Increase in chemokine CXCL1 by ERβ ligand treatment is a key mediator in promoting axon myelination

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Estrogen receptor β (ERβ) ligands promote remyelination in mouse models of multiple sclerosis. Recent work using experimental autoimmune encephalomyelitis (EAE) has shown that ERβ ligands induce axon remyelination, but impact peripheral inflammation to varying degrees. To identify if ERβ ligands initiate a common immune mechanism in remyelination, central and peripheral immunity and pathology in mice given ERβ ligands at peak EAE were assessed. All ERβ ligands induced differential expression of cytokines and chemokines, but increased levels of CXCL1 in the periphery and in astrocytes. Oligodendrocyte CXCR2 binds CXCL1 and has been implicated in normal myelination. In addition, despite extensive immune cell accumulation in the CNS, all ERβ ligands promoted extensive remyelination in mice at peak EAE. This finding highlights a component of the mechanism by which ERβ ligands mediate remyelination. Hence, interplay between the immune system and central nervous system may be responsible for the remyelinating effects of ERβ ligands. Our findings of potential neuroprotective benefits arising from the presence of CXCL1 could have implications for improved therapies for multiple sclerosis.

Significance

CXCL1 is a major neutrophil chemoattractant that binds to the chemokine receptor, CXCR2, on neutrophils and oligodendrocytes. Estrogen receptor β ligand treatment in a mouse model of multiple sclerosis induces an increase in peripheral and brain CXCL1 levels that correlate with an increase in axon remyelination. Oligodendrocyte progenitor recruitment and differentiation by CXCL1, in combination with attenuated IFN-γ production reducing apoptosis, may account for at least one avenue whereby estrogen receptor β ligands exert their clinical benefits.

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Additional information

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Previously, ERβ ligand-mediated immune effects have been assessed after peak disease when demyelination and CNS inflammation has plateaued (12, 15). To understand how ERβ ligands modify EAE pathogenesis and to expose a potential common immunomodulatory role of ERβ ligands, leukocyte populations and secreted cytokines/chemokines were analyzed from mice treated with one of several ERβ ligands from EAE onset to peak disease. In the present study, we show that therapeutic ERβ ligands enhance peripheral and CNS concentrations of the C-X-C motif chemokine, CXCL1, with similar remyelinating and neuroprotective properties.

### Results

#### Effect of Therapeutic ERβ Ligand Treatment on EAE Clinical Score and Uterine Weight

A persistent disease course starting at day 15–16 with chronic motor deficits was observed in vehicle-treated EAE mice. Therapeutic IndCl and WAY treatment administered at peak disease decreased clinical disease severity over time (15) (Fig. 2A and SI Appendix, Table S2). Not previously reported, therapeutic WAY treatment induced an increase in myelin density, increase in the OL population, and decrease in “g-ratio” of white-matter tracts (SI Appendix, Fig. S2), similar to EAE groups treated with DPN (17) or IndCl (15).

To address therapeutic response of different ERβ ligand treatment on peak EAE pathology and immune response, treatment was started at day 8 and suspended on day 21 (Fig. 2B and SI Appendix, Table S3). Estradiol pretreatment (pre-E2) completely abrogated clinical disease (15). All other treatment groups displayed onset of clinical disease beginning 7–10 d post-induction, with peak disease (defined as 2 to 3 sequential days of maximal clinical score) occurring between days 18 and 21 (Fig. 2A and B). All therapeutic ERβ ligands administered at onset of disease did not attenuate clinical symptoms by day 21 (Fig. 2B). Pre-E2 treatment and not ERβ ligands increased uterine weight by nearly fourfold (Fig. 2C), as previously observed (15).

#### Improved Axon Myelination and No Effect on Leukocyte Infiltration and Astrogliosis

Reduced axon numbers and myelination at peak EAE has been reported (17, 21). Similarly, vehicle-treated mice at peak disease (Fig. 2B) displayed extensive demyelination, indicated by reduced myelin basic protein (MBP*) staining intensity, proximal to CD45+ (a pan-leukocyte marker) perivascular cuffs and leukocyte infiltrates that were not observed in normal animals (Fig. 3A). Significant loss of MBP* and NF200* axons was observed in the vehicle-treated mice, while ERβ ligand treatment rescued both myelin and axons (Fig. 3 B and C).

There was an increase in CD45+ leukocyte infiltration in the white matter of vehicle-treated and ERβ ligand-treated mice, whereas pre-E2 treatment had minimal CD45+ immunoreactivity (Fig. 4 A and C). Similarly, immunoreactivity for the myeloid cell marker ionized calcium-binding adaptor molecule 1 Iba1 and the astrocyte marker glial fibrillary acidic protein (GFAP) was significantly elevated in vehicle- and ERβ ligand-treated mice (Fig. 4). Pre-E2 abrogated this increase (Fig. 4 D and E).

#### No Change in CNS-Infiltrating Leukocyte Populations

To profile CNS infiltrating leukocytes after ERβ ligand treatment, mono- nuclear cells were isolated at peak EAE disease and analyzed by flow cytometry. Vehicle-treated mice showed elevated CD11b+CD45R0+ activated microglia/macrophage frequency (gate R2) (Fig. 5 A and C). Pre-E2 significantly reduced CD11b+CD45R0+ frequency (Fig. 5C), while ERβ ligand and vehicle treated groups displayed similar frequency of activated microglia/macrophages (Fig. 5C). Similarly, increased CD4+ IFN-γ+ Th1 and CD4+ IL-17 Th17 frequencies were observed in vehicle-treated mice but not pre-E2 (Fig. 5 D and E). ERβ ligands did not affect Th1 or Th17 frequency (Fig. 5 D and E).

**Modification of Cytokine and Chemokine Production by Peripheral Leukocytes.** To characterize the effects of ERβ ligands on the peripheral immune system, splenocytes were harvested from mice 21 d after EAE induction and assessed by flow cytometry. Splenic CD11b+CD45R0+ macrophage/monocyte composition was attenuated by pre-E2, but not ERβ ligand treatment (Fig. 6 A and B). Th1 frequency was significantly reduced in pre-E2 and ERβ ligand treated groups vs. vehicle (Fig. 6C), while Th17 frequency was not affected (Fig. 6D and SI Appendix, Fig. S3).

Cytokine and chemokine expression was analyzed to assess the role of leukocyte effector function in ERβ ligand derived benefits during peak disease (22).

#### Proinflammatory cytokines.

The Th1 cytokine IFN-γ was significantly up-regulated in vehicle-treated mice vs. normal. In contrast, pre-E2, post-IndCl, WAY, and DPN decreased IFN-γ concentration relative to vehicle (Fig. 7 A, i). IL-2 was significantly increased in vehicle-treated mice vs. normal and was attenuated only by pre-E2 treatment (Fig. 7 A, ii). Vehicle-treated mice produced higher levels of TNF-α and IL-17 vs. normal (Fig. 7 A, iii and iv). Pre-E2, IndCl, WAY, and DPN produced similar levels of TNF-α and IL-17 as vehicle treatment (Fig. 7 A, iii and iv). Increased IL-1β was observed in vehicle-treated and pre-E2 groups vs. normal. Interestingly, all ERβ ligand groups exhibited greater IL-1β production vs. vehicle (Fig. 7 A, v).

**Fig. 2.** Therapeutic ERβ ligand treatment decreases EAE severity with no effect on uterine weight. (A) Treatment with ERβ ligands, IndCl (post-EAE+IndCl; purple), and WAY (post-EAE+WAY; orange) began on day 21 until day 40. Onset of clinical disease occurred between days 17 and 19 in vehicle-treated EAE mice (post-EAE+vehicle; red), with peak severity at day 21. Treatment with IndCl and WAY significantly decreased disease severity vs. vehicle beginning by day 20. Strong 40 (IndCl-purple), WAY-WAY+WAY stars). Differences in EAE clinical scores were determined by two-way unbalanced ANOVA with Dunnett’s multiple comparisons test (4B). n = 8–10 mice per group; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (B) Therapeutic treatment with ER ligands, IndCl, WAY and DPN (post-EAE+DPN; blue) began on day 8 until day 40. Prophylactic E2 (pre-EAE+E2; green) prevented onset of clinical disease. Vehicle-reatted EAE mice displayed onset of clinical disease symptoms between days 7 and 10 with disease severity peaking around day 17. During peak disease, IndCl, WAY, and DPN treatment did not affect EAE clinical scores. (C) Assessment of postperfusion uterus to body weight. Only pre-E2-treated female mice exhibited a 4x increase in uterine:body weight ratio. n = 8–10 mice per group.
Antiinflammatory cytokines. Skewing immune response toward production of Th2 cytokines, such as IL-4, IL-5, and IL-10, with parallel suppression of Th1 cytokines ameliorates EAE (23). IL-4 was up-regulated only in vehicle-treated mice (Fig. 7 B, i). Similarly, neither IL-5 nor IL-10 was changed by E2 or ERβ ligand treatment (Fig. 7 B, ii and iii).

Chemokines. CXCL1 was significantly up-regulated by ERβ ligands relative to vehicle (Fig. 7 C, i). No change was observed in pre-E2 groups. CXCL9 and CXCL10, closely related IFN-γ-inducible T cell and monocyte chemoattractants, were increased in vehicle-treated mice vs. normal. CXCL9 levels were similar in all treatment groups compared with vehicle; however, pre-E2 and IndCl treatment significantly reduced CXCL10 levels, with no change by WAY or DPN treatment (Fig. 7 C, ii and iii).

ERβ Ligands Up-Regulate CXCL1. Due to its role in OPC recruitment and up-regulation by splenic leukocytes in response to ERβ ligand treatment, CXCL1 expression was examined in white matter from mice at peak EAE by immunohistochemistry (IHC). In the CNS, astrocytes are a primary source of CXCL1 during white-matter development (24) and neuroinflammation (25). Compared with vehicle, IndCl- and DPN-treated mice exhibited increased CXCL1+ immunoreactivity that appeared to overlap with GFAP (Fig. 8 A–C) (26). In addition, CXCL1 immunoreactivity colocalized with IL-1β and GFAP in astrocytes during ERβ ligand treatment (SI Appendix, Fig. S4). In addition, supernatant from primary astrocyte cultures stimulated with IL-1β contained increased levels of CXCL1. The same supernatant when added to OL cultures induced an increase in MBP expression (SI Appendix, Fig. S5).

CXCR2 receptor is critical for developmental OPC positioning and differentiation in the CNS (27). OL primary cultures in the presence of CXCR2 antagonist, SB 225002 and IL-1β astrocyte-conditioned media showed decreased differentiation and enhanced OL apoptosis (SI Appendix, Fig. S5). To determine the distribution of CXCR2 during peak EAE disease in response to ERβ ligand treatment, CXCR2 expression by OPCS/OLs was assessed by colabeling with the OL lineage-specific transcription factor, Olig2 in spinal cords. Olig2+ nuclei were comparable between all groups evaluated and CXCR2 was detected in a subset of Olig2+ nuclei (Fig. 8 B and C). The fraction of CXCR2+Olig2+ cells increased in all treatment groups (Fig. 8E), consistent with previous reports that OPC/OL CXCR2 is up-regulated in MS (28).

Fig. 3. Therapeutic ERβ ligand treatment improves myelination during peak EAE disease. (A) Representative 10× magnification images of thoracic spinal cords collected at peak disease (day 21). Experimental groups shown in Fig. 2B, were immunostained for MBP (red), CD45 (green), and DAPI (blue), with 4× normal (i) and vehicle (ii) images to show pathology. (B) Magnified 40× images showing ventral column immunostained with MBP (red) and NF200 (green). (C) Quantification of MBP intensity and NF200+ axon numbers revealed significant decreases in only vehicle treated groups. n = 5 mice per group; *P < 0.05, **P < 0.01.

Fig. 4. Treatment with ERβ ligands do not exhibit significant changes in CNS immune cell populations during peak EAE disease. (A and B) Representative 40× magnification images of thoracic spinal cords collected at peak disease (day 21) from experimental groups shown in Fig. 2B were immunostained for astrocytes (GFAP, green), leukocytes (CD45, red), microglia/macrophages (Iba1, red), and DAPI (blue). (C–E) Quantification of normalized intensities of CD45, GFAP, and Iba1. Vehicle-treated mice exhibited increased CD45, Iba1, and GFAP intensity that was decreased only with pre-E2 treatment. n = 5 mice per group; *P < 0.05, **P < 0.01, ***P < 0.001.
ligands in a clinically relevant mouse model of MS. Our ligands tested in the present study may elicits OL apoptosis in vitro (32) and ligand, 4-(2-phenyl-5,7-
axis) and CD45 (CD45 CLEAR) distributions, while activated microglia/macrophages (CD11b+) were present in the CNS from vehicle, pre-E2, and therapeutic ERβ ligand treated mice collected from peak EAE mice. In the CNS, resting microglia (R1) are represented by CD11b+CD45lo populations, while activated microglia/macrophages (R2) are represented by CD11b+CD45hi (β) and C Quantification of resting (CD11b+CD45lo, R1) and activated microglia/macrophages (CD11b+CD45hi, R2) from prophylactic E2 and therapeutic ERβ ligands treated EAE mice. Pre-E2 decreased activated macrophages compared with vehicle. (D and E) Th1 (CD4+IFN-γ+) and Th17 (CD4+IL-17+) frequency in the CNS was decreased by pre-E2 treatment. ERβ ligands did not affect Th1 or Th17 frequency. n = 8–10 mice per group; *P < 0.05, **P < 0.01, ***P < 0.001.

Discussion
In this study, we characterized the therapeutic impact of three ERβ ligands in a clinically relevant mouse model of MS. Our results suggest that the protective effects of ERβ ligands on remyelination and neuroprotection may be mediated by changes in cytokine and chemokine production. Comparisons among the ERβ ligands tested show improved myelination. Consistent with previous studies (12, 15), this occurred with little impact on the composition or polarization of CNS or splenic leukocytes, but involved reduction of peripheral IFN-γ production and increased CXCL10 expression in the CNS and spleen. Individual ERβ ligands displayed differential effects on the production of the IFN-γ-inducible CXCL10, with IndCl being the only compound tested to suppress production aside from pretreatment with E2. These data indicate that individual ERβ ligands may have various effects on leukocyte activity in EAE, while also pointing to a potential immunological mechanism common to ERβ-mediated remyelination. Specifically, three findings that were observed with all ERβ ligands tested stand out: (i) increased myelination, (ii) reduced IFN-γ, and (iii) increased CXCL1. These data suggest that ERβ ligands may facilitate an environment within the inflamed CNS where OPCs are recruited to the site of ongoing demyelination by astrocytic CXCL1 and allowed to differentiate into myelinating OLs through attenuation of cytotoxic IFN-γ production (SI Appendix, Fig. S6).

Although the ligands used in this study were all selective for ERβ, their activity in the peripheral and CNS may differ (12, 15, 19, 29). For example, IndCl, but not DPN or WAY, was effective at repressing inflammatory response gene products in microglia and astrocytes (19). The differential immune effects observed by treatment with the ERβ ligands tested in the present study may be due to how these compounds recruit cofactors. As an example, IndCl promotes entry of ERβ into the C-terminal binding protein-dependent transrepression pathway while DPN does not (19). However, while transcriptional cofactor recruitment differs, these ERβ ligands converge in their ability to induce OPC/OL survival and myelination during demyelinating disease (15, 30). Importantly, this was shown to be mediated by direct action on OLs as conditional knockout of ERβ from these cells abrogated the clinical benefits of DPN (30). Quiescent microglia are distributed throughout the homeostatic CNS and are characterized by coexpression of the myeloid cell integrin/complement receptor CD11b and low levels of CD45. In the present study, unlike pre-E2, no ERβ ligand tested abrogated the rise in activated microglia/macrophages recovered from the CNS or spleen of vehicle-treated mice.

Unexpectedly, ERβ ligands had divergent effects on peripheral and CNS T lymphocyte populations. Pre-E2 reduced the frequency of Th1 cells in splenic and CNS mononuclear cells. In contrast, pre-E2 reduced CNS-infiltrating Th17 cells, but had no effect on splenic lymphocytes. Similarly, ERβ ligand treatment decreased Th1 cell frequency, as well as proinflammatory cytokine IFN-γ in the periphery, with no change in Th1 populations in the CNS. This finding may reflect CNS infiltration at disease onset or reduced Th1 differentiation resulting from decreased IFN-γ concentrations. Another ERβ ligand, 4-(2-phenyl-5,7-bis[trifluoromethyl]pyrazol-1,5,3-aryl)morpholin-3-yl)phenol (PHTPP), was shown to suppress Th1 response by acting directly on CD4+ T cell ERβ expression in vitro (16). We did not observe a suppression of Th17 frequency or peripheral IL-17 secretion with ERβ ligands. Additional study is required to assay the effect of ERβ ligands on CD8+ populations; however, E2 has been shown to increase their activity in a viral of lung infection (31).

Proinflammatory cytokines, such as IFN-γ, drive EAE by potentiation antigen presentation and secretion of additional cytokines and chemokines (22). Of potential importance to the mechanism whereby ERβ ligands promote remyelination, splenocytes from ERβ ligand treatment produced less IFN-γ in response to MOG35-55 peptide. IFN-γ elicits OL apoptosis in vitro (32) and ectopic CNS expression results in OL death and impaired myelination (33). These data indicate that IFN-γ-induced death of OLs/OPCs may represent a potential etiology for remyelination failure in EAE and MS. However, IFN-γ may possess pleiotropic effects on OLs during demyelinating disease (34). Thus, ERβ ligand-mediated peripheral IFN-γ reduction may play a key role in supporting remyelination by slowing Th1 differentiation, while lowered levels of CNS IFN-γ may improve OL survival.

Interestingly, IL-1β, which is associated with various inflammatory and demyelinating disorders (35) and responsive to E2 signaling (36), was significantly increased by ERβ ligand treatments. While IL-1β is cytotoxic to mature OLs in vitro, neither demyelination nor OL loss during cuprizone demyelination are attenuated in mice lacking IL-1β (35). However, remyelination fails due to lack of IL-1β-mediated expression of insulin growth factor-1 (37), pointing toward a complex role for this cytokine. Our results suggest that IL-1β in the presence of the ERβ ligands may be associated with myelin repair by promoting mature OL differentiation and remyelination during EAE.

Chemokines and their cognate receptors play a critical role in the recruitment and trafficking of leukocytes in the context of Th17 effector function. Despite the small number of mice in our study, these trends were also seen in the spleen, where all three compounds reduced Th17 frequencies. Fig. 6. Effect of therapeutic ERβ ligand treatment on splenic immune cell numbers during peak EAE. (A) Representative plots of splenic CD11b (y axis) and CD45 (x axis) distribution in vehicle, prophylactic E2, and therapeutic ERβ ligand treated mice collected at peak disease. Activated monocytes/macrophages (R2) are represented by CD11b+CD45lo (β) and C Quantification of activated (CD11b+CD45hi) macrophages and their cognate receptors play a critical role in the recruitment and trafficking of leukocytes in the context of IL-17 axis) is associated with various inflam- matory signaling (36), was significantly increased by ERβ ligand treatments. While IL-1β is cytotoxic to mature OLs in vitro, neither demyelination nor OL loss during cuprizone demyelination are attenuated in mice lacking IL-1β (35). However, remyelination fails due to lack of IL-1β-mediated expression of insulin growth factor-1 (37), pointing toward a complex role for this cytokine. Our results suggest that IL-1β in the presence of the ERβ ligands may be associated with myelin repair by promoting mature OL differentiation and remyelination during EAE.

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Drugs were dissolved in vehicle (10% ethanol and 90% Miglyol 812N) at the following concentrations and administered subcutaneously daily: IndCl 5 mg/kg/d, WAY 1 or 10 mg/kg/d, and DPN 8 mg/kg/d (Fig. 1). 2.5 mg/kg/d E2 was used as a positive control. Treatment was initiated at postimmunization day 0, 8, or 21.

EAE. Active EAE was induced in 8-wk-old female C57BL/6 mice, as previously described (14, 48) (SI Appendix, Fig. S1). Animals were maintained according to the University of California, Riverside Office of Research Integrity and the Institutional Animal Care and Use Committee.

disease conditions. ERβ ligands increased CXCL1 and decreased CXCL10. Abundant CXCL10 has been detected at early time points in the development of EAE and MS lesions (38) and promotes neuronal and OL apoptosis (39). Here, only IndCl decreased CXCL10 levels.

CXCL1, a major neutrophil chemoattractant expressed by astrocytes and microglia, affects chemotaxis through its receptor CXCR2, which is present on diverse cellular populations, including leukocytes, keratinocytes, dermal fibroblasts, neutrophils, and OLs (40, 41). E2 has been shown to be a negative regulator of CXCL1/CXCR2 signaling pathways (42-45) through predominantly ERα-dependent pathways (42). However, CXCL1-mediated functions extend beyond chemotaxis. In MS, both CXCL1+ astrocytes and CXCR2+ OLs (46) have been detected in lesions (46), indicating that it may play a role in recruitment of OPCs to demyelinating sites. CXCL1 overexpression by astrocytes attenuates EAE disease severity (47), while CXCL2 deficient mice exhibit reduced white-matter volume and myelin proteins in the spinal cord (48). Furthermore, CXCR2 signaling promotes OPC survival by increasing levels of the anti-apoptotic protein, Bcl-2 (39). Together, these results indicate a potential role for astrocyte derived CXCL1 in promoting OL survival in ERβ ligand treated EAE mice.

Our finding that ERβ ligand therapy promotes increased astrocytic CXCL1 production may represent an additional facet of how these compounds induce remyelination. OPC recruitment and differentiation by CXCL1, in combination with attenuated IFN-γ production reducing OL apoptosis, may account for at least one avenue whereby ERβ ligands exert their clinical benefits. In summary, our data show that ERβ ligand neuroprotection/remyelination may be partly mediated by skewing the proinflammatory phenotype to a protective phenotype. Our results demonstrate that the interplay between CNS- and immune-derived signals is central to the induction and regulation of neuroinflammatory diseases such as MS. The possibility that ERβ ligands modulate the cytokine and chemokine milieu to potentially promote repair/remyelination opens up exciting therapeutic options.

Materials and Methods

Treatment. Drugs were dissolved in vehicle (10% ethanol and 90% Miglyol 812N) at the following concentrations and administered subcutaneously daily: IndCl 5 mg/kg/d, WAY 1 or 10 mg/kg/d, and DPN 8 mg/kg/d (Fig. 1). 2.5 mg/kg/d E2 was used as a positive control. Treatment was initiated at postimmunization day 0, 8, or 21.
Mononuclear Cell Isolation. Splenocytes were isolated and stimulated in vitro using 25 μg/mL MOG35–55 (30, 49). Supernatants were collected after 48 h and cytokine levels were determined by Luminex. CNS mononuclear cells were isolated per the protocol in ref. 50.

Flow Cytometry and ELISA. Leukocytes were stained with antibodies (SI Appendix, Table S1) and flow cytometry data were acquired. CXCL1 concentrations supernatant were measured using the murine CXCL1 ELISA kit.

Cell Culture. Primary astrocytes and OPCs were isolated as described previously (51). Astrocytes were treated with IL-1β or media alone for 48 h then supernatant was applied to primary OLs + CXCR2 antagonist SB 225002.

IHC and Analysis. IHC was performed on formalin-fixed thoracic spinal cord sections and primary cell cultures with different antibodies (SI Appendix, Table S1). Images were quantified using unbiased stereology (49) and ImageJ software.

Statistical Analysis. All data were analyzed in GraphPad Prism 6. Differences in EAE clinical scores were determined by two-way unbalanced ANOVA with Dunnnett's multiple comparisons test (48). All others were analyzed by ordinary one-way ANOVA with Dunnett's multiple comparisons test or Kruskal-Wallis with Dunn's multiple comparisons test. Data are presented as mean ± SEM; n = 2–3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Data Availability. All data generated or analyzed during this study are included in the main text and in the SI Appendix.