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Laquinimod enhances central nervous system barrier functions

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ABSTRACT

Laquinimod is currently being tested as a therapeutic drug in multiple sclerosis. However, its exact mechanism of action is still under investigation. Tracking of fluorescently-tagged encephalitogenic T cells during experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, revealed that laquinimod significantly reduces the invasion of pathogenic effector T cells into the CNS tissue. T-cell activation, differentiation and amplification within secondary lymphoid organs after immunization with myelin antigen, their migratory capacity and re-activation within the nervous tissue were either only mildly affected or remained unchanged. Instead, laquinimod directly impacted the functionality of the CNS vasculature. The expression of tight junction proteins p120 and ZO-1 in human brain endothelial cells was up-regulated upon laquinimod treatment, resulting in a significant increase in the transendothelial electrical resistance of confluent monolayers of brain endothelial cells. Similarly, expression of the adhesion molecule activated leukocyte cell adhesion molecule (ALCAM) and inflammatory chemokines CCL2 and IP-10 was suppressed, leading to a significant reduction in the migration of memory T_H1 and T_H17 lymphocytes across the blood brain barrier (BBB). Our data indicate that laquinimod exerts its therapeutic effects by tightening the BBB and limiting parenchymal invasion of effector T cells, thereby reducing CNS damage.

1. Introduction

MS is an autoimmune disease induced by brain antigen-reactive T cells. These cells are believed to be activated in peripheral immune organs during the course of viral or bacterial infections, and thereby gain the ability to overcome the blood brain barrier (BBB), which normally protects the CNS against the entry of circulating immune cells and soluble factors (Kebir et al., 2007; Kebir et al., 2009; Alvarez et al., 2013). Within the CNS, these pathogenic effector T cells re-encounter their cognate antigen in the perivascular space, where they are re-activated. The consequent inflammatory process culminates in structural damage and in the acute clinical relapse of MS. In experimental autoimmune encephalomyelitis (EAE), a widely used model for MS, laquinimod was shown to protect rodents from the development of severe clinical disease (Brunmark et al., 2002; Schulze-Topphoff et al., 2012; Wegner et al., 2010; Yang et al., 2004). The proposed mechanisms underlying this therapeutic effect of laquinimod are multifold and include interference with the function of pathogenic effector T cells, alteration of antigen-presenting cell (APC) function, and protection of neurons or glial cells from structural damage (Jolivel et al., 2013; Schulze-Topphoff et al.,

Abbreviations: EAE, experimental autoimmune encephalomyelitis; BBB, blood brain barrier; APC, antigen presenting cell; MOG, myelin oligodendrocyte glycoprotein; OVA, ovalbumin; MBP, myelin basic protein; RFP, red fluorescent protein; GFP, green fluorescent protein; TCR, T cell receptor; CFSE, carboxyfluorescein succinimidyl ester; ECs, endothelial cells; TEER, transendothelial electrical resistance; TJ, tight junction; AJ, adherent junction; ALCAM, activated leukocyte cell adhesion molecule; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; MCAM, melanoma cell adhesion molecule; ACM, astrocyte-conditioned media.

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2012). One additional finding uniformly reported in all experimental studies is the substantial decrease in CNS-invading T cells following treatment with laquinimod (Schulze-Topphoff et al., 2012; Wegner et al., 2010), which was initially attributed to a direct action of laquinimod on effector T cells. To better understand the effects of laquinimod on effector T cells during the different phases of the autoimmune process, we used fluorescently-labeled T cells that can be directly visualized *in vivo* by intravital two photon microscopy and functionally characterized after *ex vivo* isolation by flow cytometry. Our data indicate that the therapeutic mode of action of laquinimod is unlikely to be solely attributed to T cell-intrinsic effects, but rather to a direct effect of laquinimod on the neurovascular unit.

2. Materials and methods

2.1. Animals

C57BL/6J mice and rats on the LEW/Crl background (*Rattus norvegicus*) were originally purchased from Charles River (Sulzfeld, Germany) and bred in the SPF animal facility of the University Medical Center Göttingen. The following mouse strains were all housed in the University Medical Center Göttingen animal facility: 2D2 MOG-specific TCR (T cell receptor) transgenic mice (Bettelli et al., 2003), MOG-specific B cell receptor knock-in mice (Th mice) (Litzenburger et al., 1998), Act-GFP mice (Okabe et al., 1997), knock-in RFP mice (Luche et al., 2007), OTII mice (Barnden et al., 1998) and FoxP3-EGFP knock-in reporter mice (Wang et al., 2008). All animal experiments were performed according to the Directive 2010/63/EU and to the Lower Saxony state regulations for animal experimentation and were approved by the responsible authorities.

2.2. Patients

Seven patients with clinically definite MS characterized by a relapse–remitting disease course were recruited from the Multiple Sclerosis Clinic of Notre-Dame Hospital (Montreal, Canada). The age of the patients ranged from 22 to 39 years, with a mean of 27 years. All the MS patients were in the acute phase of disease (relapse), defined by the occurrence of new neurological symptoms lasting at least 24 h. None of the patients received immunosuppressive, immunomodulatory, or steroid therapy for at least 6 months prior to blood collection. Two healthy women and two healthy men volunteers (mean age, 26 years) were included as controls. Informed written consent was obtained from all participating subjects, in accordance with the local ethics committee (BH 07.001).

2.3. EAE models, laquinimod treatment

For induction of active EAE in mice, 10–12 week-old animals were immunized with 50 μg $MOG_{35–55}$ in complete Freund's adjuvants and pertussis toxin was injected on days 0 and 2 as previously described (Wüst et al., 2008). Mice were treated with 30 mg/kg laquinimod dissolved in tap water orally by gavage, either beginning at the day of immunization for 14 days (preventive treatment), or starting once the mice had reached an average clinical score of 2–3 for 10 consecutive days (therapeutic treatment). Animals were weighed daily and scored for clinical signs of the disease on a scale of 0 to 10 depending on severity; scores were as follows: 0 = normal; 1 = reduced tone of tail; 2 = limp tail, impaired righting; 3 = absent righting; 4 = gait ataxia; 5 = mild paraparesis of hindlimbs; 6 = moderate paraparesis; 7 = severe paraparesis or paraplegia; 8 = tetraparesis; 9 = moribund; 10 = death. In some experiments, 6×10^6 purified T cells specific for MOG or OVA were injected intravenously (i.v.) two days before immunization.

Lewis rat adoptive transfer EAE was induced by i.v. injection of 5×10^6 myelin basic protein (MBP)-reactive T cells blasts (day 2 after re-stimulation). To address the role of non-CNS-specific T cells under steady-state conditions, 5×10^6 $T_{OVA–GFP}$ blasts were transferred as above. MBP-specific and OVA-specific $CD4^+$ T cells (T_{MBP} and T_{OVA}) were retrovirally transduced to express the fluorescent protein gene as previously described (Flügel et al., 1999). Rats were treated twice daily with 25 mg/kg laquinimod dissolved in tap water orally by gavage, starting 1.5 days after transfer. Weight and clinical scores were recorded daily (score 0 = no disease; 1 = flaccid tail; 2 = gait disturbance; 3 = complete hind limb paralysis; 4 = tetraparesis; 5 = death).

2.4. Cell cultures of human primary brain-derived endothelial cells and astrocytes

With informed consent and ethical approval (ethical approval number HD04.046), human temporal lobe material was obtained from patients who underwent surgical treatment for intractable temporal lobe epilepsy. Primary human brain-derived endothelial cells (BBB-ECs) were isolated as described previously (Alvarez et al., 2011). To harvest astrocyte-conditioned media (ACM), human fetal astrocytes (obtained from the human fetal repository in the Albert Einstein College of Medicine, Bronx, NY) were isolated and grown in complete DMEM (Invitrogen), supplemented with 10% FBS (Alvarez et al., 2011). ACM was harvested every 7 days from confluent astrocyte monolayers, and filtered when added to BBB-ECs.

2.5. In vitro cell culture

2.5.1. Mice

To assess the effect of laquinimod on cytokine production of T cells *in vitro*, MOG-specific T and B cells were isolated from naïve 2D2 and Th mice, respectively. 1×10^6 T cells and 2×10^6 B cells were co-cultured for 3 days in a 24-well plate in RPMI with MOG protein at a concentration of 0.1 or 1 μg/ml in the presence/absence of 10 μM laquinimod. The supernatant was tested for IFNγ by ELISA (ELISA MAX™ Deluxe, Biolegend, San Diego, CA, USA).

2.5.2. Rats

To assess the effect of laquinimod on T cell proliferation *in vitro*, 1×10^5 $T_{MBP\text{-}GFP}$ cells were cultured with the cognate antigen or a sham antigen in 96-well plates in the presence/absence of 10 g/ml of laquinimod. Irradiated thymocytes (1×10^6/well) were used as APCs. T cell proliferation was assessed by H^3-Thymydin incorporation, 48 h after antigen encounter. Alternatively the rate of proliferation of $T_{MBP\text{-}GFP}$ cells was assessed by flow-cytometry 2, 3 and 4 days after antigen stimulation.

2.5.3. Human T_H1 and T_H17 lymphocyte polarization

Human memory $CD4^+$ T lymphocytes isolated from the peripheral blood of healthy donors or untreated relapsing-remitting MS patients were cultured for 6 days with autologous $CD14^+$ monocytes under T_H1 or T_H17 polarizing conditions, as previously published (Kebir et al., 2009).

2.5.4. Human in vitro transmigration experiments

Transmigration assays were performed using a modified Boyden chamber as previously published (Kebir et al., 2009; Alvarez et al., 2011). Briefly, human BBB-ECs were grown to confluence on gelatin-coated 3 μm pore sized Boyden chambers (Corning) in culture medium supplemented with 40% (v/v) ACM for 3 days. Laquinimod (0.1 or 1 μM) or PBS was applied daily to BBB-ECs during the growth phase. On the day of migration, media was changed and 1×10^6 T_H1 or T_H17 lymphocytes were then allowed to migrate for 24 h across human BBB-ECs. Migrated cells were collected from the lower chamber and counted.