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Inhibition of estrogen receptor alpha stabilizes regulatory T cell function in autoimmune hepatitis

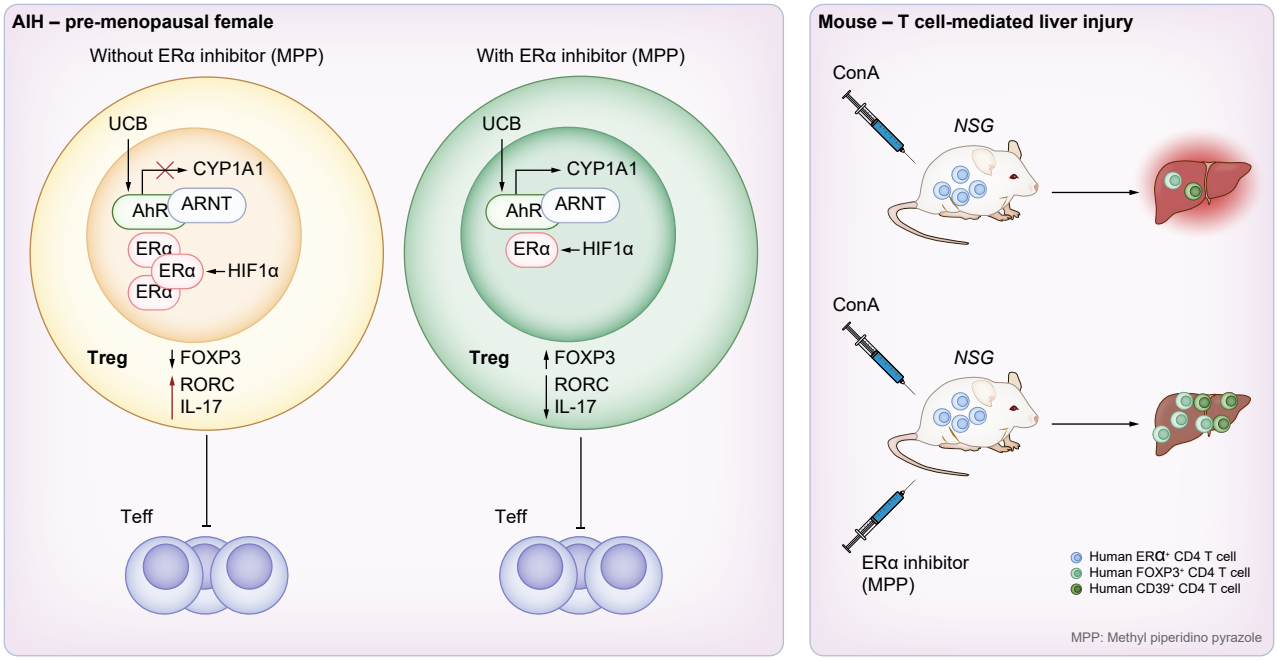
Authors

Lina Zhang, Barbora Gromova, Du Hanh Nguyen, …, Sizun Jiang, Satya K. Kota, Maria Serena Longhi

Correspondence

mlonghi@bidmc.harvard.edu (M.S. Longhi).

Graphical abstract



Highlights:

- ERα is upregulated in Tregs of pre-menopausal females with AIH.
- Blockade of ERα boosts suppression and limits plasticity in AIH Tregs.
- Blockade of ERα limits Concanavalin-A-induced liver injury in humanized mice.
- HIF-1α is the main factor inducing ERα in AIH Tregs.

Impact and implications:

Regulatory T cell (Treg) dysfunction plays a key role in the breakdown of immune tolerance in autoimmune hepatitis (AIH), a severe hepatopathy that often progresses to end-stage liver disease despite immunosuppression. Here we report that upregulation of estrogen receptor-alpha (ERα) alters Treg function and stability in pre-menopausal females with AIH, and that blockade of ERα using a specific antagonist stabilizes Tregs and curbs inflammation in humanized mice with liver injury. These findings implicate aberrant ERα signaling in the sex bias of AIH and support ERα inhibition as a potential therapeutic strategy to re-establish immune tolerance.

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Research article

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Inhibition of estrogen receptor alpha stabilizes regulatory T cell function in autoimmune hepatitis

Lina Zhang[1,2,†], **Barbora Gromova**[1,3,†], **Du Hanh Nguyen**[1,4,†], **Cortney Cagle**[1,†], **Graziela S. Gomes**[1,5,†], William Li[1], Li Gao[1,6], Wei Zhang[1,6], Jonathon J. Graham[1], Na Wang[1,7], Ahmadreza Kalbasi[1], Eva Csizmadia[1], Angelina Wei[1], Jessica Cassavaugh[1], Alan Bonder[8], Vilas Patwardhan[8], Sizun Jiang[9], Satya K. Kota[10], Maria Serena Longhi[1,*]

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Background & Aims: Regulatory T cell (Treg) dysfunction is a hallmark of autoimmune hepatitis (AIH), a serious hepatopathy often progressing to end-stage liver disease despite immunosuppression. Treg impairment in AIH has been linked to defective signaling through the aryl hydrocarbon receptor (AhR), a modulator of adaptive immunity. Herein, we investigated whether increased estrogen receptor alpha (ERα), an AhR non-canonical binding partner, impacts Treg immunity and contributes to the sex bias observed in AIH.

Methods: Tregs were isolated from the peripheral blood of pre-menopausal, post-menopausal and male patients with AIH, as well as controls. Their function, stability, transcriptome and response to AhR were assessed in the absence or presence of methylpiperidinopyrazole (MPP), an ERα-selective antagonist. Therapeutic effects of MPP were tested in a model of Concanavalin-A-induced liver injury in humanized mice.

Results: ERα was upregulated in Tregs from pre-menopausal females with AIH (1.1 ± 0.3) compared with healthy pre-menopausal females (0.4 ± 0.1; $p = 0.038$), and in pre-menopausal compared with post-menopausal females with AIH (0.3 ± 0.1; $p = 0.04$). In pre-menopausal females with AIH, MPP enhanced Treg suppressive capacity (2.5-fold increase; $p = 0.001$), restored response to AhR activation (2-fold increase; $p = 0.03$), and reduced functional plasticity, evidenced by decreased IL-17A and RORC expression under pro-inflammatory conditions. MPP also ameliorated Concanavalin-A-induced hepatitis in *NOD/scid/gamma* mice reconstituted with human CD4+ ERα+ cells, as shown by reduced ALT levels (101 ± 24 *vs*. 25 ± 8; $p = 0.024$), diminished hepatic inflammation on histology, and increased intrahepatic CD4+FOXP3+ and CD4+CD39+ T-cell frequencies. Bioinformatic and inhibition analyses identified hypoxia-inducible factor-1α as a key inducer of ERα in AIH Tregs.

Conclusions: ERα upregulation drives Treg dysfunction in pre-menopausal females with AIH and likely contributes to the sex bias in the disease. ERα blockade stabilizes Tregs and limits inflammation *in vivo*, representing a promising therapeutic strategy to enhance and sustain immune tolerance in AIH.

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Introduction

Impairment of $CD4^+CD25^{high}FOXP3^+$ regulatory T cells (Tregs) plays an important permissive role in chronic inflammation and autoimmunity by enabling effector T lymphocytes to perpetrate tissue damage, like in autoimmune hepatitis (AIH).[1–4] AIH is an organ-specific autoimmune disease mainly present in females and characterized by hypergammaglobulinemia, serum auto-antibodies, and histological evidence of interface hepatitis.[5]

We have previously shown that Tregs obtained from the peripheral blood of patients with AIH display increased plasticity in a pro-inflammatory milieu and fail to adequately suppress effector T cells.[6] In AIH, impaired Treg suppression is linked to altered response to aryl hydrocarbon receptor (AhR) activation.[7] AhR is a transcription factor that mediates toxin responses and adaptive immunity, including Treg function.[8,9] After activation in the presence of exogenous or endogenous ligands, AhR translocates to the cell nucleus where it forms a complex with aryl hydrocarbon receptor nuclear translocator (ARNT), its canonical binding partner. The AhR/ARNT complex regulates downstream genes like cytochrome P4501A1 (*CYP1A1*), the main gene regulated by the AhR/ARNT complex, *IL-10*, *IL-22*, *IL-17* and *ENTPD1*, which encodes for CD39, an ectoenzyme key to immunotolerance, and the levels of which are markedly reduced in AIH Tregs.[6] We have noted that Tregs from patients with AIH express high levels of estrogen receptor alpha (ERα), a non-canonical binding partner of AhR. In AIH, ERα binds AhR with a higher affinity than ARNT,[7] this suggesting that Treg dysfunction in AIH might be linked to aberrant ERα levels and/or signaling.

* Corresponding author. Address: Department of Anesthesia, Critical Care and Pain Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215, USA; Tel.: + 1 617-735-2905, fax: + 1 617-735-2930.
E-mail address: mlonghi@bidmc.harvard.edu (M.S. Longhi).
† These authors share first authorship
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There is evidence that several autoimmune diseases like AIH are more prevalent in females and estrogen-mediated signaling is an important factor contributing to these differences.[10] In this regard, population studies in the context of inflammatory bowel disease, a condition frequently found in association with AIH, have linked increased estrogen levels, induced by oral contraceptive pills, to heightened risk of disease and worsened clinical symptoms,[11–13] implicating a role for ERα signaling in inflammation. This is supported by evidence that T cell-specific deletion of *Era* reduces activation and increases Foxp3 expression in a mouse model of colitis.[14] In contrast to ERα, estrogen signaling through ERβ, the ER isoform that dimerizes with ERα, has been associated with immune protection in experimental colitis, suggesting differential effects of ER isoforms in inflammation.[15,16]

Here, we investigated whether aberrant ERα levels impact Treg function and ability to respond to AhR activation in AIH; and possibly account for sexual dimorphism in this autoimmune condition.

We report that Tregs from pre-menopausal females with AIH display heightened levels of ERα, which is induced by hypoxia-inducible factor 1-alpha (HIF-1α), an AhR inhibitor. Blockade of ERα using a specific antagonist boosts Treg function and ability to respond to AhR activation while stabilizing the phenotype of these cells in an inflammatory milieu. The beneficial effects of ERα blockade are also noted *in vivo*, in a model of T cell-mediated liver injury in humanized mice.

Patients and methods

Patients

Peripheral blood mononuclear cells (PBMCs) were obtained from 72 patients with type 1 AIH characterized by anti-nuclear antibodies (ANA) and/or smooth muscle antibodies (SMA). At the time of diagnosis histopathological features of interface hepatitis on liver biopsy and ANA and/or SMA autoantibodies were present in all patients. At the time of the study, 20 patients were pre-menopausal, 33 were post-menopausal, and 19 were male. Pre-menopausal females were defined as those women with regular menstrual periods and without peri-menopausal or menopausal symptoms at the time of blood collection. Post-menopausal females were those women without a menstrual period for longer than 12 months. Fifty-four patients (14 pre-menopausal females, 24 post-menopausal females and 16 males) were studied during remission (*i.e.* normal transaminase levels), while 18 (6 pre-menopausal females, 9 post-menopausal females and 3 males) were studied during an episode of relapse. Patients on immunosuppression were treated with azathioprine (50-150 mg/day) alone or in combination with prednisone (4-12.5 mg/day); budesonide (3-6 mg/day) alone or in combination with azathioprine; mycophenolate (500-3,000 mg per day); or tacrolimus (0.5-1 mg per day). Eleven patients were not on immunosuppression at the time of study. Of these, 10 were in remission and underwent treatment withdrawal because of normal transaminase levels for more than 2 years; one patient was studied during an episode of relapse due to low adherence to medications. Clinical and laboratory data of patients with AIH are summarized in Table S1. Forty-eight matched healthy individuals (17 pre-menopausal females, 16 post-menopausal females, and 15 males) served as normal controls. All female patients and controls were not undergoing hormonal therapies at the time of the study.

Studies on human samples received IRB approval (protocol # 2018P000019) at BIDMC (Beth Israel Deaconess Medical Center), Boston, MA. Written consent was obtained from all study participants.

Details about measurement of estradiol levels, cell culture, inhibition studies, quantitative reverse-transcription PCR, flow cytometry, suppression assay, chromatin immunoprecipitation and immunohistochemistry staining are provided in the supplementary materials and methods.

Cell isolation and polarization

PBMCs were obtained by density gradient centrifugation on Ficoll-Paque (GE Healthcare Life Sciences, Pittsburgh, PA). The viability of PBMCs was tested by Trypan Blue exclusion and exceeded 98% in all cases. Tregs were purified using immunomagnetic beads ($CD4^+CD25^+CD127^{dim/-}$ Regulatory T cell Isolation Kit II human, Miltenyi; purity higher than 92% in all cases) (Fig. S1). For experiments presented in Fig. 3, Tregs were polarized from naïve CD4 cells, obtained from PBMCs by negative selection (Miltenyi Biotec, San Diego, CA). In these experiments, CD4 cells were resuspended in RPMI 1640 (Gibco, Thermo Fisher Scientific, Waltham, MA), supplemented with 10% FBS, and then exposed to Treg polarizing conditions for 5 days. These consisted of TGF-β (10 ng/ml), IL-2 (100 ng/ml) and Dynabeads Human T Activator CD3/CD28 for T cell expansion (Thermo Fisher Scientific) at a bead/cell ratio of 1/2. After 5 days, Treg phenotype was validated by flow cytometry (Fig. S2). In additional experiments, CD4 naïve cells were differentiated into T helper (Th)17 or Th1 cells following 5-day culture in the presence of IL-6 (50 ng/ml), TGF-β (3 ng/ml), IL-1β (25 ng/ml) and Dynabeads Human T activator CD3/CD28 at 1:50 for Th17 cells; and IL-12 (20 ng/ml) and anti-human IL-4 antibodies (10 μg/ml, clone # 34019, R&D Systems) for Th1 cells. All cytokines were purchased from Peprotech, Thermo Fisher Scientific.

NanoString assay

Total RNA was obtained from untreated and methylpiperidinopyrazole (MPP, Tocris Bio-Techne, Minneapolis, MN)-treated Tregs, using RNeasy Mini Kit (Qiagen, Germantown, MD), according to our previous protocols.[17–19] RNA quantification was determined by Nanodrops whereas RNA integrity was verified using Agilent Bioanalyzer. Samples with DV200 higher than 70% were used in subsequent steps. NanoString profiling was performed using the nCounter CAR T Characterization Panel (NanoString Technologies, Seattle, WA). 140 ng total RNA was used as input into hybridization reactions containing reporter and capturing probes, as per manufacturer's instructions. As we previously reported, RNA samples were hybridized at 65 °C for 24 h and subsequently processed and each analyzed in a single nCounter cartridge lane.[17,19] The post-hybridization process was carried out in a nCounter MAX Analysis System (NanoString Technologies). RCC files were compiled and analyzed using the nSolver Analysis Software (version 4.0). The internal reference genes included in the probe set were used for normalization. Untreated and MPP-treated Treg samples were run in triplicate and experiments repeated independently three times.

Induction and assessment of liver injury

Acute liver injury was induced in *NOD/scid/gamma* mice as reported before.[20] *NOD/scid/gamma* mice were adoptively transferred with human CD4 cells expressing ERα, using Concanavalin-A (Con-A) to reflect T cell-mediated liver inflammation.[21,22] As before, Con-A was used for its ability to activate human T cells.[23] Six-week-old female *NOD/scid/gamma* mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and kept under pathogen-free conditions. Mice were injected with 2-3 × 10^6 CD4 T cells, obtained from one healthy donor. Prior to injection, CD4 T cells were tested for ERα expression by quantitative reverse-transcription PCR and flow cytometry. After 3 weeks, mice were checked for human chimerism and those showing more than 10% human chimerism (about 75% of the injected mice) were administered MPP at 1 mg/kg 20 h before being given Con-A at 20 mg/kg i.v. Four hours later[20] mice were euthanized.

In an additional set of experiments, MPP was administered in 8-week-old female C57BL/6 mice (Taconic Biosciences, Germantown, NY) 16 h before Con-A injection. Eight hours later, mice were euthanized.[24]

In both *NOD/scid/gamma* and C57BL/6 mice, blood was withdrawn from the vena cava. Spleen and liver were collected from each recipient. Serum alanine aminotransferase (ALT) was measured using ALT (SGPT) - IFCC Liquid Reagent Set (Teco Diagnostic, Anaheim, CA) (normal range: 5-35 IU/L). Lymphocytes were isolated from spleen using Buffer EL Erythrocyte Lysis Buffer (Qiagen, Germantown, MD). Intrahepatic lymphocytes were obtained as previously described.[25] Briefly, liver tissue was mechanically disrupted, and the resulting homogenate was centrifuged at 40 *g* to remove the hepatocyte pellet and subsequently filtered using 70 μm filters. Staining was carried on using anti-human CD3 (clone # OKT3, Biolegend), CD4 (clone # OKT4, Biolegend), CD39 (clone # A1, Biolegend), IL-17A (clone # BL168, Biolegend), IL-10 (clone number # JES3-9D7, Biolegend), FOXP3 (clone # PCH101, eBioscience) and IFNγ (clone # B27, BD Pharmingen) antibodies when human lymphocytes from *NOD/scid/gamma* mice were analyzed; or with anti-mouse CD3 (clone # 145-2C11, Biolegend), CD4 (clone # RM4-5, Biolegend), FOXP3 (clone # MF-14, Biolegend), CD39 (clone # 24DMS1, eBioscience), IL-10 (clone # JES5-16E3, Biolegend), IL-17A (clone # TC11-18H10.1, Biolegend) and IFNγ (clone # XMG1.2, Biolegend), when lymphocytes from C57BL/6 mice were tested. Flow cytometry analysis was carried out, as indicated above. The full gating strategy is shown in Figs. S3 and S4.

The animal protocols (# 049-2021 and # 052-2024) were approved by the Animal Care and Use Committee at BIDMC, Boston.

Statistics

Details about NanoString, differential gene expression and gene set-enrichment analysis (GSEA) are provided in the supplementary materials and methods.

Results are expressed as mean ± SEM unless otherwise stated. Normality of variable distribution was assessed by Kolmogorov-Smirnov Goodness of fit test. Comparisons were performed using parametric (paired or unpaired Student's *t* test) or non-parametric (Mann-Whitney) test, according to data distribution (all two-sided). One-way ANOVA, followed by Tukey's multiple comparisons test, was used when analyzing more than two sets of data. Correlation was performed using Pearson's correlation coefficient. $p \leq 0.05$ was considered significant. Differences in categorical variables were analyzed using Chi-square test. Statistical analysis was performed using GraphPad Prism, version 9.20 (GraphPad Software, San Diego, CA).

Results

ERα is aberrantly elevated in Tregs of pre-menopausal females with AIH

We have previously shown that Tregs from patients with AIH display impaired response to AhR activation[7] and that ERα binds to AhR with higher affinity than the canonical partner ARNT, possibly interfering with the ability of AhR to regulate downstream pathways.

We measured ERα levels in Tregs isolated from the peripheral blood of pre-menopausal and post-menopausal females and males with AIH and compared these with Tregs obtained from age- and sex-matched controls to establish whether heightened levels of ERα are a unique feature of AIH Tregs or whether levels change according to sex and/or menopausal status.

We found that, in pre-menopausal females, *ERa* levels were higher in AIH than in healthy individuals (Fig. 1A and S5A-C). Among patients with AIH, levels of *ERa* were higher in pre-menopausal than in post-menopausal females (Fig. 1A and S5B,C). In AIH, levels of *ERb* tended to be higher in pre-menopausal females compared to post-menopausal females and males (Fig. 1B). In post-menopausal females, *ERb* was markedly decreased in AIH compared to health (Fig. 1B). In pre-menopausal females, the *ERb*/*ERa* ratio was significantly decreased in AIH compared to health, but not in other groups (Fig. 1C). Among patients with AIH, the *ERb*/*ERa* ratio was higher in pre-menopausal than in post-menopausal females and males, and inversely correlated with total bilirubin levels in pre-menopausal females with AIH ($R = -0.71$, $p = 0.05$) (Fig. S6A).

We next tested whether exposure to propyl pyrazole triol (PPT), an ERα-specific agonist, impacts the expression of genes linked to Treg function in patients with AIH and controls.[6,26,27] In healthy individuals, exposure of Tregs obtained from post-menopausal females resulted in decreased *FOXP3* levels (Fig. 1D), while PPT did not have a significant impact on Tregs of pre-menopausal females and males (Fig. 1D-G). In pre-menopausal females with AIH, exposure of Tregs to PPT did not substantially impact the levels of *FOXP3*, *IL-10* and *LGALS9* (encoding Galectin-9) (Fig. 1D,E,G), while increasing – albeit not significantly – those of *CD39* (Fig. 1F). The addition of PPT to Tregs obtained from male patients resulted in increased *IL-10* levels (Fig. 1E), whereas no significant changes were noted following addition of PPT to Tregs obtained from post-menopausal females (Fig. 1D-G).

Overall, these data show that ERα is markedly elevated in Tregs obtained from pre-menopausal females with AIH, and that Treg responses to PPT do not result in substantial changes in Treg gene profiles between AIH and health. These findings suggest that alterations of ERα signaling rather than the response to its specific agonist PPT might account for differences in Tregs of pre-menopausal females.

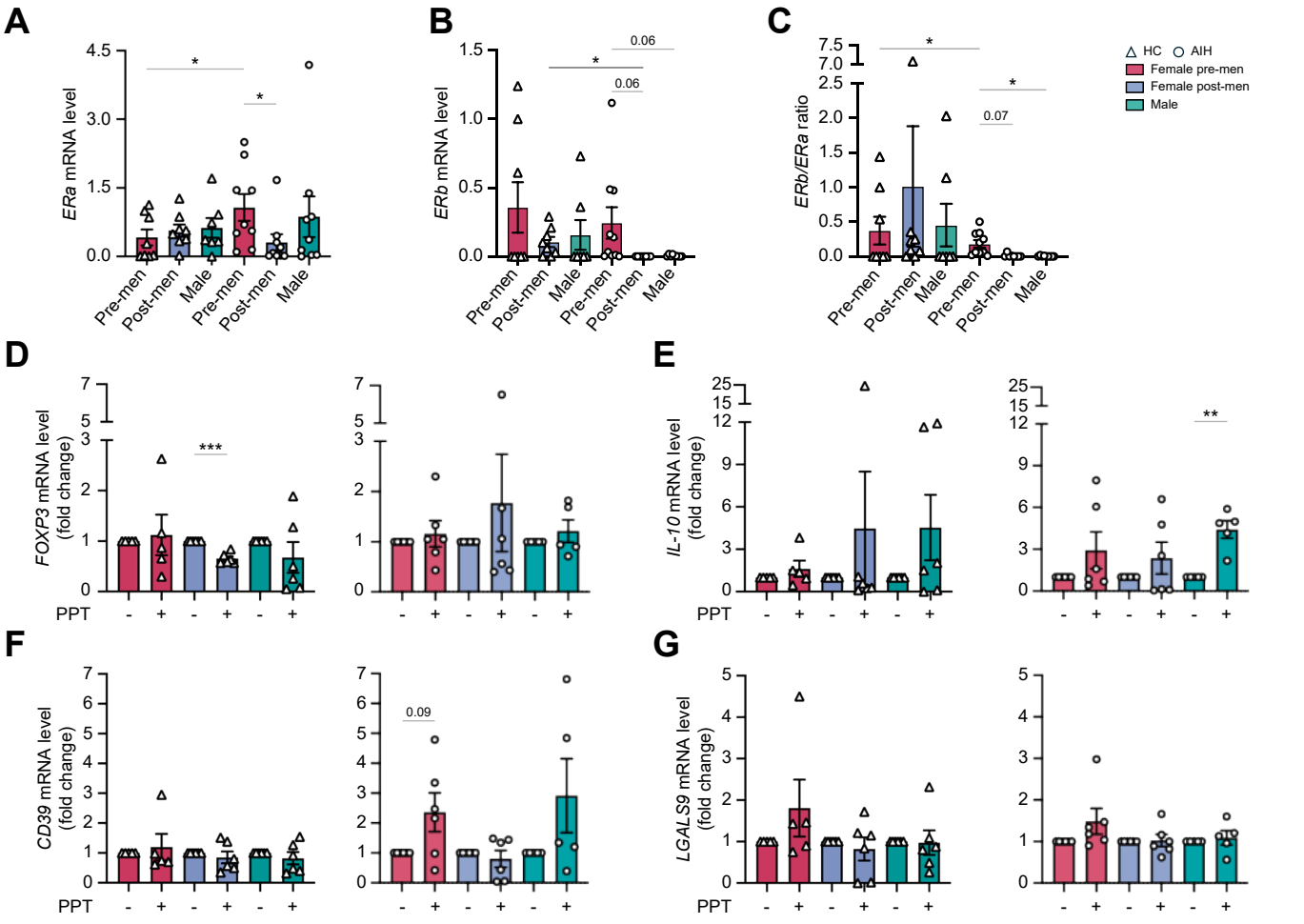


Fig. 1. Heightened ERα levels in Tregs of pre-menopausal females with AIH. (A-B) Mean ± SEM *ERa* and *ERb* mRNA levels in HCs and patients with AIH: pre-menopausal (HC, n = 8; AIH, n = 10), post-menopausal (HC, n = 8; AIH, n = 8), and males (HC, n = 7; AIH, n = 9). (C) Mean ± SEM *ERb/ERa* ratio in Tregs from HCs and patients with AIH: pre-menopausal (HC, n = 8; AIH, n = 10), post-menopausal (HC, n = 8; AIH, n = 8), and males (HC, n = 7; AIH, n = 9). (D-G) Mean ± SEM *FOXP3*, *IL-10*, *CD39* and *LGALS9* mRNA levels in Tregs of HCs and patients with AIH: pre-menopausal (HC, n = 5; AIH, n = 6), post-menopausal (HC, n = 6; AIH, n = 6) and males (HC, n = 6; AIH, n = 5), in the absence or presence of PPT. **p* ≤0.05; ***p* ≤0.01; ****p* ≤0.001 (Panels A-C: one-way ANOVA test; Panels D-G: two-sided paired *t* test). AIH, autoimmune hepatitis; ERα, estrogen receptor alpha; HCs, healthy controls; PPT, propyl pyrazole triol.

Blockade of ERα stabilizes Tregs in pre-menopausal females with AIH

We next determined whether blockade of ERα impacts Treg function, *i.e*. the ability of Tregs to suppress the proliferation of $CD4^+CD25^-$ cells. Tregs were exposed to the ERα-specific antagonist MPP prior to their co-culture with $CD4^+CD25^-$ responders. As depicted in Fig. 2A,B, addition of MPP boosted the suppression of Tregs isolated from healthy individuals and pre-menopausal females with AIH, particularly in the latter. In pre-menopausal females, enhancement of Treg suppression after exposure to MPP was successful in Tregs from all patients with AIH and in Tregs from 7 out of 13 healthy individuals (Chi-square $p = 0.031$). Treg suppression at baseline before MPP addition was impaired in AIH when compared to healthy pre-menopausal females (Fig. S7A). This difference was abrogated after Treg treatment with MPP (Fig. S7A).

Blockade of ERα tended to increase Treg suppression in healthy post-menopausal females without having significant effects on the function of Tregs isolated from post-menopausal females with AIH or males, with or without AIH (Fig. 2A,B).

In healthy pre-menopausal females and those with AIH, the addition of MPP decreased the proliferation of Tregs in the presence of IL-2 and anti-CD3/CD28 T cell activation beads (Fig. S7B). Treg proliferation in pre-menopausal females was higher in AIH than in health, both with or without MPP (Fig. S7B).

As we previously showed that AIH Tregs are plastic and tend to acquire effector cell features when exposed to a pro-inflammatory stimulus,[6,20] we exposed Tregs from pre-menopausal females to MPP in the absence or presence of IL-6 and IL-1β inflammatory challenge.[6] While in healthy individuals, Tregs did not undergo changes in the expression of FOXP3, RORC, IL-17A and IFNγ following challenge (Figs 2C-F and S7C–F), in AIH, decreased FOXP3 and increased RORC and IL-17A levels were noted after Treg exposure to IL-6 and IL-1β (Figs 2C-E and S7C–F). The addition of MPP to AIH Tregs restored FOXP3 while decreasing RORC and IL-17A to baseline levels (Figs 2C-E and S7C–F). In healthy Tregs, MPP reduced *IL-17A* and *IFNG* expression in the presence of pro-inflammatory stimulation (Fig. 2E,F).

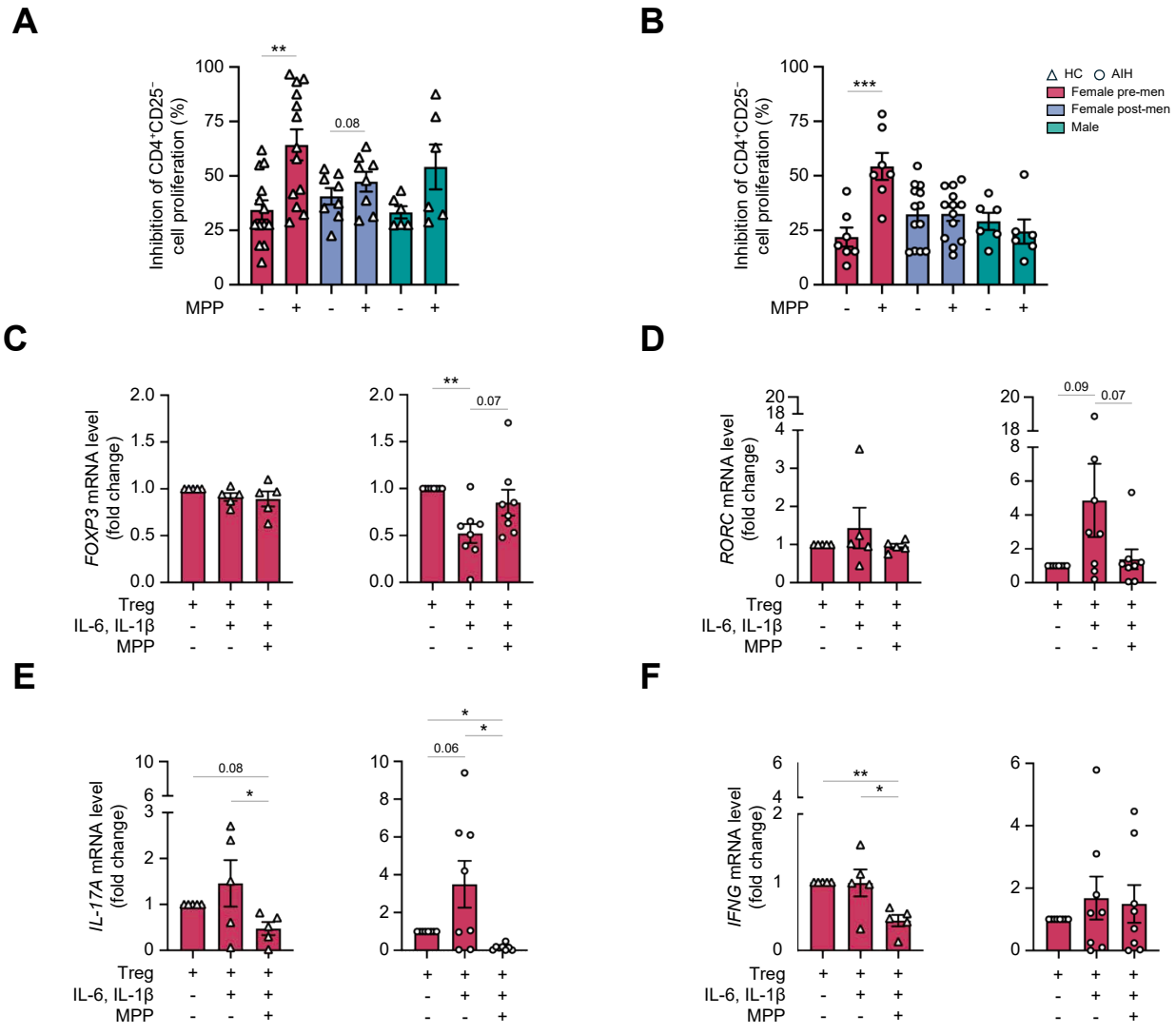


Fig. 2. Blockade of ERα ameliorates function and stability in Tregs of pre-menopausal females with AIH. Mean ± SEM percentage inhibition of $CD4^+CD25^-$ cell proliferation following addition of untreated or MPP-treated Tregs in (A) HCs and (B) patients with AIH: pre-menopausal (HC, n = 13, AIH, n = 7), post-menopausal (HC, n = 8, AIH, n = 13), and male (HC, n = 6, AIH, n = 6). Mean ± SEM (C) *FOXP3*, (D) *RORC*, (E) *IL-17A* and (F) *IFNG* mRNA levels in Treg cultures in the absence or presence of IL-6+IL-1β or with pro-inflammatory challenge and MPP. Tregs were obtained from pre-menopausal females (HC, n = 5; AIH, n = 8). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ (Panels A-B: two-sided paired *t* test; Panels C–F: one-way ANOVA test). AIH, autoimmune hepatitis; ERα, estrogen receptor alpha; HCs, healthy controls; MPP, methyl piperidino pyrazole; Treg, regulatory T cell.

No significant changes in the expression of *FOXP3*, *RORC*, *IL-17A* and *IFNG* were noted after addition of MPP in the presence of IL-6+IL-1β in Tregs obtained from post-menopausal females and males, with or without AIH (Fig. S8A–H).

To test whether the beneficial effects of ERα blockade are Treg specific, we exposed Th17 and Th1 cells, obtained from healthy individuals and pre-menopausal females with AIH, to MPP and measured levels of *IL-17A*, *RORC*, *FOXP3*, *IFNG* and *T-bet*. Treatment with MPP did not have significant effects on the expression of *IL-17A*, *RORC* and *FOXP3* in Th17 cells; and on the levels of *IFNG* and *T-bet* in Th1 lymphocytes of healthy individuals and pre-menopausal females with AIH (Fig. S9A–E).

Collectively, these data show that blockade of ERα boosts the suppressive function of Tregs in pre-menopausal females, especially in those with AIH. Notably, in AIH, blockade of ERα stabilizes Tregs in the presence of pro-inflammatory challenge.

Blockade of ERα restores response to AhR in pre-menopausal females with AIH

We next determined whether blockade of ERα impacts Treg response to AhR activation in the presence of unconjugated bilirubin (UCB), having previously shown that AIH Tregs fail to adequately respond to this AhR endogenous ligand.[7] Response to UCB was assessed in healthy and AIH Tregs, obtained from pre-menopausal and post-menopausal females and males, and measured as upregulation of *CYP1A1*, the main gene controlled by the AhR/ARNT complex, *CD39* and *IL-10*, which are also modulated by AhR/ARNT[7] (Table S2). In

healthy individuals, exposure of Tregs to UCB resulted in substantial upregulation of *CYP1A1* in pre- and post-menopausal females (Fig. 3A,C); increased *CD39* in all groups (Fig. 3A,C,E); and significantly increased *IL-10* in males (Fig. 3E). In AIH, exposure to UCB led to increased *CYP1A1* levels in males (Fig. 3F) and in heightened *CD39* and *IL-10* expression in post-menopausal females (Fig. 3D).

Exposure of Tregs to MPP resulted in increased *CYP1A1* and *CD39* levels in healthy pre-menopausal females (Fig. 3A), and in increased *CYP1A1* in pre-menopausal females and males with AIH (Fig. 3B,F). Compared to baseline levels, the combination of MPP and UCB significantly upregulated *CYP1A1* levels in pre-menopausal females with or without AIH (Fig. 3A, B) and in post-menopausal females with AIH (Fig. 3D); increased *CD39* levels in males with AIH (Fig. 3F); and resulted in upregulation of *IL-10* in pre-menopausal females with or without AIH (Fig. 3A, B) and males with AIH (Fig. 3F).

Treg exposure to UCB and MPP tended to increase *CYP1A1* in pre-menopausal and post-menopausal females with AIH (Fig. 3B,D); boosted *CD39* in post-menopausal females with AIH (Fig. 3D); and increased *IL-10* in pre- and post-menopausal females with AIH (Fig. 3B, D), compared to MPP alone, suggesting a synergistic effect of the two compounds.

Following treatment with MPP and UCB, we noted an increase in *CYP1A1* levels only in pre-menopausal females with AIH, compared to UCB alone (Fig. 3B). In pre-menopausal females with AIH there was an inverse correlation between *CYP1A1* expression in the presence of UCB and MPP and total bilirubin levels ($R = -0.859$, $p = 0.028$) (Fig. S6B).

Overall, these data show that response to AhR following exposure to UCB is impaired in pre-menopausal females with AIH but is restored when ERα is inhibited by MPP. Blockade of ERα using MPP boosts *CYP1A1* levels in healthy and AIH pre-menopausal females and in AIH males, suggesting a role for ERα in the control of AhR, also in the absence of activation.

Blockade of ERα limits liver inflammation *in vivo*

Given the beneficial effects of ERα blockade over Treg immunophenotypic and functional properties, we tested the effects of MPP *in vivo*, in a model of T cell-mediated liver injury induced by Con-A in *NOD/scid/gamma* mice repopulated with blood donor-derived CD4 cells expressing ERα[20] (Fig. 4A,B). After verifying CD4 T cell reconstitution 3 weeks after adoptive transfer, mice were treated with MPP, exposed to Con-A 20 h later, and sacrificed after 4 h (Fig. 4A). There was a decrease in serum ALT levels (Fig. 4C) and reduced inflammation on histology (Fig. 4D) in mice treated with MPP, compared to recipients exposed to Con-A and treated with vehicle (Fig. 4C,D). When analyzing the phenotypic profile of human intrahepatic and splenic lymphocytes, we noted that MPP-treated mice displayed increased proportions of intrahepatic $CD4^+FOXP3^+$ and $CD4^+CD39^+$ cells (Fig. 4E,F), and higher frequencies of splenic $CD4^+FOXP3^+$ and $CD4^+IL\text{-}10^+$ cells (Fig. 4J,L). No differences in the frequency of human CD4 cells positive for IL-17A or IFNγ were noted between mice treated with MPP or vehicle (Fig. 4H,I,M,N). Comparable results were obtained when using C57BL/6 mice, treated with MPP and then exposed to Con-A 16 h before the harvest (Fig. S10A). Treatment with MPP reduced ALT serum levels and histological inflammation (Fig. S10B and C), while boosting the frequency of intrahepatic $CD4^+CD39^+$ and $CD4^+IL\text{-}10^+$ lymphocytes (Fig. S10E and F).

Overall, these data indicate that MPP reduces liver inflammation *in vivo* while boosting intrahepatic CD4 lymphocytes with immunoregulatory properties.

HIF-1a induces ERα in pre-menopausal females with AIH

We next tested the effects of ERα blockade on the transcriptional profile of Tregs obtained from the peripheral blood of healthy individuals and pre-menopausal females with AIH. Using the nCounter CAR-T Characterization Panel (NanoString) as a T cell-targeted transcriptomic approach, we found that in Tregs obtained from healthy pre-menopausal females, treatment with MPP resulted in marked upregulation of IFN-related genes including IFN-induced protein with tetratricopeptide repeats (*IFIT2*), which encodes for a protein with anti-proliferative properties (Fig. 5A; Table S3); and in downregulation of *TYK2*, a gene implicated in autoimmune and inflammatory conditions[28] (Fig. 5A; Table S3). In pre-menopausal females with AIH, treatment of Tregs with MPP resulted in downregulation of genes like *CTSW*, which encodes for cathepsin W, a cysteine proteinase usually expressed by effector cells[29] that is also involved in restraining peripheral Tregs;[30] *CD96* that encodes for a molecule involved in T cell activation; and *CD84*, encoding for a membrane glycoprotein, which is a member of the signaling lymphocyte activation family (SLAM) (Fig. 5B; Table S4).

GSEA indicated that, in pre-menopausal females with AIH, MPP treatment was negatively correlated with gene sets associated with cell cycle, p53 pathway and endocytosis (Fig. 5C,D) and positively correlated with gene sets linked with MAPK and PI3K signaling pathways (Fig. 5C,D). No correlation between MPP treatment and gene sets was noted, when analyzing Tregs from healthy pre-menopausal females.

To determine the factors most likely involved in transcriptional induction of *ERa*, we conducted bioinformatic analysis of the *ERa* promoter using JASPAR and identified multiple predicted binding sites for HIF-1α, a transcription factor that was previously found to inhibit regulatory cell immunity under chronic inflammatory conditions;[7,31,32] Krüppel-like factor 6 (KLF6), an AhR non-canonical binding partner, which is upregulated in AIH Tregs;[7] and for signal transducer and activator of transcription 3 (STAT3), which has been linked to Th17 cell immunity.[33–35] Based on this prediction analysis, we tested the effects of *HIF-1a*, *KLF6* and *STAT3* knockdown on *ERa* levels in Tregs obtained from pre-menopausal females with or without AIH. While no changes in *ERa* levels were noted in Tregs from healthy individuals after silencing of *HIF-1a* and *KLF6* (Fig. 6A,B), in AIH, silencing of *HIF-1a* resulted in a marked decrease in the levels of *ERa* (Fig. 6D). Blockade of *KLF6* in AIH Tregs tended to decrease *ERa* without, however, reaching statistical significance (Fig. 6E). Silencing of *STAT3* did not change *ERa* levels in Tregs from healthy individuals or patients with AIH (Fig. 6C,F). Blockade of *HIF-1a*, *KLF6* or *STAT3* did not result in significant changes of *ERa* levels in Tregs from post-menopausal females and males with or without AIH (Fig. S11A-L).

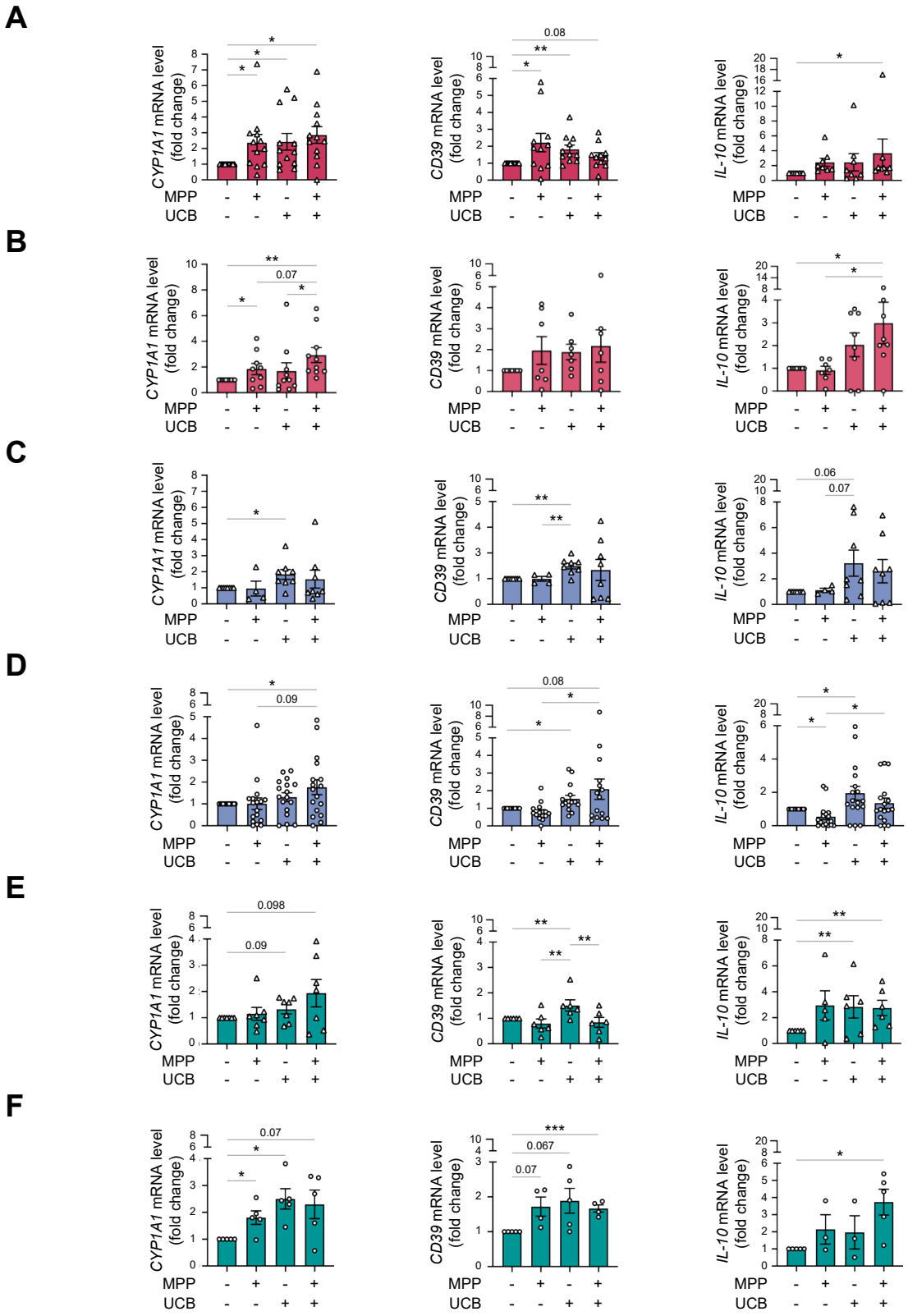


Fig. 3. Blockade of ERα restores the ability to respond to AhR activation in pre-menopausal females with AIH. Mean ± SEM *CYP1A1*, *CD39* and *IL-10* mRNA levels in untreated, MPP, UCB and UCB plus MPP-treated Tregs of HCs and patients with AIH: (A, B) pre-menopausal (HC, n = 8-12; AIH, n = 7-10), (C, D) post-menopausal (HC, n = 8; AIH, n = 15-17), and (E, F) males (HC, n = 6-7; AIH, n = 5). In Panels B, C, E and F some Treg samples could not be used for all culture conditions due to the limited number of cells available. **p* ≤0.05; ***p* ≤0.01; ****p* ≤0.001 (one-way ANOVA test). AIH, autoimmune hepatitis; ERα, estrogen receptor alpha; HCs, healthy controls; MPP, methyl piperidino pyrazole; Treg, regulatory T cell; UCB, unconjugated bilirubin.

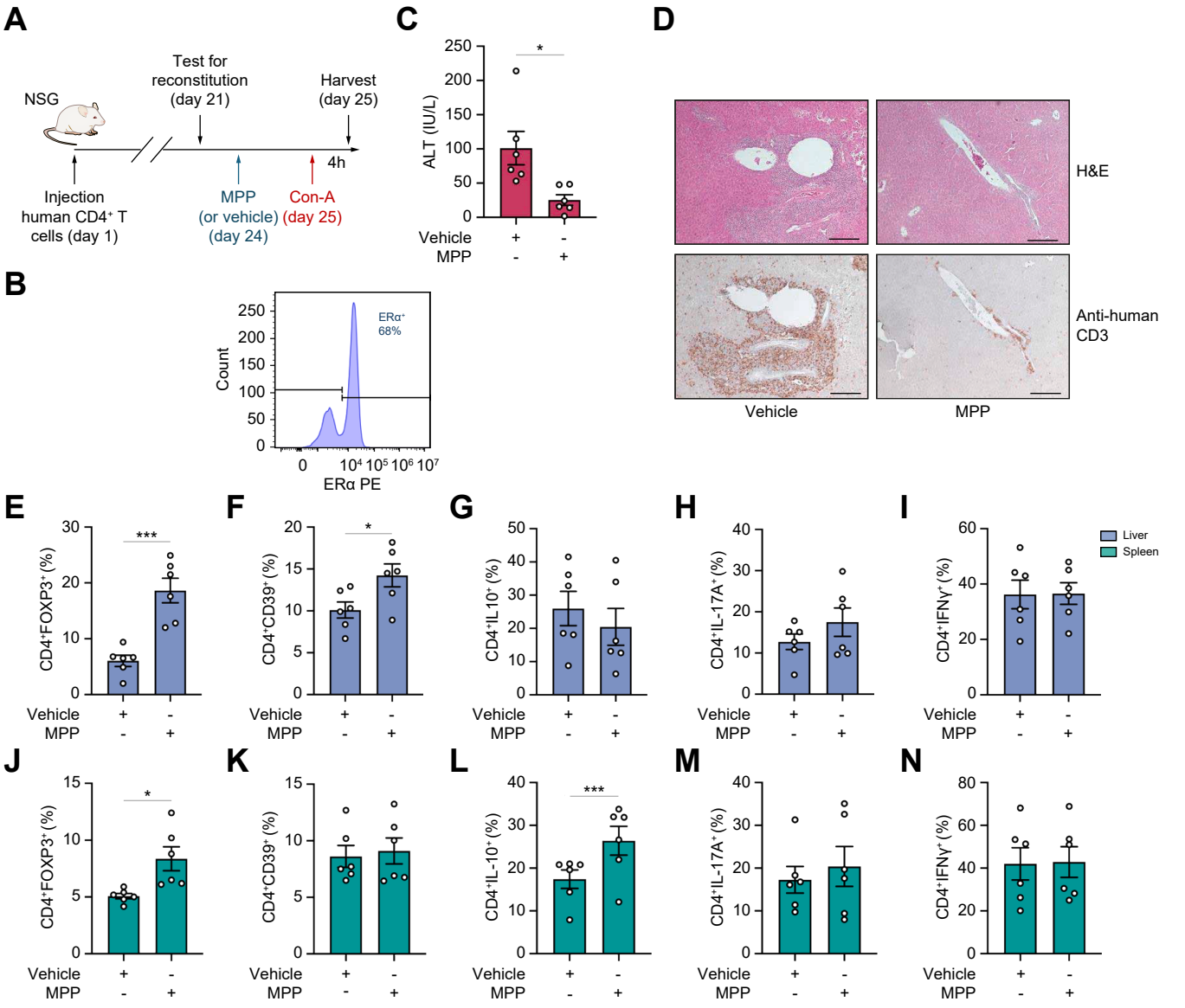


Fig. 4. Blockade of ERα ameliorates liver damage *in vivo*. (A) Experimental schematic. (B) Frequency of ERα$^+$ lymphocytes in donor's CD4. (C) Mean ± SEM serum ALT levels in vehicle (n = 6, all females) or MPP-treated mice (n = 6, all females). (D) H&E and human CD3 staining of liver sections (original magnification, × 10, scale bar: 200 μm), obtained from representative mice treated with vehicle or MPP. Mean ± SEM frequency of intrahepatic and splenic human CD4 lymphocytes positive for (E, J) FOXP3, (F, K) CD39, (G, L) IL-10, (H, M) IL-17A and (I, N) IFNγ. *p ≤0.05; ***p ≤0.001 (two-sided unpaired t test). ALT, alanine aminotransferase; ERα, estrogen receptor alpha; MPP, methyl piperidino pyrazole.

To further prove the HIF-1α/*ERa* interaction, we performed chromatin immunoprecipitation on Tregs obtained from pre-menopausal females with or without AIH. Sequence analysis of -2.5 Kb of the *ERa* promoter (chr6:151,805,182-151,807,681) indicated two closely spaced consensus sequences (S-1-151,805,600-151,805,604), (S-2-151,805,666-151,805,671) as putative binding sites for HIF-1α (see supplemental material). A region (chr6:151,805,556-151,807,699) of the *ERa* promoter spanning these two sites was analyzed for HIF-1α enrichment by chromatin immunoprecipitation. After Treg exposure to hypoxic conditions (*i.e.* 2% O_2) to favor HIF-1α upregulation, chromatin was extracted and then immunoprecipitated with IgG control or HIF-1α antibodies. Chromatin immunoprecipitation PCR using primers spanning the predicted HIF-1α binding sites revealed significant enrichment of HIF-1α antibody binding to the *ERa* promoter only in Tregs derived from patients with AIH but not from healthy individuals (Fig. 6G,H). Further, exposure of Tregs derived from patients with AIH, but not from healthy individuals, to hypoxic conditions (*i.e.* 2% O_2) boosted ERα expression levels (Fig. S12A and B).

Collectively, enrichment of HIF-1α antibody binding to the *ERa* promoter along with reduced RNA expression upon silencing of HIF-1α and increased ERα levels under hypoxic conditions show an important role for HIF-1α in the transcriptional induction of *ERa* in pre-menopausal females with AIH.

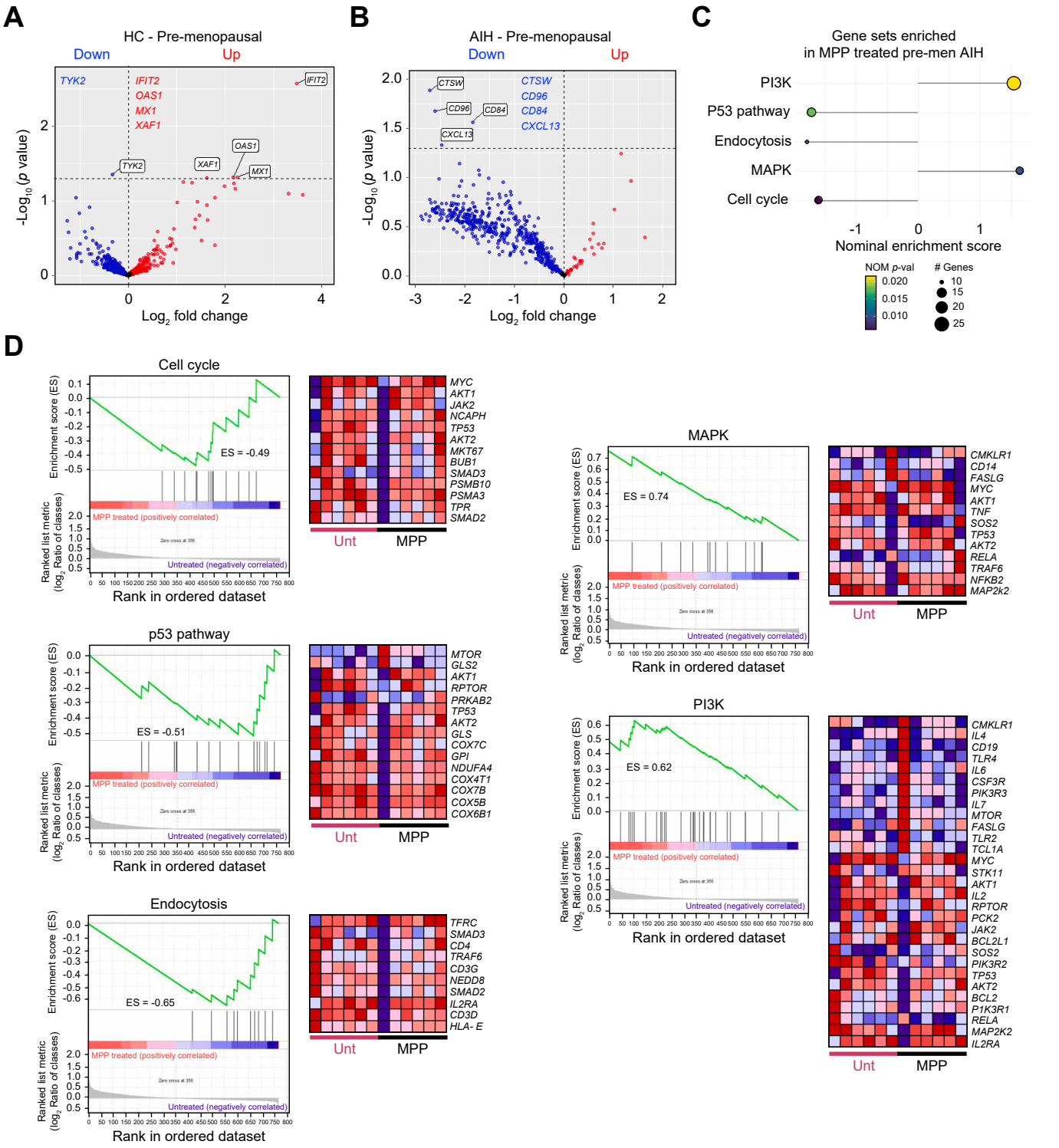


Fig. 5. Effects of ERα blockade on Treg cell transcriptome. (A-B) Volcano plots showing upregulated (red) and downregulated (blue) genes in MPP *vs*. vehicle-treated Tregs (HC, n = 6; AIH, n = 6). X and Y axes correspond to Log_2 fold-change and $-log_{10}$(*p* value) respectively. (C) Enriched gene sets in MPP- *vs.* vehicle-treated Tregs in pre-menopausal females with AIH. (D) GSEA plots showing 'Cell cycle', 'p53 pathway', 'Endocytosis' gene sets being negatively correlated, and 'MAPK' and 'PI3K' gene sets being positively correlated with MPP treatment in AIH Tregs. Heatmaps representing expression of genes included in 'Cell cycle', 'p53 pathway', 'Endocytosis', 'MAPK' and 'PI3K' gene sets in untreated and MPP-treated Tregs in pre-menopausal females with AIH. AIH, autoimmune hepatitis; ERα, estrogen receptor alpha; GSEA, gene set-enrichment analysis; HCs, healthy controls; MPP, methyl piperidino pyrazole; Treg, regulatory T cell.

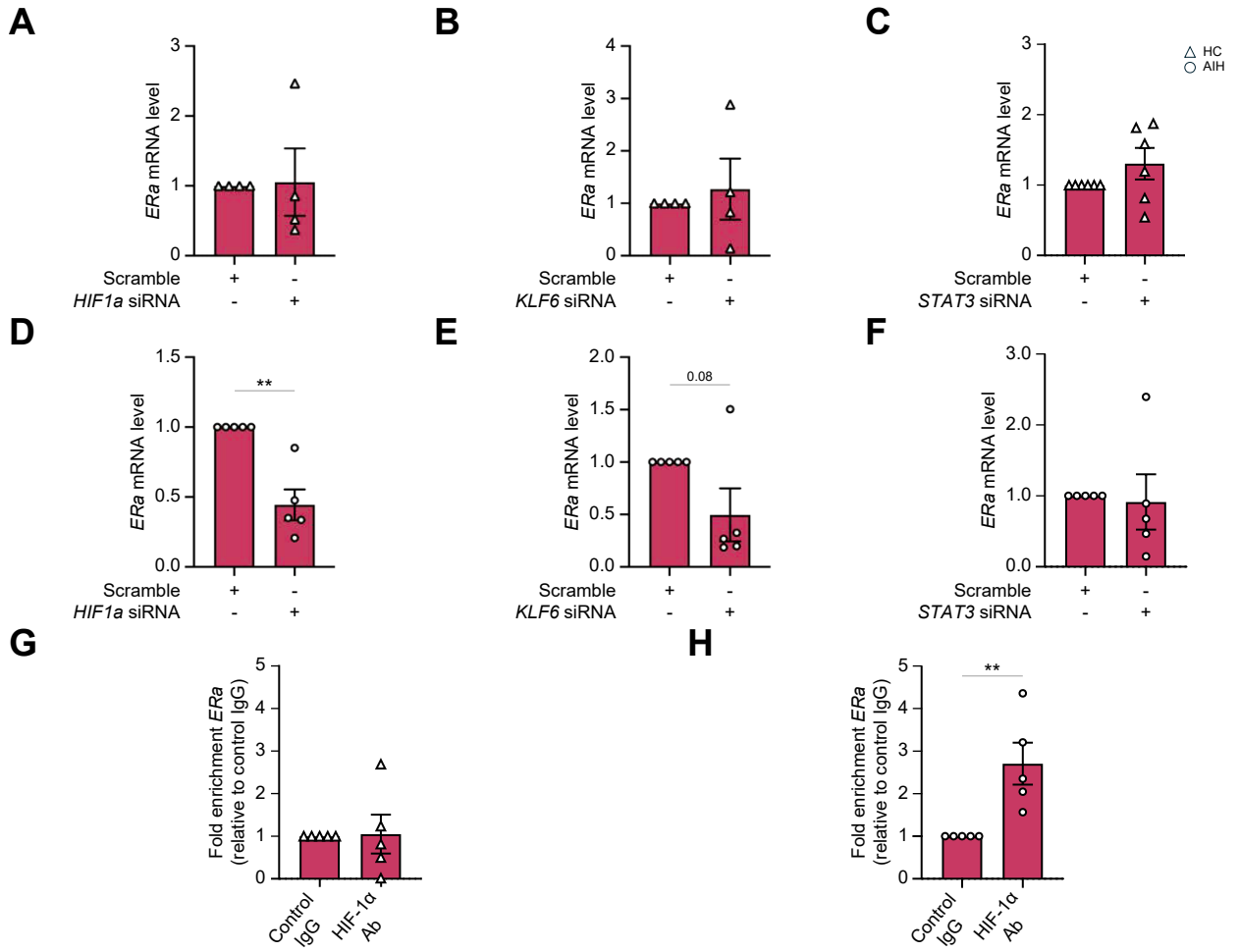


Fig. 6. HIF-1α promotes ERα transcription in Tregs of pre-menopausal females with AIH. Mean ± SEM *ERa* mRNA levels in scrambled (scr) and *HIF-1a* (A, D), *KLF6* (B, E) or *STAT3* (C, F) siRNA treated Tregs from HCs (n = 4-6) and pre-menopausal females with AIH (n = 5). $^{**}p \leq 0.01$ (two-sided paired *t* test). (G-H) Enrichment of HIF-1α antibody binding to the *ERa* promoter, compared to IgG negative control. Mean ± SEM is indicated (HC, n = 5; AIH, n = 5). $^{**}p \leq 0.01$ (two-sided paired *t* test). AIH, autoimmune hepatitis; ERα, estrogen receptor alpha; HCs, healthy controls; Treg, regulatory T cell.

Discussion

Our manuscript shows that ERα is increased in Tregs obtained from pre-menopausal females with AIH and that blockade of this factor stabilizes Tregs while boosting their function and ability to effectively respond to AhR activation. These data indicate an important role for ERα in limiting Treg function in AIH and suggest that Treg impairment in this autoimmune setting might be driven by aberrant ERα levels in pre-menopausal females.

Our results indicate that blockade of ERα using a specific antagonist boosts Treg function in pre-menopausal females with AIH, while also restoring the ability of these cells to respond to AhR activation in the presence of the endogenous ligand UCB. This results in increased levels of *CYP1A1*, the main gene regulated by the AhR/ARNT complex, and in increased expression of IL-10, another gene controlled by AhR. These findings support a role for ERα signaling in inflammation. How, and to what extent, Treg responses to ERα blockade are affected by immunosuppression remains unclear, as only a small number of the patients with AIH were not receiving immunosuppressive therapy at the time of the study. Future investigations should address this important point.

Our data showing that the response of Tregs to PPT is comparable in AIH and healthy individuals, regardless of sex and menopausal status, suggest that Treg function in pre-menopausal females with AIH is not impacted by estrogen binding to ERα but, instead, by non-canonical interactions of ERα with AhR.

Tregs from post-menopausal females and males with AIH maintain – at least to some extent – the ability to respond to AhR activation, suggesting that, in these patient groups, Treg impairment is linked to defects in other immunoregulatory pathways. These might be related to markedly decreased levels of Galectin-9, which upon interaction with Tim-3 on effector T cells induces their apoptosis;[27] inability to respond to IL-2, the main survival factor for Tregs;[36] or, alternatively, to altered CD39 regulation, as we have shown in other inflammatory contexts.[37,38]

That ERα plays an inflammatory role in Tregs from pre-menopausal females is also corroborated by the evidence that

ERα blockade stabilizes the phenotype of Tregs following challenge in the presence of IL-6 and IL-1β, two of the cytokines favoring CD4 cell polarization into Th17 cells in humans. This is reflected by an increase in FOXP3 levels and a decrease in RORC and IL-17A levels to baseline. These findings support previous work showing that T lymphocyte-specific deletion of *Era* decreases their activation and augments expression of Foxp3 in the setting of experimental colitis[14]; whereas ERα deficiency in females of the lupus prone (NZB × NZW) F(1) background results in an attenuated clinical phenotype.[39]

Our data indicate that Tregs from pre-menopausal females display higher sensitivity to MPP, possibly as a result of heightened expression of ERα. In turn this might derive from increased estrogen levels and/or exposure to hypoxic conditions linked to chronic inflammation that favor upregulation of ERα.

Blockade of ERα in pre-menopausal females impacts gene expression in healthy individuals and patients with AIH in a comparable manner, with downregulation of genes linked with pro-inflammatory cell phenotypes and T cell activation. Importantly, GSEA of Tregs from pre-menopausal females with AIH has revealed that ERα blockade results in downregulation of gene sets associated with cell cycle and endocytosis; and in upregulation of gene sets linked to MAPK and PI3K signaling pathways. Previous work has shown that MAPK triggers activation of the transcription factor c-AMP response element-binding protein that induces cytokine genes like *IL-2* and *TGF-β* leading to Treg differentiation.[40,41] Whether this signaling cascade is also activated in AIH awaits further investigations.

Importantly, our data indicate HIF-1α as a chief inducer of *ERa* transcription, as also supported by chromatin immunoprecipitation studies. As highlighted above, HIF-1α is known to limit regulatory cell immunity by directly interfering with AhR, or by impacting cell responses to AhR activation, as we previously found in Th17 cells in the context of AIH and inflammatory bowel disease.[7,31] Our new findings propose another mechanism by which HIF-1α regulates AhR activation and signaling, *i.e.* through the induction of ERα which, by serving as non-canonical binding partner for AhR, might limit Treg function and response to ligands.

Previous studies suggesting an anti-inflammatory role for ERβ are supported by our data, which show a decreased ERβ/ERα ratio in AIH Tregs from pre-menopausal females, and a negative correlation of that ratio with total bilirubin levels.

Our data have important therapeutic implications given the beneficial effects of MPP *in vitro* and *in vivo*, as confirmed by Treg stabilization in a pro-inflammatory environment and by amelioration of liver injury in *NOD/scid/gamma* mice reconstituted with human CD4 cells. These findings emphasize the importance of targeting ERα signaling, especially in females of reproductive age, and corroborate previous studies showing that exposure to estrogen increases the expression of pro-inflammatory cytokines in female mouse biliary epithelial cells[42] and that a combination of estrogen and IL-6 contributes to sex bias in drug-induced liver injury.[43] Blockade of ERα in pre-menopausal females might also have beneficial effects in other autoimmune diseases with female preponderance like systemic lupus erythematosus[44] and rheumatoid arthritis.[45]

Since the first reports,[46,47] AIH treatment has been based on corticosteroids and other immunosuppressants, which do not always prevent disease progression and, importantly, do not restore immune tolerance. Therefore, targeting ERα in pre-menopausal females with AIH might represent a novel therapeutic strategy to control inflammation and impede tissue damage perpetuation. This approach would favor the reconstitution and maintenance of immune tolerance by boosting Treg function and stability. Additional pre-clinical investigations are needed to determine how long the beneficial effects of ERα antagonists can be maintained, also in the presence of an inflammatory environment; and how Treg-specific delivery of the drug could be achieved *in vivo*, *e.g.* using functionalized nanoparticles. These steps would be essential to facilitate translation into the clinical setting. Further pre-clinical optimization of dose and dosing frequency is required, considering potential side effects of ERα antagonism, such as menopausal symptoms and bone loss.

Our study has limitations as further work is warranted to understand how ERα impacts Treg functional stability at the molecular level. As the experiments in this study have been conducted in polyclonal Tregs, future investigations should establish whether polyclonal and liver autoantigen-specific Tregs express ERα at comparable levels and are therefore equally impacted by its interference.

Collectively, our data show that aberrant expression of ERα, likely enhanced by hypoxia-induced inflammation, unleashes Treg dysfunction in pre-menopausal females with AIH. Specific blockade of ERα stabilizes phenotype, ameliorates suppressive function and effectively controls inflammation *in vivo*, therefore supporting the use of ERα signaling inhibitors in these patients. Importantly, these data propose that alterations in ERα signaling might be responsible for sex bias in AIH, especially in younger individuals.

Affiliations

[1]*Department of Anesthesia, Critical Care and Pain Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA;* [2]*School of Medicine, Tufts University, Boston, MA, USA;* [3]*Institute of Molecular Biomedicine, Faculty of Medicine, Comenius University, Bratislava, Slovakia;* [4]*Department of Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany;* [5]*Graduate Program in Pharmaceutical Sciences, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre RS, Brazil;* [6]*Department of Endocrinology and Metabolism, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Jinan, Shandong, China;* [7]*Department of Hematology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan, Shandong, China;* [8]*Division of Gastroenterology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA;* [9]*Center for Virology and Vaccine Research, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA;* [10]*Harvard School of Dental Medicine, Department of Oral Medicine, Infection and Immunity, Boston, MA, USA*

Abbreviations

AIH, autoimmune hepatitis; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CYP1A1, cytochrome P4501A1; ERα, estrogen receptor alpha; HIF-1α, hypoxia-inducible factor 1-alpha; KLF6, Krüppel-like factor 6; MPP, methyl piperidino pyrazole; PPT, propyl pyrazole triol; STAT3, signal transducer and activator of transcription 3; Th, helper T; Treg, regulatory T cell; UCB, unconjugated bilirubin.

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Conflict of interest

The authors declare no conflicts of interest pertaining to this manuscript. Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Concept and design: MSL; Experiments and procedures: LZ, BG, DN, CC, GGS, LG, WZ, WL, JJG, NW, AK, EC, AW; Funding acquisition: MSL, SKK, BG, DN; Writing - original draft: LZ, BG, DN, CC, GGS; Writing - review and editing: MSL, SKK, AB, VP, SJ, JC.

Data availability

NanoString bulk transcriptomics data are provided in Tables S3 and S4. All other data reported in this paper are available from the lead contact upon request.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2025.101678.

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Author names in bold designate shared co-first authorship

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