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The immunomodulatory effect of laquinimod in CNS autoimmunity is mediated by the aryl hydrocarbon receptor

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ABSTRACT

Though several functional properties of laquinimod have been identified, our understanding of the underlying mechanisms is still incomplete. Since the compound elicits similar immunomodulatory effects to ligands of the aryl hydrocarbon receptor (AhR), we compared the efficacy of laquinimod in experimental autoimmune encephalomyelitis (EAE)-afflicted wild-type and AhR-deficient mice. Laquinimod failed to ameliorate clinical symptoms and leukocyte infiltration in AhR-deficient mice; however, treatment exerted neuroprotection by elevation of brain-derived neurotrophic factor (BDNF) independent of genetic profile. Thus, our data identify the AhR pathway in these mutant mice as crucial for the immunomodulatory, but not neuroprotective, efficacy of laquinimod in EAE.

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1. Introduction

Multiple sclerosis (MS) is one of the most common chronic, autoimmune-mediated, neurodegenerative diseases leading to neurological disability in young adults. The pathology of MS is primarily characterized by the formation of inflammatory demyelinating lesions within the central nervous system (CNS), resulting in neuronal tissue damage and functional deficits. Current models of disease etiology theorize that disease immunopathology is dependent upon peripheral activation of myelin-specific T cells and their subsequent migration into the CNS by surpassing the blood brain barrier (Fletcher et al., 2010). Following reactivation by local antigen-presenting cells (APCs), namely dendritic cells (DCs), pathogenic T cells then not only promote secretion of chemokines and vasoactive molecules, thereby recruiting several other leukocytes to the CNS, but also produce pro-inflammatory cytokines to stimulate local microglia and astrocytes (Bailey et al., 2007; Kawakami et al., 2004), further exacerbating the autoimmune response (Maggi et al., 2014).

Much of our present knowledge regarding the immunological mechanisms underlying MS pathogenesis is derived from the rodent model experimental autoimmune encephalomyelitis (EAE) (Gold et al., 2006). Extensive studies in EAE not only elucidated the principal aspects of T cell-mediated CNS inflammation, but also provided a system within which to both identify and validate disease-modifying agents and therapeutic strategies (Haghikia et al., 2013). Such a process led to the discovery of laquinimod for the treatment of MS.

An orally-administered quinoline-3-carboxamide, laquinimod has been described in preclinical studies as more potent than its structurally-related predecessor, linomide, for the inhibition of disease incidence and relapses (Brunmark et al., 2002; Yang et al., 2004). And while several phase II and phase III clinical trials demonstrated lesion and annual relapse rate reduction, a decrease of cerebral atrophy, and a favorable safety profile (Comi et al., 2008, 2012; Filippi et al., 2014; Polman et al., 2005; Vollmer et al., 2014), less is known about the mechanistic underpinnings of the molecule.

EAE studies investigating laquinimod have shown that administration either ameliorates clinical symptoms or completely suppresses manifestation of disease in a dose-dependent manner, these effects being associated with reduced numbers of infiltrating leukocytes in the CNS (Brunmark et al., 2002; Toubi et al., 2012; Wegner et al., 2010). Additionally, it has been shown that laquinimod can differentially impact several myeloid cell populations: though treatment reduces T cell-activating $CD4^+$ DCs, it also promotes the development of anti-inflammatory type II, tolerogenic DCs and monocytes (Schulze-Topphoff et al., 2012; Thöne et al., 2012). In turn, laquinimod-induced effects on lymphoid cells include the elevation of

Abbreviations: AhR, aryl hydrocarbon receptor; APC, antigen-presenting cell; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; KO, knockout; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; WT, wild-type.

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regulatory T cells (Treg), the reduction of pro-inflammatory Th1 and Th17 responses, and a shift of cytokine production towards an anti-inflammatory phenotype in T and B cells (Aharoni et al., 2012; Schulze-Topphoff et al., 2012; Toubi et al., 2012; Wegner et al., 2010; Yang et al., 2004). Adoptive transfer studies with laquinimod-treated DCs indicate that these effects are mediated by laquinimod-induced alterations of DC phenotype rather than being a direct impact on lymphocytes (Brück and Wegner, 2011; Jolivel et al., 2013).

In EAE, the aryl hydrocarbon receptor (AhR) is critical for immunomodulatory effects similar to those of laquinimod. AhR activation can promote either Treg or Th17 differentiation in a ligand-specific manner, resulting in either amelioration or exacerbation of clinical symptoms respectively (Quintana et al., 2008). AhR signaling has also been implicated in disease amelioration through either the induction of tolerogenic DCs, as has laquinimod (Quintana et al., 2010), or activation of mucosal DCs, promoting type 1 regulatory (Tr1) cell differentiation (Apetoh et al., 2010; Wu et al., 2011). Due to these similarities, we hypothesized that AhR activation comprises a crucial aspect of the laquinimod-mediated mechanism in EAE.

The AhR belongs to the family of basic helix-loop-helix/Per-Arnt-Sim transcription factors. While it has previously been associated with the mediation of the adaptive response to environmental chemicals, such as environmental pollutants, most notably that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCCD) (Denison et al., 2002), analysis of the AhR binding pocket suggests the plausible accommodation of many variable xenobiotic ligands, with polycyclic aromatic hydrocarbons – molecules structurally related to laquinimod (Tuvesson et al., 2005) – demonstrating elevated binding affinity (Waller and McKinney, 1995). Additionally, a growing number of studies have described new endogenous ligands, further highlighting the impact of the AhR pathway on disease tolerance and immunity (García-Lara et al., 2015; Henry et al., 2006; Lowe et al., 2014; Mezrich et al., 2010).

By conducting AhR activation reporter assays, mapping clinical EAE disease course and analyzing histological spinal cord stainings of wild-type and AhR knockout mice, we demonstrate in the following experiments that AhR signaling is indeed integral for the efficacy of laquinimod in EAE.

2. Material and methods

2.1. Laquinimod binding assay

To test for the AhR-activating potential of laquinimod, a reporter vector assay was used. 5×10^4 HepG2 cells with functional AhR were plated and 24 h later co-transfected with either specific reporter pGL3-5xDRE (containing five dioxin responsive elements (DREs) from rat *cyp1a1* promoter), or the empty vector pGL3. Twenty-four hours after transfection with reporter constructs, cells were treated with increasing concentrations of laquinimod (1–50 μM) or solvent H_2O for another 24 h. Following the lysis of the cells, luciferase activity was determined by using the Dual-Luciferase® Reporter Assay System (Promega, Fitchburg, USA), and read with Tecan Reader Infinite M200 PRO (Tecan Trading AG, Männedorf, Switzerland). Firefly luciferase activity derived from the reporter plasmids was normalized to *Renilla reniformis* luciferase activity derived from co-transfected pRL-SV40 (Promega, Fitchburg, USA).

2.2. Mice

Eight- to ten-week-old C57BL/6J wildtype (WT) mice and AhR knockout (KO; $Ahr^{tm1Bra/J}$; Jackson Stock No.: 002831) mice on the same genetic background were bred and housed under pathogen-free conditions in the animal facility at the Ruhr-Universität Bochum, Germany. Animals were kept in a 12 hour day-night cycle and received standard chow and water ad libitum.

2.3. MOG-EAE

For EAE induction, myelin oligodendrocyte glycoprotein 35–55 peptide (MOG_{35-55}; Institute of Medical Immunology, Charité, Berlin, Germany) was emulsified in Complete Freund's Adjuvant (Difco Laboratories, Detroit, USA) to reach a concentration of 1 mg/ml.

Each animal was subcutaneously injected with 50 μl emulsion in each flank at the tail base, followed by intraperitoneal application of 100 μl PBS containing 200 ng Pertussis toxin (EMD Chemicals, San Diego, USA) immediately and 48 h post-immunization (p.i.). Beginning from the day of immunization, all mice were treated daily with 200 μl laquinimod (5 mg/kg body weight; Teva Pharmaceutical Industries Ltd., Netanya, Israel) or vehicle H_2O (control) via oral gavage.

Animals were weighed and scored for disease severity on a daily basis. Clinical symptoms were assessed via a scale ranging from 0 to 10, as thus modified from Linker et al. (2002): 0, normal; 1, reduced tone of tail; 2, limp tail; 3, mild ataxia; 4, moderate ataxia or mild paresis of hind limbs; 5, paraparesis of the hind limbs; 6, severe paresis of both or paralysis of one hind limb; 7, paralysis of both hind limbs; 8, tetraparesis; 9, moribund; 10, death.

2.4. Evaluation of immune cell infiltration and axonal damage in the CNS

Animals were perfused with 0.9% sodium chloride and 4% paraformaldehyde on day 23 post-immunization. The spinal cord and spleen were prepared, dehydrated and embedded in paraffin. All histopathological and immunohistochemical examinations were performed on 5 μm sections. For study of immune cell infiltration antigens were unmasked by incubation in boiling citrate buffer, and then blocked with 10% bovine serum albumin (BSA). Primary antibody labeling of T cells (rat α-CD3 1:200, Serotec, Puchheim, Germany) and macrophages (rat α-Mac-3 1:200, BD Pharmingen) occurred overnight at 4 °C. Following the blockade of endogenous peroxidase by H_2O_2, visualization was facilitated by the use of a biotin conjugated anti-rat secondary antibody (1:200, Linaris, Dossenheim, Germany), the Vectastain ABC detection system (Vector Laboratories, Burlingame, USA), and diaminobenzidine (Merck, Darmstadt, Germany). Nuclei were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, USA). Tissue sections omitting the primary antibody served as a negative control.

Demyelination of white matter was assessed by incubation of slides in Luxol Fast Blue solution (Morphisto, Frankfurt, Germany) overnight at 60 °C followed by counterstain with Periodic Acid-Schiff (Avantor Performance Materials, Center Valley, USA). Bielschowsky silver impregnation was performed to determine the axonal density.

The histological quantifications were performed on serial sections, with three sections per mouse from the cervical, thoracic and lumbar spinal cord. Areas with complete demyelination and homogenous immune cell infiltration were identified and defined as lesions and quantified by blinded observers. Analysis procedures were performed as previously described (Linker et al., 2011).

2.5. RNA isolation and gene expression analysis

Total RNA was purified from brain and spinal cord using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and stored immediately at −80 °C until further processing. Reverse transcription into cDNA was performed with the qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, USA). Real-time PCR amplifications were carried out on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, California, USA) using the GoTaq qPCR Master Mix (Promega, Fitchburg, USA) as reaction reagent.

The mouse brain-derived neurotrophic factor (BDNF) gene was amplified using designed forward primer 5′-GCCCTGCGGAGGCTAAGTGG-3′ and reverse primer 5′-AGGGTGCTTCCGAGCCTTCC-3′.

For each sample, mRNA abundance was performed in triplicates and normalized to the level of housekeeping gene β-actin. Data were

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