modifying therapies [70].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Drs. Sylvia Hewitt and Ken Korach of the NIEHS for providing the estrogen receptor gene-targeted mice, as well as for their advice and consultation on the project. The Vermont Genomics Integrative Resource core facility is acknowledged for the support with transcriptional profiling experiments. This work was supported by research grant RR-1602–07780 from the National Multiple Sclerosis Society to DNK and a University of Vermont Fostering Interdisciplinary Scholarship, Arts & Research Grant to DNK.

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Highlights

- Sex differences in MS are well documented but poorly understood
- p38 MAP kinase signaling in myeloid cells regulates EAE in a sex specific manner
- The sex-specific role of p38 MAP kinase in EAE is regulated by estrogen receptor alpha, as demonstrated by genetic depletion of *Esr1*
- Estrogen receptor alpha and p38 MAP kinase co-regulate the transcriptional landscape of myeloid cells

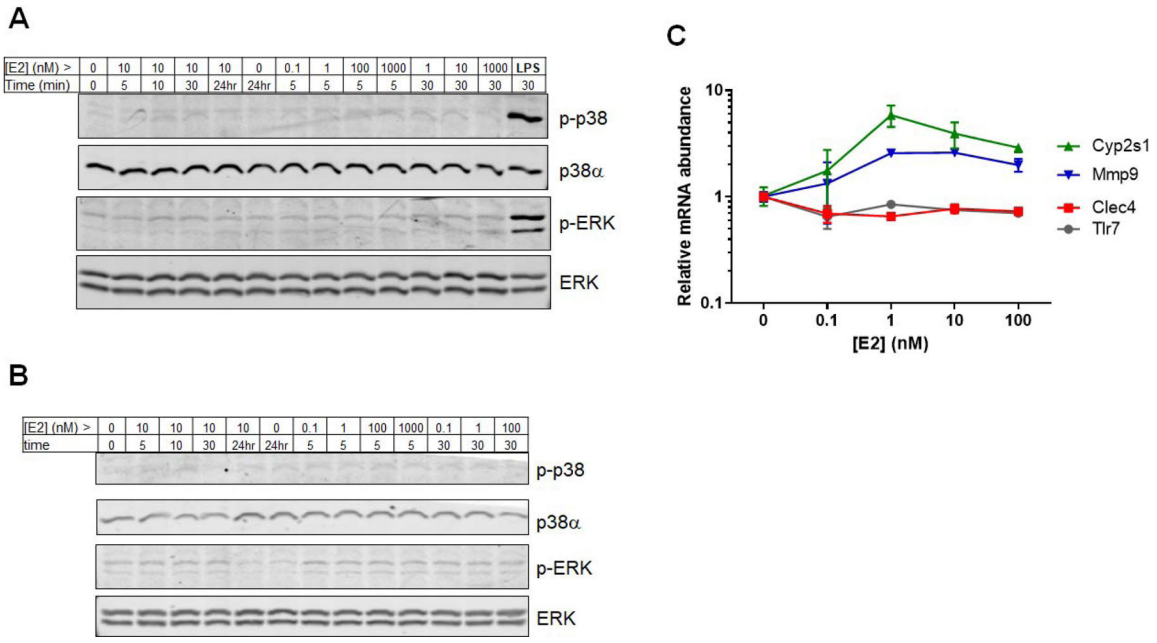

Figure 1. Estradiol (E2) does not induce phosphorylation of p38 or ERK MAPKs in macrophages.

(**A**) Peritoneal macrophages or (**B**) Bone marrow derived macrophages (BMDM) from WT C57BL/6J mice cultured in hormone-free media were stimulated with the indicated concentrations of E_2 for the indicated period of time, or with 100 ng/mL LPS for 30 minutes as a positive control. Cells were lysed and subjected to immunoblot for phosphorylated p38 (p-p38) or ERK (p-ERK), or total p38α and ERK. (**C**) WT BMDM were stimulated with the indicated concentration of E_2 for 24 hrs, followed by RNA extraction, cDNA synthesis, and qPCR measurement of expression of the indicated genes, normalized by the expression of house-keeping gene *B2m* and further normalized to the level of unstimulated cells.

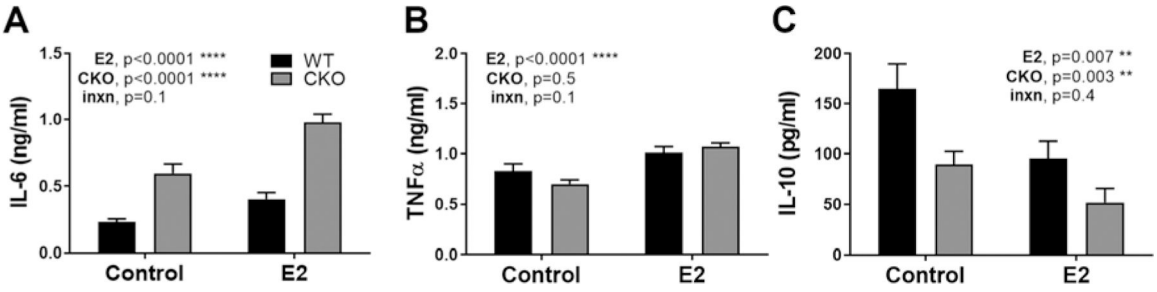

Figure 2. Estradiol (E2) enhances *in vitro* macrophage pro-inflammatory cytokine production independent of p38α.

BMDM from WT or p38αCKO^{Lysm} (CKO) mice were differentiated in cultured in hormone-free media in the presence 10 nM E_2 or ethanol vehicle (control) and stimulated with 100 ng/mL LPS. Production of IL-6 (**A**), TNFα (**B**), and IL-10 (**C**) was measured using ELISA. Significance of effect of genotype (WT or CKO), E_2 treatment, or their interaction was determined by two-way ANOVA.

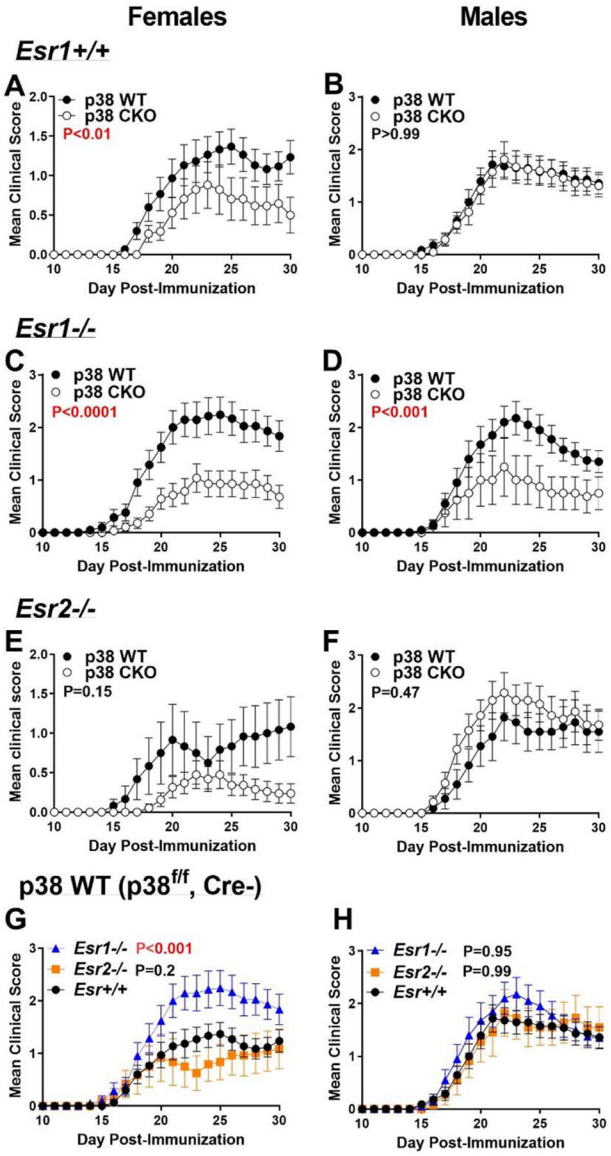

Figure 3. Global *Esr1* ablation modulates the effects of p38α deletion in males.

EAE was induced in female (left panels) and male (right panels) *Esr+/+* (**A** and **B**), *Esr1−/−* (**C** and **D**), and *Esr2−/−* (**E** and **F**), $p38^{f/l}$ littermate (p38 **WT**) and $p38\alpha CKO^{LysM}$ (p38 **CKO**) mice as in described in the Materials and Methods. In (**G**) and (**H**), data for p38αWT mice from (**A**) – (**F**) were reanalyzed for effect of *Esr1* and *Esr2* deficiency. Data represent 3–4 pooled independent experiments for each strain combination. Data were analyzed by Friedman's non-parametric two-way ANOVA for effect of strain over time (strain * time interaction), the significance of which is indicated in the panels (in red font if P<0.05). For panels (**G**) and (**H**), the P values indicate comparisons between *Esr1−/−* and *Esr1/2+/+*, and *Esr2−/−* and *Esr1/2+/+*, respectively. *Esr1/2+/+* mice were generated in parallel with *Esr1−/−* and *Esr2−/−* deficient mice from the same heterozygous × heterozygous crosses. See Table 1 for additional description of genotypes.

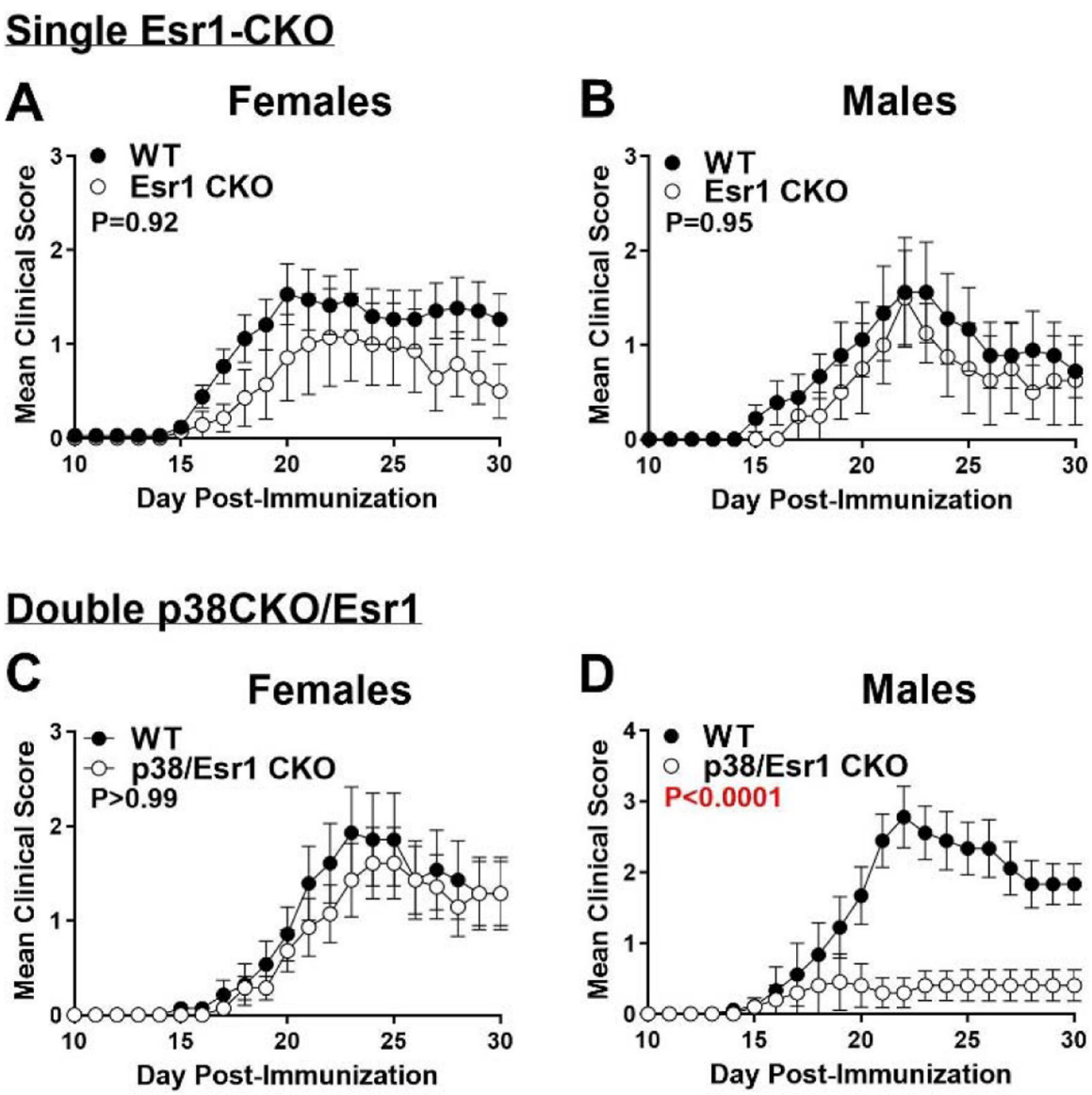

Figure 4. Myeloid cell-intrinsic *Esr1* signaling regulates the role of p38α in EAE in males.

EAE was induced in Esr1-CKO^{LysM} and WT littermate control (Cre-negative *Esr1*$^{f/f}$) female (**A**) and male (**B**) mice. EAE was induced in p38α/*Esr1*-CKO^{LysM} and littermate WT controls (Cre-negative p38$^{f/f}$ *Esr1*$^{f/f}$) female (**C**) and male (**D**) mice. Data represent 3 pooled independent experiments for each strain combination. Data were analyzed by Friedman's non-parametric two-way ANOVA for effect of strain over time (strain * time interaction), the significance of which is indicated in the panels (in red font if P<0.05). See Table 2 for additional description of genotypes.

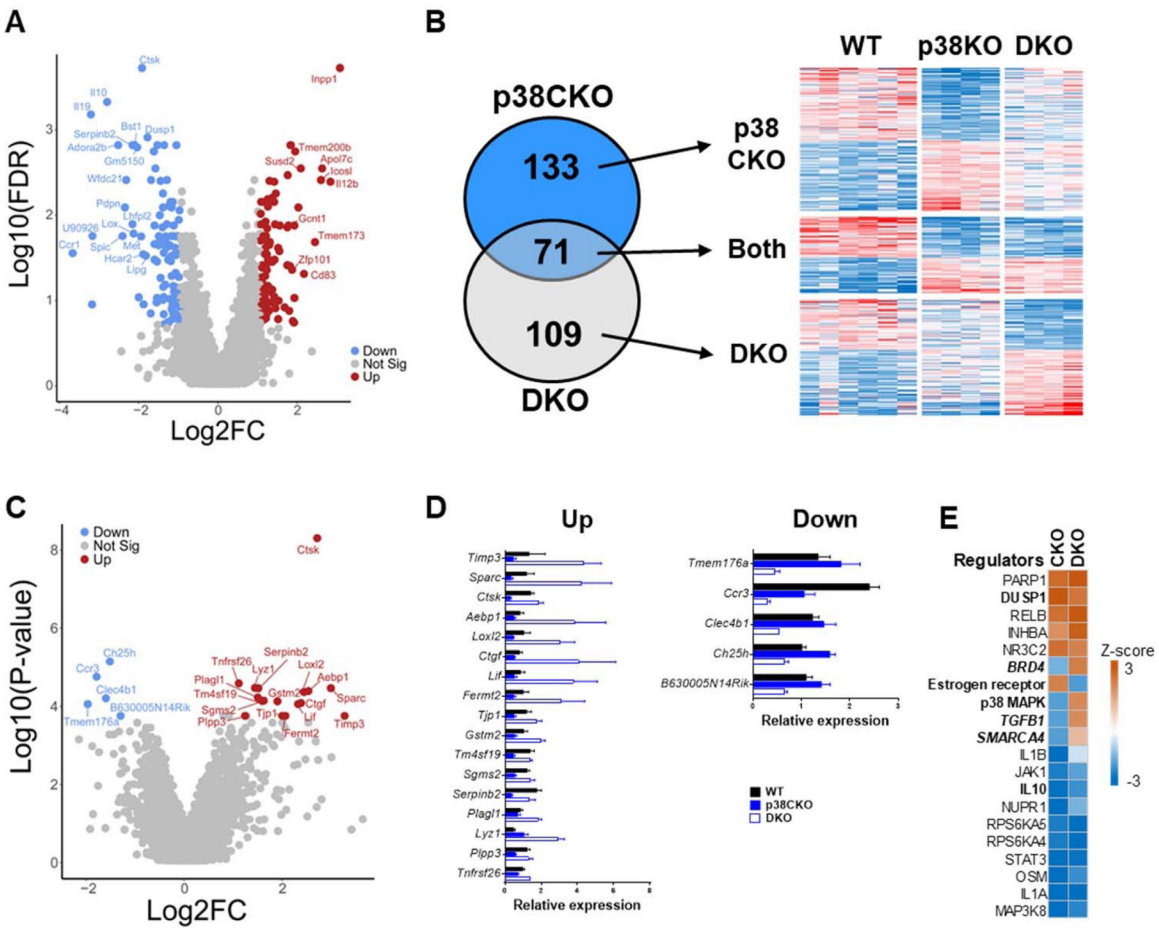

Figure 5. *Esr1* enables transcriptional reprogramming of p38α-dependent genes and pathways in macrophages.

BMDM were isolated from p38αCKO^{LysM} (n=4; CKO) and p38α/Esr1-CKO^{LysM} (n=4; DKO) male mice, or control male WT littermates (Cre-negative, p38αf/f and p38αf/f/Esr1f/f, n=4 and n=2, respectively; WT). Cells were stimulated for 4 hrs with 100 ng/mL LPS, followed by RNA extraction and microarray analysis, as detailed in the Materials and Methods. (**A**) Volcano plot showing DEG between WT and p38αCKO (p38CKO) macrophages (fold change (FC) expressed as p38CKO - WT). Colored genes indicate upregulated (red) and downregulated (blue) genes in p38CKO cells relative to WT passing the DE threshold (|FC|>2; FDR<0.2). The labeled genes represent the most highly differentially expressed genes (|FC|>3.5; FDR<0.05). Heatmap showing row-normalized expression for DEGs across WT, p38KO, and p38α/Esr1-CKO (DKO) cells, displaying significant DEGs in WT vs. p38CKO (top), WT vs. DKO (bottom), or in both, as indicated in the Venn diagram. (**C**) Volcano plot showing DEGs between DKO and p38CKO macrophages (FC expressed as DKO - p38CKO). Colored genes indicate upregulated (red) and downregulated (blue) genes in DKO cells relative to CKO passing the DE threshold (|FC|>2; FDR<0.2). (**D**) Row normalized expression of DEGs from in WT, p38CKO, and DKO macrophages. (**E**) Upstream regulator analysis was performed as described in the Materials and Methods. Z scores for the top 20 upstream regulators are shown. The sign and magnitude of the Z scores are indicative of the predicted strength and direction of

the genotype (p38CKO or DKO vs. WT) effect. See Table 2 for additional description of genotypes.

Table 1.

Nomenclature and details of knockout mouse genotypes, related to Figure 3. Abbreviations are as follows, +/+: homozygous wild type allele; −/−: homozygous knockout allele, f/f: homozygous floxed allele.

Nomenclature	Group (Esr phenotype)	p38α phenotype	Genotype			
			p38α	*LysM-Cre*	*Esr1*	*Esr2*
Esr+/+ p38αWT	*Esr+/+*	p38α WT	f/f	−	+/+	+/+
Esr+/+ p38αCKO		p38α CKO	f/f	+	+/+	+/+
Esr1−/− p38αWT	*Esr1−/−* (global)	p38α WT	f/f	−	−/−	+/+
Esr1−/− p38αCKO		p38α CKO	f/f	+	−/−	+/+
Esr2−/− p38αWT	*Esr2−/−* (global)	p38α WT	f/f	−	+/+	−/−
Esr2−/− p38αCKO		p38α CKO	f/f	+	+/+	−/−

Table 2.

Nomenclature and details of knockout mouse genotypes, related to Figures 4 and 5. Abbreviations are as follows, +/+: homozygous wild type allele; f/f: homozygous floxed allele.

Nomenclature	Group (*Esr1* phenotype)	*Esr1* phenotype	p38α phenotype	Genotype		
				p38α	*Esr1*	*LysM-Cre*
Esr1-WT	Single Esr1-CKO	WT	WT	+/+	f/f	−
Esr1-CKO		CKO	WT	+/+	f/f	+
p38αWT	Single p38α-CKO	WT	WT	f/f	+/+	−
p38αCKO (CKO)		WT	CKO	f/f	+/+	+
p38α/*Esr1*-WT	Double p38α/*Esr1*-CKO	WT	WT	f/f	f/f	−
p38α*/Esr1*-CKO (DKO)		CKO	CKO	f/f	f/f	+