Laquinimod dampens IL-1β signaling and Th17-polarizing capacity of monocytes in patients with MS

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Abstract

Objective
To assess the impact of laquinimod treatment on monocytes and to investigate the underlying immunomodulatory mechanisms in MS.

Methods
In this cross-sectional study, we performed in vivo and in vitro analyses of cluster of differentiation (CD14+) monocytes isolated from healthy donors (n = 15), untreated (n = 13), and laquinimod-treated patients with MS (n = 14). Their frequency and the expression of surface activation markers were assessed by flow cytometry and the viability by calcein staining. Cytokine concentrations in the supernatants of lipopolysaccharide (LPS)-stimulated monocytes were determined by flow cytometry. The messenger ribonucleic acid (mRNA) expression level of genes involved in cytokine expression was measured by quantitative PCR. The LPS-mediated nuclear factor kappa-light-chain-enhancer of activated B-cell (NF-κB) activation was determined by the quantification of the phosphorylation level of the p65 subunit. Laquinimod-treated monocytes were cocultured with CD4+ T cells, and the resulting cytokine production was analyzed by flow cytometry after intracellular cytokine staining. The interleukin (IL)-17A concentration of the supernatant was assessed by ELISA.

Results
Laquinimod did not alter the frequency or viability of circulating monocytes, but led to an upregulation of CD86 expression. LPS-stimulated monocytes of laquinimod-treated patients with MS secreted less IL-1β following a downregulation of IL-1β gene expression. Phosphorylation levels of the NF-κB p65 subunit were reduced after laquinimod treatment, indicating a laquinimod-associated inhibition of the NF-κB pathway. T cells primed with laquinimod-treated monocytes differentiated significantly less into IL-17A–producing T helper (Th)-17 cells.

Conclusions
Our findings suggest that inhibited NF-κB signaling and downregulation of IL-1β expression in monocytes contributes to the immunomodulatory effects of laquinimod and that the impairment of Th17 polarization might mediate its disease-modifying activity in MS.

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In MS, the complex orchestration of various cell types of the innate and the adaptive immune system leads to an autoimmune-mediated inflammation of the CNS.1 De-myelination and the consecutive tissue damage are largely mediated by encephalitogenic T helper (Th)-1 and interleukin (IL)-17–producing Th17 cells, which are key players of the proinflammatory adaptive immune system.2 However, the pathogenic role of the innate immune system, consisting of myeloid cells such as monocytes, dendritic cells, macrophages and local microglia, is increasingly recognized.

Recent data suggest that myeloid cells not only mediate the expansion of antigen-specific lymphocytes via their antigen-presenting function but that they also promote proinflammatory immune cell responses through cytokine and chemokine secretion.3 Furthermore, tissue-resident monocytes may even directly contribute to lesion formation by the adoption of an inflammatory phenotype as macrophages4 and by the production of reactive oxygen species.1,4,5 The importance of myeloid cells in MS pathogenesis is further underlined by the fact that they constitute the predominant immune cell subset in active MS lesions.6 Infiltrating monocytes are also evident in different animal models of MS such as experimental autoimmune encephalomyelitis (EAE) or cuprizone-induced demyelination,7 where they were identified as a pathogenic granulocyte-macrophage colony-stimulating factor (GM-CSF)–responsive cell type for the development of tissue inflammation.8 Furthermore, the blocking of monocyte recruitment to the CNS inhibits EAE development.9

Laquinimod is a quinoline-3-carboxamide derivative, which has been evaluated as an orally administered medication for the treatment of relapsing-remitting MS (RRMS). In EAE and the cuprizone model, laquinimod ameliorated the severity of clinical signs and prevented demyelination.10–12 In the 2 phase 3 trials, ALLEGRO and BRAVO, laquinimod was well tolerated, yet at the dosage tested (0.6 mg/d) only led to moderate effects on the reduction of relapse rates as primary study end points.13–15 Although data indicated beneficial effects on disability progression, brain atrophy, and the risk of sustained disability,13,14 the Committee for Medicinal Products for Human Use refused marketing authorization for RRMS based on the assessment of the risk-benefit ratio with regard to safety data from animal studies.16 In the phase 2 trial ARPEGGIO, laquinimod did not demonstrate a significant effect on brain volume loss in primary progressive MS (PPMS).17 Furthermore, laquinimod treatment of patients with Huntington disease did not improve total motor scores in the LEGATO-HD trial.18 Subsequently, its development for treatment of MS and Huntington disease was discontinued. Still, the potential of laquinimod for the treatment of Crohn disease19 and uveitis is currently being evaluated.

Although recent data suggest various mechanisms of action by which laquinimod exerts its immunomodulatory effects, much still remains to be elucidated, particularly with regard to the impact of laquinimod on the complex interplay of different immune cell types. Several preclinical studies have demonstrated that laquinimod treatment inhibits the production of proinflammatory cytokines like interferon (IFN-γ), tumor necrosis factor (TNF)-α, IL-12, and IL-17 and increases the production of IL-4, IL-10, and transforming growth factor (TGF)-β, leading to a downregulation of encephalitogenic Th1 and Th17 immune responses and to an upregulation of regulatory Th2 immune response, respectively.20–22 It has further been shown that the downregulation of the reactivity of autoimmune T cells can partly be attributed to the modulation of proinflammatory signaling at the level of antigen-presenting cells (APCs), such as dendritic cells.23,24 Murine experiments also implicated a contribution of monocytes to this immunomodulatory effect.25 On a molecular basis, an upstream inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway might be one of the underlying pathomechanisms for the aforementioned immunomodulatory effects of laquinimod.24,26,27

Besides these anti-inflammatory effects, laquinimod also seems to display neuroprotective properties, as it was found to increase the level of brain-derived neurotrophic factor in the serum of patients with MS,25 to exert direct antiapoptotic activity on neurons,28 to protect against glutamate excitotoxicity,29 and to improve remyelination.30

Given the critical role exerted by myeloid cells in MS pathophysiology, this study aimed to assess the impact of laquinimod on monocytes, to illustrate the underlying molecular pathomechanisms, and to investigate the effect of monocyte...
modulation on proinflammatory T-cell responses. We here report that laquinimod dampens IL-1 signaling and the Th17-polarizing capacity of monocytes in patients with MS.

Methods

Study design, sample collection, and processing
A cross-sectional analysis of monocyte phenotype, cytokine expression, and interaction with T-cell populations was performed in healthy donors (n = 15), untreated patients with RRMS (n = 13), and laquinimod-treated patients with RRMS (n = 14) (table). Treated patients received 0.6 mg laquinimod once daily for an average duration of 4.2 years. A comparable level of disability, as assessed by the Expanded Disability Status Scale,31 was found between the laquinimod-treated patients with MS and the untreated patients with MS.

Heparinized blood samples were collected from healthy donors, untreated, and laquinimod-treated patients with MS. Cluster of differentiation (CD14+) monocytes were magnetically sorted from peripheral blood mononuclear cells with human CD14 MicroBeads (Miltenyi Biotec). Purity and surface expression of CD86, human leukocyte antigen (HLA)-DR, and CD14 of isolated monocytes were assessed by flow cytometry. Thereafter, these cells were plated in a 48-well plate (1 × 10⁶ cells/well) in X-VIVO 15 medium. For messenger ribonucleic acid (mRNA) analysis, monocytes were snap frozen after 4 hours.

Standard protocol approvals, registrations, and patient consents
Informed written consent was obtained by all patients and healthy donors, and the study was approved by a local ethical committee (number 837.019.10). The laquinimod-treated patients were enrolled in open-label extensions of phase III trials.

Flow cytometric analysis
Cells were stained with the following antibody conjugates: anti-CD14-APC, anti-CD14-V500, anti-HLA-DR-V450, anti-CD3-APC-H7, anti-CD4-V450, anti-CD4-PerCP, anti-CD80-fluorescein isothiocyanate (FITC), anti-CD83-PE, anti-CD86-PE, anti-IL-17A-APC, anti-IL-17A-alexa fluor (AF) 647, anti-IFN-γ-FITC, anti-IFN-γ-V450, and anti-NF-κB p65-phosphothanolamine N-carboxyfluorescein (PE-CF) 594. Antibodies were obtained from Becton Dickinson (BD) Biosciences or eBioscience. Intracellular stainings for IFN-γ and IL-17A were performed using the Cytofix/Cytoperm kit from BD Biosciences. Stained cells were measured by FACSCanto II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Viability measurements
Isolated CD14+ monocytes from healthy donors were cultured with or without laquinimod (10 and 100 μM) in X-VIVO 15 medium. After 24 hours of culture, the monocytes were harvested and counted. Moreover, their viability was assessed by calcein (Invitrogen) that only stains living cells. Propidium iodide (PI, Sigma-Aldrich) was used to stain the dying cells. Calcein was used in a concentration of 1 μg/mL, whereas PI was used in a concentration of 0.5 μM.

Cytokine and chemokine assays
To stimulate the secretion of cytokines and chemokines, the CD14+ monocytes were incubated with 1 μg/mL lipopolysaccharide (LPS) for 24 hours. Thereafter, the supernatant was collected to determine the levels of cytokines and chemokines produced. Cytokine concentrations were measured with the FlowCytomix (eBioscience) Human Th1/Th2/Th9/Th17/Th22 13plex kit (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p70, IL-13, IL-17A, IL-22, and TNF-α). Chemokine concentrations were measured with the FlowCytomix Human Chemokine 6plex kit (G-CSF, IL-8, monocyte chemoattractant protein (MCP)-1, MIG, macrophage inflammatory protein (MIP)-1α, and MIP-1β). Data were analyzed with FlowCytomix Pro 2.4. The experimental results represent the fold induction of protein secretion after stimulation with LPS.

RNA isolation and quantitative PCR analysis
The gene expression level of cytokines (IL-1β and TNF-α) and chemokines (MIP-1α and MIP-1β) in isolated CD14+ monocytes following 4-hour stimulation with LPS was measured by quantitative PCR. Total RNA from cultured CD14+ monocytes was isolated using the RNeasy Mini Kit (Qiagen). DNase-I treatment was additionally performed to avoid genomic DNA contamination. The total RNA was used to obtain complementary deoxyribonucleic acid (cDNA) by the SuperScript III First-Strand Synthesis System and random hexamer primers (Invitrogen). Real-time PCR was performed with amplification primers by using iQ SYBR Green supermix (BioRad) and the iCycleriQ (BioRad). Relative changes in gene expression were

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<td><strong>Group</strong></td>
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<td>Healthy donors (n = 15)</td>
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<td>Untreated patients with MS (n = 13)</td>
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<td>Laquinimod-treated patients with MS (n = 14)</td>
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Abbreviation: n.d. = not determined.
Age, disease duration, and laquinimod treatment duration are presented as mean ± SD (range) expressed in years.
determined using the ΔΔCt method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, and EF1α as reference genes. The experimental results represent the fold induction of the gene expression after stimulation with LPS normalized to GAPDH expression.

**Quantitative assessment of NF-κB activation in monocytes**

CD14⁺ monocytes from healthy donors were magnetically isolated as described above and pretreated with 10 and 100 μM of laquinimod and activated with LPS (1 μg/mL) for 30 minutes in vitro. Monocyte fixation and permeabilization were performed according to BD Phosflow Protocol III for human peripheral blood mononuclear cells (BD Phosflow Protocol III 2011). To ensure permeabilization of both cell and nuclear membranes, the BD Phosflow Perm Buffer III was used. After permeabilization, the cell samples were washed twice with BD Pharmingen Stain Buffer and resuspended in 100 μL of the buffer. Then, 5 μL aliquots of anti-NF-κB p65-PE-CF594 were added to LPS-treated samples and respective reference samples without stimulus. After incubation for 30 minutes in the dark, samples were analyzed by flow cytometry to assess phosphorylation of p65.

**Differentiation of T cells by autologous monocytes**

CD14⁺ monocytes from healthy donors were magnetically isolated as described above. Autologous T cells were purified using the human Pan T Cell Isolation Kit (Miltenyi Biotec). The isolated monocytes were treated with or without laquinimod (10 μM) and LPS (1 μg/mL) for 18 hours in X-VIVO 15 medium, washed, and subsequently cocultured with isolated 10⁵ autologous T cells with or without tetanus toxin (5 μg/mL) in a...
final volume of 200 μL X-VIVO 15 medium. After 3 days of coculture, cytokine production was analyzed by flow cytometry after intracellular cytokine staining and by ELISA. For the latter, IL-17A in the supernatant was measured using human IL-17A–specific (homodimer) ELISA Ready-SET-Go! (eBioscience).

Statistical analysis
All data were analyzed using PRISM6 (Graphpad software). Data are presented as mean ± standard error of mean from at least 3 independent experiments (in vitro experiments). We used the nonparametric Mann-Whitney U test to compare differences of the mean between 2 independent groups and the Kruskal-Wallis test in case of more than 2 independent groups. To account for errors due to multiple testing, these were followed by a Dunn multiple comparison test.

Data availability
The raw data used in preparation of the figures and tables will be shared in anonymized format on request of a qualified investigator to the corresponding author for purposes of replicating procedures and results.

Results
Laquinimod does not alter the frequency of circulating monocytes in patients or their viability in vitro
There was no significant difference in the CD14+ monocyte counts between healthy donors, untreated, and laquinimod-treated patients with MS (figure 1A).

The treatment of CD14+ monocytes isolated from healthy donors with laquinimod in a dose of 10 or 100 μM in vitro did not modify the expression of CD14 (figure 1B) or the percentages of PI- (figure 1C) and calcein-positive cells (figure 1D). The cell yield at the end of the culture period reflecting the survival and the proliferation was also not modified by laquinimod (figure 1E).

Laquinimod modulates the expression of activation markers on monocytes
Ex vivo, the expression of CD86 was higher on monocytes of laquinimod-treated patients than on monocytes of healthy donors and untreated patients with MS, whereas there was no difference of the expression of major histocompatibility complex (MHC-II) (figure 2A).

However, in vitro treatment of HD monocytes with 10 and with 100 μM of laquinimod did not lead to significant changes of the expression of CD80, CD83, and CD86, whereas here, we observed a slight upregulation of MHC-II expression after incubation with 10 μM laquinimod but not after incubation with 100 μM laquinimod (figure 2B).

Laquinimod reduces the IL-1β secretion of monocytes via downregulation of gene expression
LPS stimulation of isolated CD14+ monocytes of laquinimod-treated patients with MS led to a significant downregulation of IL-1β secretion compared with LPS-stimulated monocytes of HDS and untreated patients with MS (figure 3A). Furthermore,
Laquinimod inhibits NF-κB signaling in monocytes

In vitro treatment of monocytes isolated from HDs with 10 μM laquinimod led to a reduction of LPS-induced phosphorylation levels of the NF-κB p65 subunit compared with untreated monocytes, whereas 1 μM laquinimod did not demonstrate relevant effects (figure 4A). This indicates a dose-dependent inhibition of the NF-κB signaling pathway by laquinimod.

Laquinimod reduces the Th17-polarizing capacity of monocytes

Intracellular staining revealed that T cells primed with laquinimod-treated monocytes loaded with tetanus toxin differentiated significantly less into IL-17A–producing T cells compared with T cells, which were primed with untreated monocytes (figure 4B). This was confirmed by lower IL-17A levels in the coculture supernatant (figure 4C). In contrast, the differentiation into IFN-γ–producing T cells was not influenced by laquinimod treatment (figure 4D).

Discussion

Myeloid cells, including monocytes, are known to exert prominent roles in the pathology of MS. In this cross-sectional study, we analyzed the effect of laquinimod therapy on circulating monocytes of patients with RRMS and investigated the underlying pathomechanisms by performing in vitro experiments.

In line with previous reports, our study confirmed that laquinimod treatment does not change the absolute count of circulating monocytes of patients with MS in vivo. Moreover, the absence of any antiproliferative activity coupled to the innocuity exerted on monocytes cultured in vitro underlines the hypothesis that the effects of laquinimod treatment are not mediated by immunosuppression.

Because of their function and expression kinetics, CD86 and CD80 affect the immune response in different ways. CD86 delivers the main costimulatory signal in the early stage, whereas CD80 is expressed later after previous proinflammatory

Figure 3 Laquinimod reduces the secretion of IL-1β and downregulates its gene expression in patients with MS

(A) The concentrations of IL-1β in the supernatants of CD14+ monocytes cultured with or without LPS for 24 hours were assessed by flow cytometry, and the fold induction of cytokine secretion after stimulation with LPS was determined. Monocytes of laquinimod-treated patients with MS secreted significantly less IL-1β than those of untreated patients with MS and healthy donors. Mean values ± SEM are shown (n = 10); **p < 0.01. (B) The gene expression level IL-1β in isolated CD14+ monocytes following 4-hour stimulation with LPS was measured by quantitative PCR. The experimental results represent the fold induction of the gene expression after stimulation with LPS normalized to GAPDH expression. Laquinimod-treated patients with MS had lower IL-1β expression levels than healthy donors. Results are presented as mean ± SEM (n = 10); *p < 0.05. CD = cluster of differentiation; HD = healthy donor; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; IL = interleukin; Laq = laquinimod; LPS = lipopolysaccharide; SEM = standard error of mean.
stimulation. Defects of proteins involved in these pathways have been associated with the development of MS.

In the ex vivo assessment of costimulatory profiles, we observed an elevated expression of CD86 on unstimulated monocytes of laquinimod-treated patients in comparison to monocytes of healthy donors and untreated patients with MS. This parallels previous studies reporting that laquinimod increases the CD86 expression on unstimulated murine monocytes in EAE and unstimulated human DCs in MS. The in vitro laquinimod exposition of unstimulated monocytes from healthy donors did not change the expression of CD86 and CD80 in the current study. In contrast, it has been shown recently that monocytes of laquinimod-treated patients with MS express lower levels of CD86 than those of untreated patients with MS after stimulation with LPS, whereas CD80 expression was not affected. This apparent discrepancy to our data might indicate that the impact of laquinimod on CD86 expression of monocytes depends on their state of activation.

Although we did not observe a difference in CD86 expression on monocytes of untreated patients with MS and healthy donors, Chuluundorj et al. have previously reported an elevated CD86 expression in a larger cohort of untreated patients with MS, which led them to hypothesize that inflammatory monocytes from patients with MS might have a higher capability to costimulate T-cell activation.

Monocytes are recognized as major sources of cytokines and chemokines in MS and are sensitive to LPS stimulation because CD14 represents a high-affinity receptor for LPS. As the expression levels of CD14 in our study showed no differences between monocytes cultivated in the absence or in the presence of laquinimod, the responsiveness to LPS was unlikely affected by the treatment with laquinimod.

The monocytes of the laquinimod-treated patients with MS in our study released significantly lower levels of various cytokines and chemokines (TNF-α, IL-6, IL-10, MIP-1α, and MIP-1β) in comparison to healthy donors, but not compared with untreated patients with MS. Therefore, it is not possible to attribute these effects solely to the treatment with laquinimod, and disease-specific differences in the monocytes’ response to stimulation need to be taken into consideration.

However, we did observe a significant downregulation of IL-1β secretion in laquinimod-treated patients compared with both healthy controls and untreated patients with MS, which strongly indicates that the suppression of the IL-1β secretion in monocytes is caused by laquinimod. This assumption could be supported at the transcriptomic level as the mRNA expression of IL-1β was also significantly reduced on laquinimod therapy in patients with MS. The mRNA expression of other genes relevant to IL-1β cleavage or IL-1β receptor signaling like Caspase-1, IL-1R1, IL1-R2, or MyD88 was not altered by laquinimod treatment. Thus, it can be hypothesized that laquinimod leads to a reduction of IL-1β secretion through the selective inhibition of IL-1β gene expression.

The NF-κB pathway is known to play an important role in the regulated expression of cytokines and chemokines in monocytes. Here, we demonstrated that in human monocytes, laquinimod reduced the p65 phosphorylation after stimulation with LPS. Phosphorylation of the p65 subunit in the cytosol of monocytes leads to a translocation of the transcription factor NF-κB into the nucleus, where it induces the expression of cytokines, including IL-1β. Our current finding supports an
earlier report that laquinimod prevents the loss of inhibitory IκB protein in laquinimod-treated monocytes on LPS stimulation, reflecting an inhibition of NF-κB pathway activation.44 Furthermore, a laquinimod-mediated downregulation of NF-κB activation has been demonstrated previously for other cell types such as dendritic cells or astrocytes.11,24

Of interest, similar effects of laquinimod treatment have recently been demonstrated in experimental models of other neurologic and systemic inflammatory diseases. For example, laquinimod treatment of monocytes taken from patients with Huntington disease was also found to secrete lower levels of inflammatory cytokines, including IL-1β,45 and in osteoarthritis, laquinimod ameliorated the IL-1β–induced generation of reactive oxygen species via a suppression of the NF-κB pathway.46

The functional relevance of laquinimod-induced alteration of cytokine secretion pattern in MS pathology was confirmed in our in vitro coculture experiments. Here, laquinimod-treated monocytes had a reduced capacity to polarize CD4+ T cells into Th17 cells and led to lower levels of IL-17A secretion. A similar observation has been reported in EAE mice where laquinimod reduced the capacity of isolated monocytes to promote a proinflammatory T-cell response in vitro.25

The role of IL-1β in the generation of Th17 cells, which are major culprits in the pathogenesis of EAE and are considered to play a pathogenic role in causing sustained tissue damage in neuroinflammation,47,48 has been proposed previously.22,49 IL-1β can act on subsets of human Th cells expressing the IL-1R,50 and the in vitro polarization of naive human Th cells in a mixture containing IL-1β induced the expression of IL-17A.51 Furthermore, the importance of the IL-1R1 in the generation of proinflammatory Th17 cells regulated by GM-CSF–driven monocyte-derived DCs has also been highlighted.8

Two limitations of this study have to be addressed. First, the findings have to be interpreted with care as the observed effects are based on a small sample size. Second, our study lacks data from patients with PPMS, in which laquinimod has been tested as well.17 Further investigations need to elucidate how monocyte subsets relevant to MS9,52 are differentially affected by laquinimod.

Our current findings suggest that the immunomodulatory effects of laquinimod involve decreased phosphorylation of p65 in NF-κB pathway and downregulation of IL-1β expression in monocytes. The consecutive decrease in IL-1β levels might further translate to an impaired differentiation and maintenance of inflammatory Th17 cells. Although the admission procedure of laquinimod for the treatment of MS has been discontinued, these findings are still of importance as they offer valuable insights into the pathogenesis of MS and for the evaluation of the potential of laquinimod for the treatment of other disease.

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Disclosure
K. Rosenfeld performed her MD thesis on the topic of the present study. S. Engel, V. Jolivel, S. H.-P. Kraus, M. Zayoud, K. Rosenfeld, F. Kurschus, and A. Waisman report no disclosures. H. Tumani received speaker honoraria from Bayer, Biogen, Fresenius, Genzyme, Merck, Novartis, Roche, Siemens, and Teva; serves as section editor for the Journal of Neurology, Psychiatry, and Brain Research; and receives research support from Fresenius, Genzyme, Merck, and Novartis. R. Furlan received honoraria for serving on scientific advisory boards or as a speaker from Biogen, Novartis, Roche, and Merc and funding for research from Merck. F. Luessi served on the advisory board of Roche and received travel funding from Teva. Go to Neurology.org/NN for full disclosures.

Publication history

Appendix
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<td>Acquisition of data and analysis of data and revising the manuscript</td>
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Website Disclosure
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