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Liver X receptor beta deficiency attenuates autoimmune-associated neuroinflammation in a T cell-dependent manner

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ABSTRACT

The initiation and progression of autoimmune disorders such as multiple sclerosis (MS) is linked to aberrant cholesterol metabolism and overt inflammation. Liver X receptors (LXR) are nuclear receptors that function at the crossroads of cholesterol metabolism and immunity, and their activation is considered a promising therapeutic strategy to attenuate autoimmunity. However, despite clear functional heterogeneity and cell-specific expression profiles, the impact of the individual LXR isoforms on autoimmunity remains poorly understood. Here, we show that LXRα and LXRβ have an opposite impact on immune cell function and disease severity in the experimental autoimmune encephalomyelitis model, an experimental MS model. While *Lxrα* deficiency aggravated disease pathology and severity, absence of *Lxrβ* was protective. Guided by flow cytometry and by using cell-specific knockout models, reduced disease severity in *Lxrβ*-deficient mice was primarily attributed to changes in peripheral T cell physiology and occurred independent from alterations in microglia function. Collectively, our findings indicate that LXR isoforms play functionally non-redundant roles in autoimmunity, potentially having broad implications for the development of LXR-based therapeutic strategies aimed at dampening autoimmunity and neuroinflammation.

1. Introduction

Multiple sclerosis (MS) is a debilitating neurological disease and one of the most prevalent autoimmune disorders in the Western world. In MS, an autoimmune response develops against antigens derived from the central nervous system (CNS), causing neuroinflammation, demyelination, and neuronal loss [1]. Ample evidence indicates that environmental determinants, in concert with genetic predisposition, trigger loss of immunological self-tolerance in MS [2]. Of particular interest, cholesterol and its cellular metabolism are increasingly being acknowledged to regulate the fate of immune cells, autoimmunity, and MS disease progression [3–5]. Yet, to date, the molecular mechanisms that underlie the impact of cholesterol on the immunopathology of MS are not fully elucidated.

Liver X receptors (LXRs) are members of the nuclear receptor family of ligand activated transcription factors [6]. Two LXR isoforms exist, α and β, which couple sensing of intracellular oxysterols and intermediates of the cholesterol biosynthetic pathway to induction of their

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target genes. Upon activation, LXRs control expression of genes involved in cholesterol metabolism, *i.e.* uptake, processing, and efflux [6]. In addition to their role in cholesterol homeostasis, several studies have demonstrated that LXR have a profound impact on immune cells and inflammatory responses. For instance, LXRs act as an essential metabolic checkpoint that regulates lymphocyte proliferation and differentiation [7,8]. Likewise, LXR activation dampens the inflammatory phenotype of macrophages and microglia via transrepression of nuclear factor-κB and activator protein-1 [9,10]. Consistent with its immunomodulatory properties, LXR activation attenuates disease severity and the neuroinflammatory burden in animal models of MS [11–13]. Reciprocally, global *Lxr* deficiency exacerbates CNS pathology and disease severity [12]. These findings indicate that LXR activation functions at the crossroads of inflammation and cholesterol metabolism, and acts as a hub and driver of the immunopathology in MS.

Emerging evidence indicates that despite considerable sequence homology LXRα and LXRβ are not functionally redundant [14–17]. We recently showed that absence of *Lxrα*, but not *Lxrβ*, decreases endothelial cell integrity and that endothelial cell-specific *Lxrα* deficiency worsens neuroinflammation in experimental autoimmune encephalomyelitis, the most commonly used experimental model to study the immunopathology of MS [14]. An additional layer of complexity is presented by tissue- and cell-specific expression profiles and functions of LXRs. Highlighting the latter, in contrast to global *Lxr* deficiency [12], T cell-specific *Lxr* deficiency ameliorates EAE disease severity [18]. Collectively, these studies support the idea that the disease-promoting and -resolving functions of LXRs are cell type- and isoform-specific.

In this study, we therefore set out to define the LXR isoform-specific influence on autoimmunity and neuroinflammation in the EAE model. We show that LXR isoforms have a different effect on EAE severity and pathology, with deficiency of *Lxrα* and *Lxrβ* being detrimental and protective, respectively. By using cell type-specific knockout models, we found that the protective effect of *Lxrβ* deficiency largely depends on changes in the peripheral T cell compartment and occurs independent of changes in microglia physiology. Collectively, our findings highlight that LXR*α* and LXRβ oppositely affect the autoimmune response in MS. By doing so, this study provides increased fundamental insight in LXR biology but also stresses the importance of therapeutic strategies aimed at targeting LXRs in an isoform- or cell type-specific manner.

2. Materials and methods

2.1. Mice

Lxrα$^{-/-}$, *Lxrβ*$^{-/-}$, and *Lxrβ* loxP/loxP (*Lxrβ*$^{fl/fl}$) mice on a C57BL/6 background were used to study the impact of LXRs on immunity and neuroinflammation [19]. Wild-type (wt) C57BL/6JBomTac mice were purchased from Taconic (Sweden). To generate mice for microglia-specific knockout of *Lxrβ*, *Lxrβ*$^{fl/fl}$ mice were crossed with B6.129P2(Cg)-Cx3cr1tm2.1(cre/ERT2) mice (*Cx3cr1*^{CreERT2}), kindly provided by prof. Dr. S. Jung (Weizmann Institute of Science, Israel) [20]. *Lxrβ*$^{fl/fl}$ and *Cx3cr1*$^{CreERT+-/-}$ littermates were used as controls. To achieve microglia-specific *Lxrβ* deficiency, recombination was induced by peritoneal administration of 100 μl tamoxifen (Sigma-Aldrich; 20 mg/ml in corn oil), 28 and 26 days prior to EAE immunization. This treatment regime allows for recombined circulating monocytes, and not tissue-resident microglia, to be replaced by non-recombined monocytes from the bone marrow. Mice were maintained on a 12 h light/dark cycle with free access to water and a standard chow diet. All animal procedures were conducted in accordance with the institutional guidelines and approved by the Ethical Committee for Animal Experiments of Hasselt University.

2.2. Experimental autoimmune encephalomyelitis (EAE)

At the age of 10–16 weeks, female or male mice were immunized subcutaneously with myelin oligodendrocyte glycoprotein peptide 35–55 (MOG_{35-55}) emulsified in complete Freund's adjuvant supplemented with *Mycobacterium tuberculosis* according to manufacturer's guidelines (EK-2110 kit; Hooke Laboratories)

Directly after immunization and after 24 h, mice were intraperitoneally injected with 100 ng pertussis toxin. Mice were weighed and clinically evaluated for neurological signs of the disease on a daily basis following a five-point standardized rating of clinical symptoms (0: no clinical symptoms; 1: tail paralysis; 2: tail paralysis and partial hind limb paralysis; 3: complete hind limb paralysis; 4: paralysis to the diaphragm; 5: death by EAE).

For the adoptive transfer experiments, inguinal lymph nodes were isolated from EAE donor mice at day 10 post-immunization. Single-cell suspensions were obtained by pushing the tissue through a 70 μm cell strainer and subsequently cultured at 7×10^6 cells/ml in stimulation medium consisting of RPMI1640 supplemented with 0.5% penicillin/streptomycin (Gibco), 10% FCS (Gibco), 1% non-essential amino acid (NEAA, Sigma-Aldrich), 1% sodium pyruvate (Sigma-Aldrich), 20 ng/ml IL23 (BioLegend), and 20 μg/ml $rMOG_{35-55}$ (Hooke Laboratories). After 3 days, 10×10^6 cells were intraperitoneally injected into wt or *Lxrβ*$^{-/-}$ recipient mice.

For bone-marrow transplantation (BMT) experiments, lethally irradiated wt recipient mice were grafted with bone marrow cells of wt and *Lxrβ−/−* mice (10×10^6 cells intravenously). Recipient mice were exposed to 8 Gy total body irradiation. After 6 weeks recovery, EAE was induced in recipient mice. During recovery, mice were treated with Neomycin (FSA Chemicals NV, Belgium) and Polymyxini B sulfas (Fagron, the Netherlands) added to the drinking water.

2.3. Microglia isolation and culture

Microglia cultures were prepared from postnatal P3 C57BL/6J mouse pups. Isolated forebrains were placed in L15 Leibovitz medium (Gibco) containing 1:10 Trypsin (Sigma-Aldrich, Diegem, Belgium) (37 °C, 15 min). Next, high glucose DMEM medium (Invitrogen) supplemented with 10% FCS, 50 U/ml penicillin, 50 U/ml streptomycin, (DMEM 10:1 medium), and 100 μl/ml DNase I (Sigma-Aldrich) was added to the forebrain tissue. Nervous tissue was dissociated by trituration with serum-coated Pasteur pipettes (Sigma-Aldrich). The dissociated mix was passed through a 70 μm cell strainer, rinsed with 5 ml of DMEM 10:1 medium, and centrifuged (170 g, 10 min, RT). After 2 days, DMEM 10:1 medium was changed and after reaching confluence (±6 days later), 2/3 DMEM 10:1 medium containing 1/3 L929-conditioned medium was added. Six days later, microglia isolation was performed using the shake-off method (200 rpm, 2 h, RT). Microglia were centrifuged (170 g, 10 min, RT), suspended in DMEM 10:1 medium containing B27 supplement (Invitrogen), and cultured at 250.000 cells/well in poly-l-lysine (Sigma-Aldrich)-coated 24-well plates. For inflammatory phenotyping, microglia were stimulated with GW3965 (1 μM, Sigma-Aldrich) in combination with LPS (100 ng/ml, Sigma-Aldrich) or IFNγ/TNFα (100 ng/ml, Peprotech) for 18 h.

2.4. Quantitative PCR (qPCR)

Tissue or cells were lysed using QIAzol (Qiagen). RNA extraction and synthesis of complementary DNA was performed as described previously [21]. qPCR was subsequently conducted on a StepOnePlus or QuantStudio 3 detection system (Applied Biosystems). Data was analyzed using the ΔΔCt method and normalized to the most stable reference genes, as described previously [22,23]. Primer sequences are available on request.

2.5. Immunofluorescence microscopy and image analysis

Cryosections were fixed in acetone for 10 min. Immunostaining and analysis of cryosections were performed as described previously [24].

Briefly, immune cell infiltrates were stained using the following antibodies: anti-CD3 (1:150; Bio-Rad) and anti-F4/80 (1:100; Bio-Rad), combined with the secondary Alexa Fluor 488 or 555-labeled anti-rat IgG antibody (1:400; Invitrogen). Analysis was carried out using a Nikon eclipse 80i microscope and ImageJ software.

2.6. T cell reactivity

Inguinal lymph nodes and spleens were isolated from mice 9 days post-immunization (dpi) of EAE. T cells were isolated from lymph nodes and cultured in RPMI medium containing 20 μM β mercaptoethanol (βME), 2% mouse serum, 1% non-essential amino acids (NEAA), 1% sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin (P/S). Cells were plated in a 96 well plate at a density of 3.10^5 cells/well and subsequently stimulated with 20 μg/ml recombinant $MOG_{35\text{-}55}$ (Hooke Laboratories, DS-0111) for 48 h. Next, 1 μCi [^{3}H] thymidine (Amersham biosciences, UK) was added for 18 h after which cells were harvested using an automatic cell harvester (Pharmacia, Uppsala, Sweden). A β-plate liquid scintillation counter (PerkinElmer, lifesciences, Wessesly, USA) was used to quantify radioactivity and results are expressed as stimulation index (% of proliferated cells relative to the mean % of unstimulated control cells).

2.7. Flow cytometry

Blood was taken from the hind limb using EDTA lined Microvette tubes (Sarstedt) and at sacrifice by cardiac puncture. Blood was transferred to a Trucount absolute count tube (BD) containing FC-receptor block (anti-CD16/CD32) before adding a cocktail of antibodies (Suppl. Table www.nature.com/articles/s41598-018-33661-y?proof=trueMay.1). Hereafter, erythrocytes were lysed using lysis buffer (8.4 g/L NH4CL and 0.84 g/L NaHCO3 in H2O, pH 7.4) before measurement and staining. For the staining of blood-derived lymphocytes, the following antibodies were used: CD45 PerCp (103130, 1:100, Biolegend), CD3e eFLUO 450 (48-0032-82, 1:100, eBioscience), NK1.1 PE (557391, 1:100, BD), CD4 APC-H7 (560181, 1:100, BD), CD8 V500 (560776, 1:200, BD), Ly6G APC-Cy7 (560600, 1:100, BD), CD11b PE-Cy7 (552850, 1:300, BD), Ly6C APC (130-102-391, 1:100, Miltenyi), Siglec-F PE (552126, 1:100, BD), and B200 V500 (561226, 1:50, BD). For detailed T cell phenotyping, the following antibodies were used: CD45 PerCp (103130, 1:100, Biolegend), CD3e eFLUO 450 (48-0032-82, 1:100, eBioscience), CD4 FITC (11-0042-82, 1:50, eBioscience), CD8 V500 (560776, 1:200, BD), CD25 APC (17-0251-82, 1:300, eBioscience), CD62L PE-Cy7 (25-0621-82, 1:800, eBioscience), CD44 APC-H7 (560568, 1:200, BD), and FOXP3 PE (72-5575-40, 1:40, eBioscience). The Anti-Mouse/Rat Foxp3 Staining Set (Ebioscience) was used to assess FOXP3 expression. Flow cytometry was performed using a BD FACSCANTO II running FACS Diva 8.0.1 software which was also used for all analyses.

2.8. Statistical analysis

Data was analyzed for statistical significance using GraphPad prism and are reported as mean ± SEM. Data were tested for normal distribution using the d'Agostino and Pearson omnibus normality test. When data sets were normally distributed, an ANOVA (Tukey's post hoc analysis) or a two-tailed unpaired student T-test (with Welch's correction if necessary) was used to determine statistical significance between groups. If data sets were not normally distributed, the Kruskal-Wallis or Mann-Whitney analysis was used. Significant differences were identified by p values < 0.05 (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

3. Results

3.1. *Lxrβ* deficiency reduces neuroinflammation and disease severity in the EAE model

To address the LXR isoform-specific roles in neuroinflammation, we first determined if constitutive *Lxrα* or *Lxrβ* deficiency differentially impacts EAE disease severity. In line with our earlier report in endothelial cells [14], we found that *Lxrα* deficiency worsened EAE disease severity, in particular in the subacute disease stage (Fig. 1A–C). While *Lxrα*$^{-/-}$ mice showed a reduced infiltration of CD3$^+$ T cells and F4/80$^+$ macrophages and microglia in the spinal cord at the peak of the disease (Fig. 1D and S1A), an increased abundance of these immune cells was observed in the chronic phase (Fig. 1E and S1B). Mirroring this, *Lxrα*$^{-/-}$ mice showed a trend towards a decreased expression of inflammatory cytokines and chemokines (*Nos2*, *Tnfa*, *Il6, Ccl2*, *Cxcl1*, and *Cxcl2*) at the peak of the disease, and increased expression at the chronic stage (Fig. 1F and G).

In stark contrast to *Lxrα* mice, absence of *Lxrβ* markedly attenuated disease severity and delayed disease onset (Fig. 1A–C). Reduced disease severity in *Lxrβ*$^{-/-}$ mice was paralleled by a decreased presence of CD3$^+$ T cells and F4/80$^+$ macrophages and microglia in the CNS, both at the peak of the disease as well as during the chronic disease phase (Fig. 1D and E). At the peak of disease (17 dpi), *Lxrβ*$^{-/-}$ mice displayed a substantially reduced expression of inflammatory cytokines and chemokines in the spinal cord (Fig. 1F). The expression of these inflammatory cytokines and mediators was comparable to that of wt EAE mice during the chronic disease stage (Fig. 1G). Counterintuitively, analysis of peripheral T cell reactivity 9 days post-immunization, a pathological parameter that often predicts disease development, showed an increased cognate antigen-specific reactivity of lymph node cells of *Lxrβ*$^{-/-}$ mice compared to wt EAE mice (Fig. 1H). In line with increased EAE disease severity, spleen and lymph node cells of *Lxrα*$^{-/-}$ mice demonstrated increased cognate antigen-specific reactivity compared to wt EAE mice 9 days post-immunization (Fig. 1H). Notably, although studies reported transcriptional compensation in single knockouts, we did not detect changes in the expression of *Lxrβ* and *Lxrα* in the spinal cord of *Lxrα*$^{-/-}$ and *Lxrβ*$^{-/-}$ EAE mice, respectively (Fig. 1I). Collectively, these findings provide evidence that LXRs affect EAE disease severity and pathology in an isoform-dependent fashion, with *Lxrα* and *Lxrβ* deficiency being detrimental and protective, respectively.

To further define the isoform-dependent impact on neuroinflammation, the LPS-induced murine neuroinflammation model was used. In this model, acute intracerebroventricular LPS administration provokes a prominent neuroinflammatory response, eventually resulting in neurodegeneration and cognitive impairment [25]. In contrast to the EAE model, the inflammatory response induced after intracerebroventricular LPS administration was not diminished in *Lxrβ*$^{-/-}$ compared to wt mice, but was instead significantly increased as is evident from the increased expression of *Nos2*, *Il6*, *Cxcl1*, and *Ccxl2* (Fig. 2A and B). Consistent with worsened EAE disease severity, *Lxrα*$^{-/-}$ mice showed an increased inflammatory burden in the brain following LPS exposure (Fig. 2A and B). These findings support the notion that the anti-inflammatory impact of *Lxrβ* deficiency is context-dependent, and may vary between experimental animal models and disease-specific cues.

Next, we sought to determine how LXR isoform specific activation affects EAE disease severity. Given that isoform-specific agonists are unavailable, *Lxrα*$^{-/-}$ and *Lxrβ*$^{-/-}$ mice were treated with the LXR pan-agonist GW3965 (experimental design in Fig. S2A). Pharmacological activation of LXRα in *Lxrβ*$^{-/-}$ mice decreased EAE disease severity (Fig. S2B-D). In contrast, LXRβ activation in *Lxrα*$^{-/-}$ mice was unable to attenuate EAE disease severity and onset (Fig. S2E-G). These findings suggest that LXRα activation is protective, consistent with increased EAE disease severity in *Lxrα*$^{-/-}$ mice, and that LXRβ activation right before disease onset does not affect EAE disease severity, opposed to the

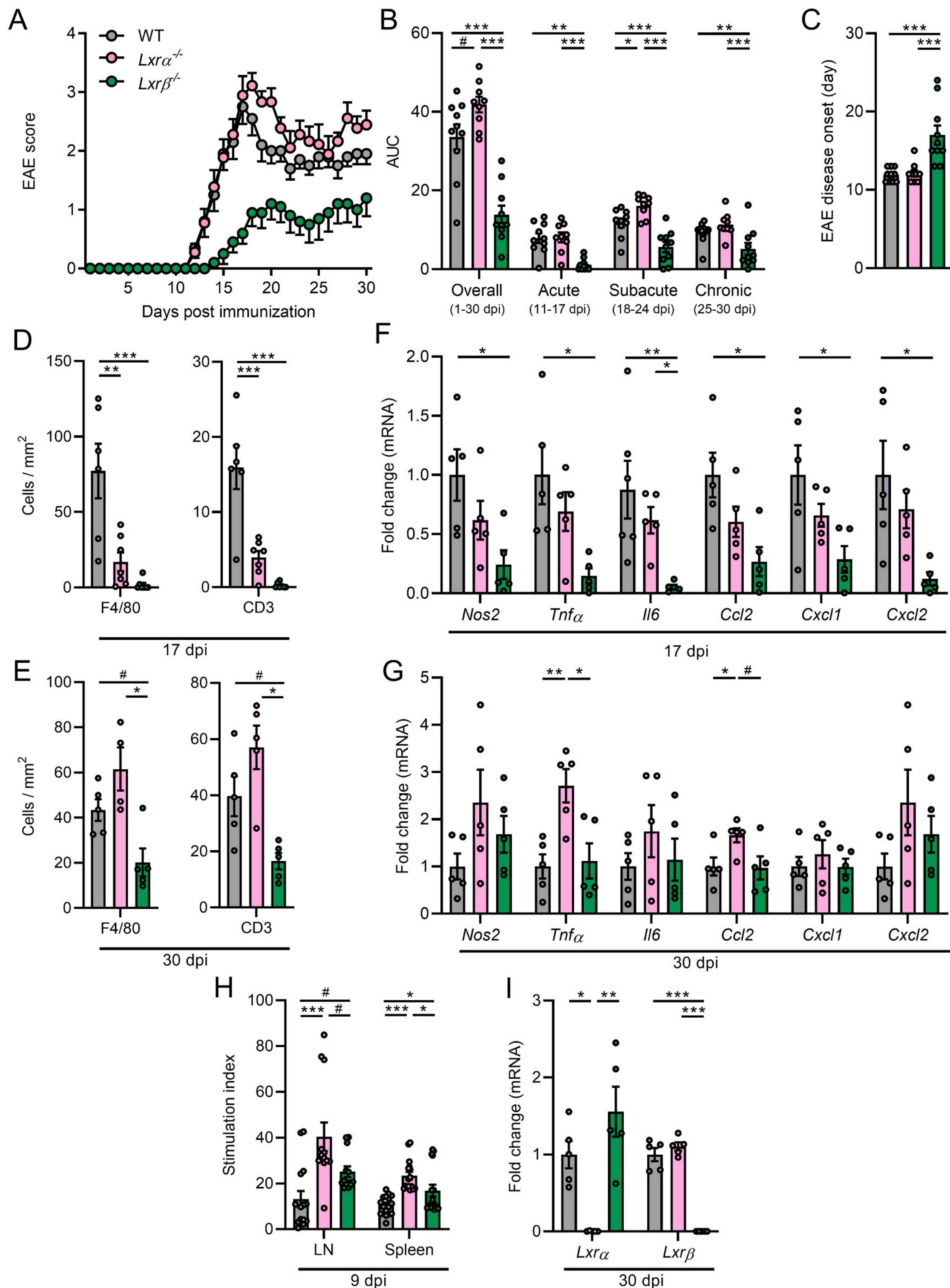

Fig. 1. LXRβ deficiency reduces neuroinflammation and disease severity in the EAE model. (**A**) EAE disease course of wild-type (wt), $Lxr\alpha^{-/-}$, and $Lxr\beta^{-/-}$ mice (n = 10 mice/genotype). (**B**) Cumulative disease severity (area under the curve, AUC) of immunized wt, $Lxr\alpha^{-/-}$, and $Lxr\beta^{-/-}$ mice at different disease stages: acute (11–17 days post-immunization, dpi), subactute (18–24 dpi), and chronic stage (25–30 dpi). (**C**) Disease onset of immunized wt, $Lxr\alpha^{-/-}$, and $Lxr\beta^{-/-}$ mice. (**D** and **E**) Number of $CD3^{+}$ T cells and F4/80+ macrophages and microglia in spinal cord tissue of immunized wt, $Lxr\alpha^{-/-}$, and $Lxr\beta^{-/-}$ mice. Immune cell abundance was determined at 17 (D) and 30 dpi (E). (**F** and **G**) mRNA expression of *Nos2*, *Tnfα*, *Il6*, *Ccl2*, *Cxcl1*, and *Cxcl2* in spinal cord tissue of immunized wt, $Lxr\alpha^{-/-}$, and $Lxr\beta^{-/-}$ mice. The transcriptional profile was determined at 17 (F) and 30 dpi (G). (**H**) Antigen-specific proliferation of splenic and lymph node-derived cells isolated from wt, $Lxr\alpha^{-/-}$, and $Lxr\beta^{-/-}$ EAE mice (9 dpi). Results are depicted as stimulation index (% of proliferated cells relative to the mean % of unstimulated control cells). (**I**) mRNA expression of *Lxrα* and *Lxrβ* in spinal cord tissue of immunized wt, $Lxr\alpha^{-/-}$, and $Lxr\beta^{-/-}$ mice (30 dpi). Results are pooled from independent biological replicates and statistically analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Data are represented as mean ± SEM. $^{\#}p < 0.1$, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

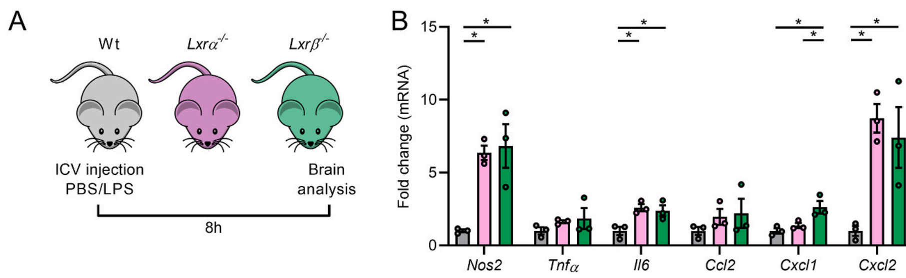

Fig. 2. *Lxrβ* deficiency worsens LPS-induced neuroinflammation. (**A**) Experimental setup of the LPS-induced neuroinflammation model. Vehicle (PBS) or LPS was stereotactically administrated in the cerebrospinal fluid of wt, *Lxrα, and Lxrβ*$^{-/-}$mice. After 8 h animals were sacrificed for transcriptional analysis (n = 3/group). (**B**) mRNA expression of *Nos2*, *Tnfα*, *Il6*, *Ccl2*, *Cxcl1*, and *Cxcl2* in brain tissue of LPS-treated wt, *Lxrα*$^{-/-}$, and *Lxrβ*$^{-/-}$ mice. To correct for potential basal genotype differences, values were corrected for genotype-specific basal expression. Results are pooled from independent biological replicates and statistically analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Data are represented as mean ± SEM. *p < 0.05.

protective impact of *Lxrβ* deficiency.

3.2. Changes in microglia physiology do not account for reduced EAE disease in *Lxrβ*$^{-/-}$ mice

Previously, we demonstrated that *Lxrα* deficiency worsens EAE

Fig. 3. Changes in microglia physiology do not account for reduced EAE disease in *Lxrβ*$^{-/-}$ mice. (**A-B**) mRNA expression of *Nos2*, *Tnfα*, *Il6*, *Ccl2*, *Ccl5*, and *Cxcl2* in wt, *Lxrα*$^{-/-}$, and *Lxrβ*$^{-/-}$ microglia upon stimulation with LPS (A) and IFNγ/TNFα (B). (**C**) Experimental setup used to assess the impact of microglia-specific *Lxrβ* deficiency on EAE disease severity. *Cx3cr1*$^{CreERT2+/-}$ and *Lxrβ*$^{fl+/+}$ mice were pooled (wt) and compared to *Lxrβ*$^{fl+/+}$*Cx3cr1*$^{CreERT2+/-}$ mice (n = 10 females and males/genotype). Four weeks prior to EAE induction animals were injected twice with tamoxifen (day −28 and −26). (**D**) EAE disease course of female wt and *Lxrβ*$^{fl+/+}$*Cx3cr1*$^{CreERT2+/-}$ mice. (**E**) Cumulative disease severity (area under the curve, AUC) of immunized female wt and *Lxrβ*$^{fl+/+}$*Cx3cr1*$^{CreERT2+/-}$ mice at different disease stages: acute (12–22 days post-immunization, dpi), subactute (23–28 dpi), and chronic stage (29–35 dpi). (**F**) Disease onset of immunized female wt and *Lxrβ*$^{fl+/+}$Cx3cr1$^{CreERT2+/-}$ mice. All results are pooled from independent biological replicates and statistically analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test (A and B) or the Mann-Whitney test (E and F). Data are represented as mean ± SEM. #p < 0.1, *p < 0.05, **p < 0.01, and ***p < 0.001.

disease severity through modulating endothelial cell physiology [14]. Here, we aimed to identify the cell type responsible for the protective impact of *Lxr*β deficiency on EAE disease severity. Given the essential role of microglia in the immunopathology of EAE and MS [26,27], we first assessed to what extent *Lxrα* and *Lxrβ* deficiency affects their inflammatory phenotype *in vitro*. To mimic the pro-inflammatory lesional microenvironment, the microglial inflammatory phenotype was assessed following exposure to the prototypical inflammatory stimulus LPS or the disease-relevant inflammatory cytokines IFNγ and TNFα. Primary $Lxr\alpha^{-/-}$ and $Lxr\beta^{-/-}$ microglia both showed reduced expression of inflammatory cytokines and chemokines upon stimulation with LPS compared to wt microglia (Fig. 3A). Stimulation with IFNγ and TNFα largely mimicked this less-inflammatory transcriptional profile in $Lxr\alpha^{-/-}$ but not $Lxr\beta^{-/-}$ microglia (Fig. 3B). *Nos2* and *Il6* even showed an increased expression in IFNγ/TNFα-stimulated $Lxr\beta^{-/-}$ microglia compared to wt and $Lxr\alpha^{-/-}$ microglia.

To determine the significance of these findings *in vivo* and to assess to what extent microglia are responsible for the reduced EAE disease severity in whole-body $Lxr\beta^{-/-}$ mice, EAE was induced in microglia-specific $Lxr\beta^{-/-}$ mice. To this end, homozygous loxP-flanked LXRβ mice were crossed with mice containing tamoxifen-inducible cre (Cre-ERT2) under control of Cx3cr1. While both microglia and circulating monocytes express CX3CR1, a timely pre-experimental administration of tamoxifen allows for recombined circulating monocytes, and not tissue-resident microglia, to be replaced by non-recombined monocytes from the bone marrow (experimental design in Fig. 3C). Despite the role of LXRβ in controlling microglia physiology *in vitro*, microglia-specific deficiency of *Lxrβ* did not reduce EAE disease severity (Fig. 3D). Instead, we observed a slightly, yet nevertheless significant, earlier disease onset and increased disease severity at the acute disease stage in female mice (Fig. 3E and F). Male littermates displayed a similar disease course (Fig. S3A-C). Collectively, these findings indicate that *Lxrβ* deficiency affects the inflammatory features of microglia *in vitro* but that these changes do not underlie reduced EAE disease severity in whole-body *Lxrβ* knockout mice.

3.3. *Lxrβ deficiency attenuates EAE disease severity in a T cell dependent manner*

As our result largely rule out the involvement of microglia in *Lxrβ* knockout mice, we next sought to investigate which other cell type may be involved. It is well established that alongside CNS-resident microglia, peripheral immune cells such as monocytes, neutrophils, natural killer (NK) cells, B cells, T cells are major drivers of CNS pathology in MS patients and EAE animals [1]. Accordingly, their infiltration into and accumulation in the CNS is considered a pathological hallmark of MS. As a first step, we used multicolor flow cytometry to interrogate whether *Lxrα* and *Lxrβ* deficiency affects the absolute number and composition of peripheral immune cells. Our data indicates that *Lxrβ* deficiency does not markedly alter the basal absolute number of B cells, NK cells, T cells, cytotoxic $CD8^+$ T cells, and helper T cells, with the exception of an increased monocyte count (Fig. 4A). In contrast, *Lxrα* deficiency increased the basal number of circulating haematopoietic cells, B cells, monocytes, neutrophils, and T cells (Fig. 4A). Interestingly, changes in the number of circulating immune cells in non-immunized $Lxr\alpha^{-/-}$ $Lxr\beta^{-/-}$ mice were reversed 9 days post EAE immunization (Fig. 4A).

Analysis of the relative abundance of immune cell subsets showed major changes were primarily apparent in non-immunized $Lxr\beta^{-/-}$ and not $Lxr\alpha^{-/-}$ mice. While the relative abundance of $CD3^+$ T cells and helper T cells was decreased in $Lxr\beta^{-/-}$ non-immunized animals, a 4-fold increase in regulatory T cells was observed (Fig. 4B). Quantitative PCR analysis demonstrated that lymph nodes of non-immunized $Lxr\beta^{-/-}$ mice showed a decreased expression of *Il1β* and *Il6*, and an increased expression of *Tgfβ and Il17* compared to wt animals (Fig. S4). These findings suggest that the increase of Tregs in $Lxr\beta^{-/-}$ mice occurs at the expense of Th17 cells [28,29]. Flow cytometric analysis further showed marked changes in the basal activation status of $CD4^+$ and $CD8^+$ T cells in $Lxr\beta^{-/-}$, but not $Lxr\alpha^{-/-}$ mice. In particular, the relative abundance of naïve $CD4^+$ and $CD8^+$ T cells was markedly decreased in healthy $Lxr\beta^{-/-}$ mice compared to wt and $Lxr\alpha^{-/-}$ mice (Fig. 4C). The opposite was observed for $CD4^+$ and $CD8^+$ effector memory T (Temra) cells (Fig. 4C). Yet again, changes the relative number of circulating immune cells in healthy $Lxr\alpha^{-/-}$ $Lxr\beta^{-/-}$ mice were largely reversed 9 days post EAE immunization (Fig. 4A), with the exception of a decreased helper T cell count (Fig. 4B and C). Collectively, these findings indicate that deficiency of *Lxrα* and *Lxrβ* affects the composition of circulating immune cells, with $Lxr\beta^{-/-}$ mice displaying major changes in the relative abundance of naïve, effector memory, and regulatory T cells.

Having established that *Lxrβ* deficiency has a substantial effect on the peripheral T cell compartment, we reasoned that reduced EAE disease severity in whole-body $Lxr\beta^{-/-}$ mice may be T cell-dependent. To test this notion, we used the adoptive transfer EAE model. Here, lymph node-derived encephalitogenic T lymphocytes from wt mice were transferred to $Lxr\beta^{-/-}$ and wt recipient mice (experimental design Fig. 5A). In contrast to our findings in whole-body $Lxr\beta^{-/-}$ animals (Fig. 1A–C), $Lxr\beta^{-/-}$ animals that received encephalitogenic wt T cells did not show reduced EAE disease severity or delayed disease onset (Fig. 5B–D). Consistent with these findings, bone marrow transplantation experiments demonstrated that absence of *Lxrβ* in haematopoietic cells ameliorated EAE disease severity and delayed disease onset (Fig. S5A-D). Collectively, these findings suggest that absence of *Lxrβ* ameliorates EAE disease severity in a T cell-dependent manner.

4. Discussion

In this study, we show that LXRα and LXRβ oppositely impact disease severity and pathology in the EAE model. While absence of *Lxrα* was detrimental, consistent with our previous findings [14], mice lacking *Lxrβ* displayed an unexpected decreased neuroinflammatory burden and markedly attenuated disease severity. By using cell-specific knockout models, T cells were identified as the main culprit cell type in $Lxr\beta^{-/-}$ mice. Our data underscore the complexity of LXR signaling in autoimmunity and neuroinflammation and highlight its ability to exert harmful and beneficial effects depending on dominant isoform and cell-type involved.

Ample evidence indicates that activation and deficiency of LXRs suppresses and worsens neuroinflammation, respectively [11–13]. Yet, a recent study found that T cell-specific deficiency of both LXR isoforms attenuates EAE disease severity [18]. In accordance with the latter, we now provide evidence that *Lxrβ* deficiency ameliorates EAE disease severity in a T cell-dependent manner. These findings argue for LXRβ being the responsible isoform for reduced EAE disease severity in T cell-specific $Lxr\alpha\beta^{-/-}$ mice [7]. Despite showing reduced disease severity, lymph node- and spleen-derived cells of $Lxr\beta^{-/-}$ EAE mice displayed enhanced cognate antigen-specific proliferation. In support of this finding, a previous study found that loss of LXRβ confers a proliferative advantage to T cells [7]. In the context of autoimmunity, increased reactivity against autoantigens is generally associated with an enhanced self-reactivity and aggravated disease severity, as observed for the $Lxr\alpha^{-/-}$ mice in this study. Based on observed increased self-reactivity in lymphoid tissue (1), the absence of marked changes in the quantity and quality of circulating T cell subsets (2), and the CNS being almost completely devoid of immune cells (3), we speculate that loss of LXRβ reduces EAE disease severity by impairing T cell egress from lymph nodes. In support of an essential role of LXRs in cellular migration, *Lxr*-deficient dendritic cells (DCs) display defective stimulus-induced migration *in vitro* and *in vivo* [30]. A reduced expression of the LXR-responsive protein CD38, an ectoenzyme essential for leukocyte trafficking [31], was found to underlie impaired DC migration in the absence of LXRs [30]. Alternatively, sphingosine-1-phosphate and its analog fingolimod (FTY720), metabolites known to promote sequestration of lymphocytes to secondary lymphoid organs, are linked

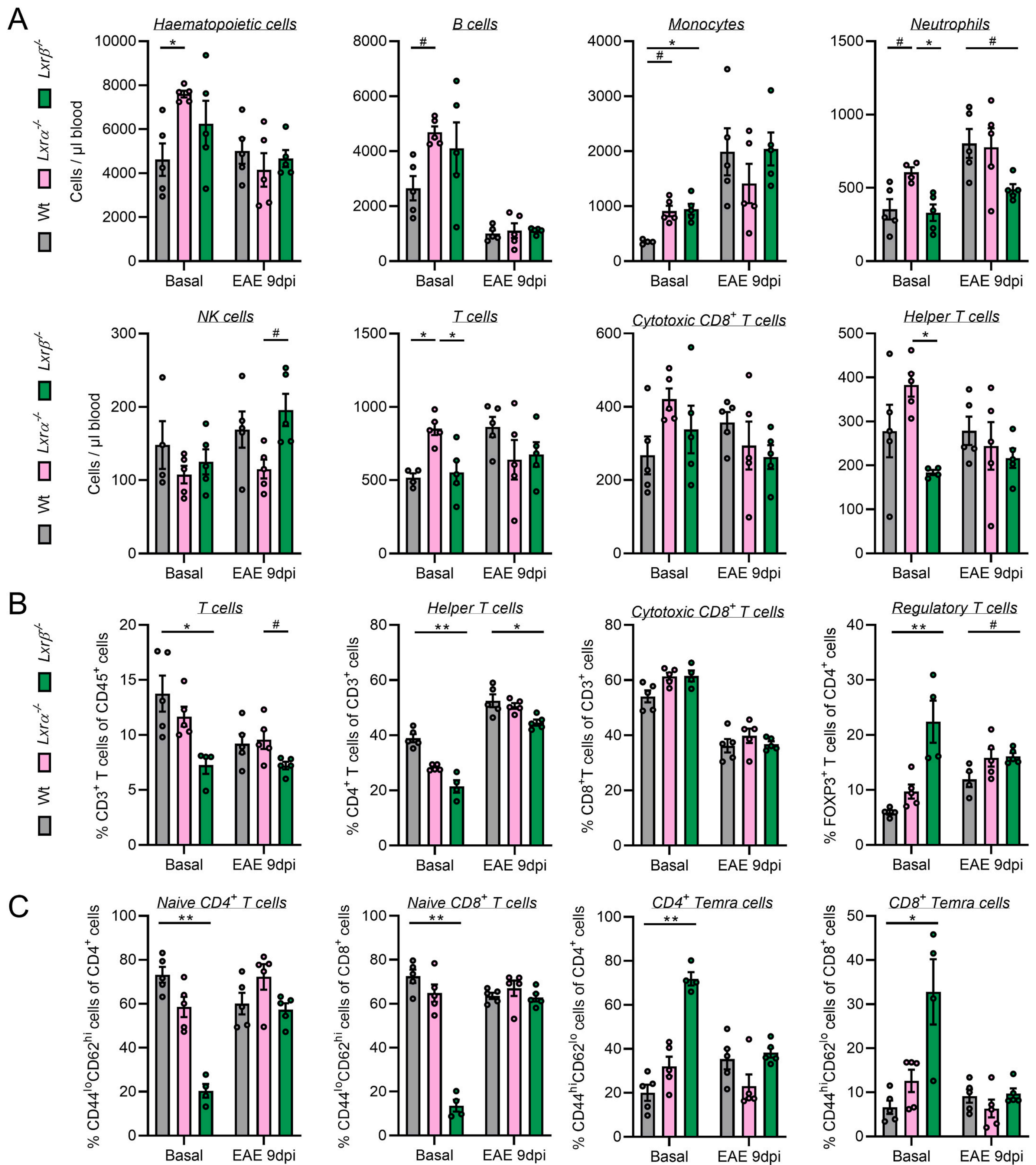

Fig. 4. ***Lxrβ* deficiency impacts the activation and relative abundance of circulating T cells**. (**A**) Absolute number of circulating immune cells in healthy and EAE animals (9 days post-immunization (dpi)). The following markers were used for immunophenotyping: haematopoietic cells ($CD45^{+}$), B cells ($CD45^{+}B220^{+}$), monocytes ($CD45^{+}CD11b^{+}NK1.1^{-}Ly6G^{-}Siglec\text{-}F^{-}$), NK cells ($CD45^{+}CD3^{-}NK1.1^{+}$), T cells ($CD45^{+}CD3^{+}$), cytotoxic T cells ($CD45^{+}CD3^{+}CD8^{+}$), and helper T cells ($CD45^{+}CD3^{+}CD4^{+}$). (**B**) Relative number of T cells (% $CD3^{+}$ cells of total $CD45^{+}$ cells), helper T cells (% $CD4^{+}$ cells of total $CD45^{+}CD3^{+}$ cells), cytotoxic T cells (% $CD8^{+}$ cells of total $CD45^{+}CD3^{+}$ cells), and regulatory T cells (% $FOXP3^{+}$ cells of total $CD45^{+}CD3^{+}CD4^{+}$ cells) in the blood of healthy and EAE animals (9 dpi). (**C**) Relative number of $CD4^{+}$ and $CD8^{+}$ naïve and effector memory T cells in the blood of healthy and EAE animals (9 dpi). The following markers were used for immunophenotyping: naive T cells ($CD44^{lo}CD62^{hi}$) and Temra cells ($CD44^{hi}CD62^{lo}$). Results represent 4 or 5 independent biological replicates (n = 4/5 mice) and were statistically analyzed using the Kruskal-Wallis test followed by Dunn's multiple comparison test. Data are represented as mean ± SEM. $^{\#}p < 0.1$, $^{*}p < 0.05$, and $^{**}p < 0.01$.

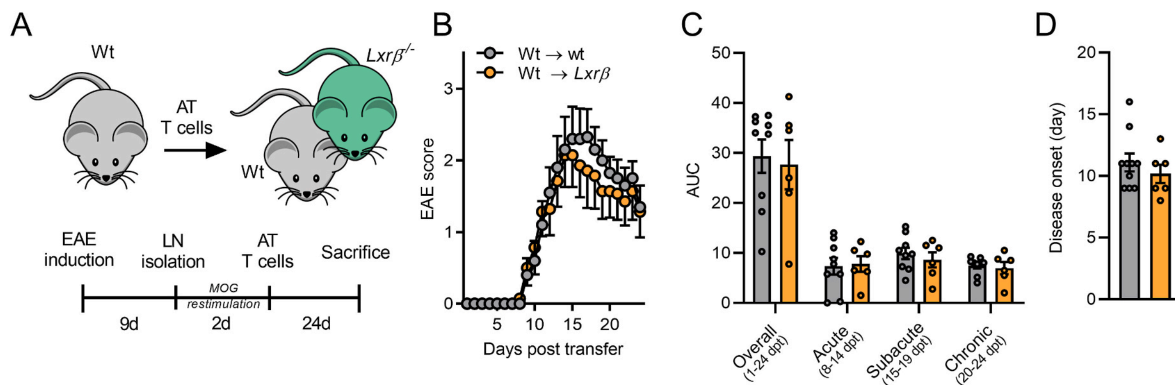

Fig. 5. ***Lxrβ*** **deficiency attenuates EAE disease severity in a T cell dependent manner.** (**A**) Experimental setup used to assess the impact of *Lxrβ* deficiency in T cells on EAE disease severity. Lymph node (LN)-derived T cells isolated from immunized wt mice (9 dpi) were reactivated ex vivo with recombinant myelin oligodendrocyte glycoprotein (MOG) and subsequently adoptively transferred to wt and $Lxr\beta^{-/-}$ mice. (**B**) EAE disease course of wt and $Lxr\beta^{-/-}$ mice that received LN-derived T cells of immunized wt mice. (**C**) Cumulative disease severity (area under the curve, AUC) of wt and $Lxr\beta^{-/-}$ mice that received LN-derived T cells of immunized wt mice at different disease stages: acute (8–14 days post transfer, dpt), subactute (15–19 dpt), and chronic stage (20–24 dpt). (**D**) Disease onset of wt and $Lxr\beta^{-/-}$ mice that received LN-derived T cells of immunized wt mice. All results are pooled from independent biological replicates and statistically analyzed using the Mann-Whitney test. Data are represented as mean ± SEM.

to LXR signaling [32,33]. Future studies should assess to what extent sequestration of autoreactive T cells in secondary lymphoid organs, as well as the involvement of CD38 and S1P signaling herein, causes the beneficial impact of LXRβ deficiency on the pathology and severity of EAE.

In contrast to EAE affected mice, non-immunized $Lxr\alpha^{-/-}$ and $Lxr\beta^{-/-}$ mice demonstrated marked changes in the absolute and relative frequency of circulating immune cells. In particular, $Lxr\beta^{-/-}$ mice demonstrated a 4-fold increase in circulating Tregs and Temra cells, and a matching decrease in naïve T cells. Akin to our findings, $CD4^+$ and $CD8^+$ T cells were previously found to acquire an effector memory phenotype in non-immunized $Lxr\alpha\beta^{-/-}$ and $Lxr\beta^{-/-}$ mice [7,18], and loss of *Lxrβ* but not *Lxrα* increases RORγt $^+$ Tregs in mesenteric lymph nodes [8]. To date, the molecular and cellular mechanisms that cause changes in peripheral immune cell frequencies in $Lxr\beta^{-/-}$ mice await experimental validation. Evidence indicates that absence of *Lxrβ* in antigen-presenting cells may underlie alterations in the abundance of Tregs [8], and potentially also naïve and effector memory T cells. In parallel, changes in thymic output and negative selection, processes essential in shaping the peripheral immune cell repertoire, are potentially involved [18]. It also remains unclear why homeostatic changes in peripheral immune composition in $Lxr\beta^{-/-}$ mice are nullified after EAE induction. Of interest, thymic involution and loss of cellularity are not only apparent in $Lxr\beta^{-/-}$ mice but also after EAE immunization [18,34]. Moreover, *Lxrαβ*-deficient mice show spontaneous breakdown in self-tolerance and develop age-dependent systemic autoimmune disease [35]. Hence, pre-existent autoimmunity and consequent changes in thymic physiology and peripheral immunity in $Lxr\beta^{-/-}$ mice could have prohibited or masked the induction of alike changes upon EAE immunization. Finally, the molecular mechanisms underlying the increased number of monocytes in non-immunized $Lxr\beta^{-/-}$ mice remain unclear. With respect to the latter, future studies should define if *Lxrβ* deficiency reduces the production of monocytes in the bone marrow and/or their subsequent release in the blood stream. Collectively, while more research is warranted, our results indicate that LXRβ controls the number and phenotype of circulating monocytes and T cells in non-immunized mice.

Microglia play an integral role in the immunopathology of MS [26]. Consistent with the importance of LXRs in driving microglia function [13,36], we found that lack of both LXR isoforms resulted in a less-inflammatory microglia phenotype, with the exception of $Lxr\beta^{-/-}$ microglia exposed to MS-relevant cytokines. $Lxr\beta^{-/-}$ microglia showed a more inflammatory phenotype upon exposure to TNFα and IFNγ, corresponding to increased EAE disease severity in microglia-specific $Lxr\beta^{-/-}$ mice. The latter findings point towards the importance of LXRβ in suppressing inflammatory microglia function, but refute the causal role of microglia in the reduced disease severity in whole-body $Lxr\beta^{-/-}$ mice. The cellular and molecular mechanisms that underlie this redundancy remain to be determined. Peripheral anti-inflammatory effects of whole-body *Lxrβ* deficiency may simply outweigh the inflammatory impact of *Lxrβ* deficiency in microglia.

We find that *Lxrβ* deficiency impacts neuroinflammation differently in the EAE model and after LPS-induced neuroinflammation. We postulate that inherent methodological and pathological differences between these models can explain these findings. In the EAE model, experimentally-induced systemic autoimmunity drives disease pathology in the CNS. In the LPS-induced neuroinflammation model, intracerebroventricular LPS administration provokes a neuroinflammatory response that relies predominantly on the inflammatory activation of glial cells, i.e. astrocytes and microglia, with little to no involvement of peripheral immunity [25]. Given the essential role of peripheral immune cells in suppressing neuroinflammation in $Lxr\beta^{-/-}$ EAE mice, the increased neuroinflammatory burden in $Lxr\beta^{-/-}$ mice upon ICV LPS mice could reflect redundancy of peripheral immunity in this model.

Our findings indicate that forced activation of LXRα in the absence of LXRβ enhances the protective effect of *Lxrβ* deficiency on EAE disease severity. Given that T cells barely express LXRα [7], this beneficial effect unlikely depends solely on changes in T cell physiology. Based on our previous study, we postulate that LXRα activation in $Lxr\beta^{-/-}$ mice may maintain or improve brain endothelial cell integrity, thereby further preventing accumulation of autoreactive immune cells into the CNS. However, changes in the physiology of other effector cells in EAE pathogenesis, i.e. astrocytes [37], DC [38], macrophages [17], and B cells [39], as well as thymic derived epithelial cells and thymocytes [18], can account for the reduced disease severity in $Lxr\beta^{-/-}$ mice upon exposure to the LXR agonists as well. Moreover, while LXRβ is more ubiquitously expressed, LXRα expression predominates in metabolic tissues such as the liver and adipose tissue. Hence, metabolic changes originating in these tissues and spreading to the circulation and other tissues, *i.e.* lymphoid organs and the CNS, may also contribute to changes in EAE disease severity. Thus, the culprit cell types and molecular mechanisms that drive the protective impact of forced activation of LXRα in $Lxr\beta^{-/-}$ EAE mice remain to be determined.

Overall, using whole-body and cell-specific knockout models, we show that LXRα and LXRβ play functionally divergent roles in autoimmunity and neuroinflammation, with *Lxrα* and *Lxrβ* deficiency being

harmful and benign, respectively. Furthermore, we provide evidence that lack of *Lxrβ* attenuates EAE disease severity in a T cell-dependent manner. Understanding the mechanisms by which LXR isoforms control neuroinflammation may help in the development of therapeutic strategies, *i.e.* targeted delivery or selective activation of LXR isoforms, to prevent autoimmunity and MS disease progression.

Author statement

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Declaration of competing interest

All authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2021.102723.

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