RESEARCH ARTICLE OPEN ACCESS

Increased NLRP3 Inflammasome Activation and Pyroptosis in Patients With Multiple Sclerosis With Fingolimod Treatment Failure

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Neurol Neuroimmunol Neuroinflamm 2023;10:e200100. doi:10.1212/NXI.0000000000200100

Abstract

Background and Objectives

Inflammasomes are involved in the pathogenesis of different neuroimmune and neurodegenerative diseases, including multiple sclerosis (MS). In a previous study by our group, the nucleotide-binding oligomerization domain, leucine-rich repeat receptor and pyrin-domain–containing 3 (NLRP3) inflammasome was reported to be associated with the response to interferon-beta in MS. Based on recent data showing the potential for the oral therapy fingolimod to inhibit NLRP3 inflammasome activation, here we investigated whether fingolimod could also be implicated in the response to this therapy in patients with MS.

Methods

NLRP3 gene expression levels were measured by real-time PCR in peripheral blood mononuclear cells at baseline and after 3, 6, and 12 months in a cohort of patients with MS treated with fingolimod (N = 23), dimethyl fumarate (N = 21), and teriflunomide (N = 21) and classified into responders and nonresponders to the treatment according to clinical and radiologic criteria. In a subgroup of fingolimod responders and nonresponders, the percentage of monocytes with an oligomer of ASC was determined by flow cytometry, and the levels of interleukin (IL)-1β, IL-18, IL-6, tumor necrosis factor (TNF)α, and galectin-3 were quantified by ELISA.

Results

NLPR3 expression levels were significantly increased in fingolimod nonresponders after 3 (p = 0.03) and 6 months (p = 0.008) of treatment compared with the baseline but remained similar in responders at all time points. These changes were not observed in nonresponders to the other oral therapies tested. The formation of an oligomer of ASC in monocytes after lipopolysaccharide and adenosine 5′-triphosphate stimulation was significantly decreased in responders (p = 0.006) but increased in nonresponders (p = 0.0003) after 6 months of fingolimod treatment compared with the baseline. Proinflammatory cytokine release from stimulated peripheral blood mononuclear cells was comparable between responders and nonresponders, but galectin-3 levels on cell supernatants, as a marker of cell damage, were significantly increased in fingolimod nonresponders (p = 0.02).

Discussion

The differential effect of fingolimod on the formation of an inflammasome-triggered ASC oligomer in monocytes between responders and nonresponders could be used as a response biomarker after 6 months of fingolimod treatment and suggests that fingolimod may exert their beneficial effects by reducing inflammasome signaling in a subset of patients with MS.

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The Article Processing Charge was funded by the authors.

Glossary

ASC = apoptosis-associated speck-like protein containing a caspase recruitment domain; **ATP** = adenosine 5′-triphosphate; **DMF** = dimethyl fumarate; **IFN-β** = interferon-beta; **IL** = interleukin; **LPS** = lipopolysaccharide; **MS** = multiple sclerosis; **NF-κB** = nuclear factor kappa B; **PBMCs** = peripheral blood mononuclear cells; **RRMS** = relapsing-remitting MS; **S1P** = sphingosine-1-phosphate; **TNF** = tumor necrosis factor.

Inflammasomes are multiprotein complexes activated by a wide range of pathogen-associated and damage-associated molecular patterns. Once activated, inflammasomes result in large oligomeric structures of the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain)[1-3] that recruit and facilitate self-cleavage of pro–caspase-1 to generate active caspase-1, which subsequently converts the proinflammatory cytokines interleukin (IL)-1β and IL-18 into their active mature secreted forms and induces pyroptosis, a type of inflammatory necrotic cell death.[4-6] Current interest in inflammasomes comes from the fact that they are emerging as therapeutic targets in a number of neuroimmune and neurodegenerative diseases, including, among others, Alzheimer disease, amyotrophic lateral sclerosis, Parkinson disease, and multiple sclerosis (MS).[7,8] In a previous study conducted by our group, the NLRP3 (nucleotide-binding oligomerization domain, leucine-rich repeat receptor and pyrin-domain–containing 3) inflammasome, the best characterized inflammasome complex, was found to be associated with the response to interferon-beta (IFN-β) in patients with MS based on the findings of significantly increased baseline *NLRP3* and *IL-1β* mRNA expression levels in peripheral blood mononuclear cells (PBMCs) of IFN-β nonresponders compared with responders according to different response criteria after 12 and 24 months of treatment.[9]

Recent studies showed that fingolimod (also known as FTY720), an oral sphingosine-1-phosphate (S1P) receptor modulator indicated for the treatment of patients with relapsing-remitting MS (RRMS), reduced microglial activation in rodent models of Parkinson disease and chronic unpredictable mild stress through the inhibition of NLRP3 inflammasome activation.[10,11] The potential for fingolimod to inhibit NLRP3 inflammasome activation prompted us to investigate whether the NLRP3 inflammasome could also be playing a role in the response to fingolimod in treated patients with MS.

Methods

Patients and Treatment Response Criteria

Patients with RRMS treated with fingolimod, teriflunomide, or dimethyl fumarate (DMF) at the outpatient clinic of the Centre d'Esclerosi Multiple de Catalunya (Cemcat, Barcelona) were included in the study. Classification of patients into responders and nonresponders was performed after 1 year of treatment according to the following criteria (Rio score)[12]: presence or absence of clinical relapses, progression or lack of progression on neurologic disability, and presence or absence of radiologic activity on the 12-month brain MRI. Patients having no relapses, no progression, and lack of MRI activity during the first year of treatment were labeled as responders. Patients satisfying any of the following 3 criteria were labeled as nonresponders: (1) presence of one or more relapses, (2) increase of 1 or more points in the Expanded Disability Status Scale score, and (3) presence of ≥3 active lesions (new or enlarging T2 lesions or gadolinium-enhancing lesions) on brain MRI. A total of 58 patients with RRMS participated in the study, of whom 23 patients were treated with fingolimod (8 responders and 15 nonresponders), 14 with DMF (7 responders and 7 nonresponders), and 21 with teriflunomide (11 responders and 10 nonresponders). A summary of the main baseline demographic and clinical characteristics of patients with MS is shown in Table.

PBMCs from 6 healthy donors (mean age [SD]: 36.0 [8.1] years; female/male [% women]: 2/4 [40%]) were also included in the study for comparison purposes.

Real-Time PCR to Measure *NLRP3* Inflammasome Expression Levels

NLRP3 mRNA expression levels were determined by real-time PCR in PBMCs of patients with MS at baseline and after 3, 6, and 12 months of treatment with fingolimod, DMF, and teriflunomide. Total RNA was extracted from PBMCs previously frozen in liquid nitrogen. RNA was extracted using an RNeasy Kit (Qiagen), followed by synthesis of cDNA with the High-Capacity cDNA Archive Kit (Applied Biosystems). mRNA expression levels were measured by real-time PCR using TaqMan probes specific for *NLRP3*. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control (Applied Biosystems). Data were analyzed by using a standard $2^{-\Delta\Delta CT}$ method.[13] Results were expressed as fold change in gene expression in nonresponders relative to responders (calibrators).

Cells and Treatments

Human PBMCs were cultured in Opti-MEM Reduced-Serum Media (Gibco). PBMCs from patients were left unstimulated or stimulated at 37°C during 2 hours with 1.6 μg/mL of *E coli* lipopolysaccharide (LPS) serotype 0111:B4 (InvivoGen) and then subsequently treated or not for 30 minutes with 3 mM adenosine 5′-triphosphate (ATP, Sigma-Aldrich).

Quantification of Monocytes With an Oligomer of ASC

Intracellular ASC oligomer formation was evaluated by seeding an individual's PBMC samples from responders, nonresponders, and healthy donors in polystyrene flow

Table Demographic and Baseline Clinical Characteristics of Patients With MS Included in the Study

	Fingolimod		Dimethyl fumarate		Teriflunomide	
Baseline characteristics	**Responders**	**Nonresponders**	**Responders**	**Nonresponders**	**Responders**	**Nonresponders**
n	8	15	7	7	11	10
Age (y)	35.0 (5.5)	36.1 (9.3)	40.0 (9.6)	46.1 (9.4)	45.1 (4.4)	40.9 (9.8)
Female/male (% women)	6/2 (75.0)	10/5 (66.6)	5/2 (71.4)	5/2 (71.4)	8/3 (72.7)	8/2 (80.0)
Duration of disease (y)	7.7 (4.7)	11.1 (7.3)	7.6 (8.2)	7.7 (6.9)	10.3 (7.2)	10.8 (8.6)
Percentage of naive patients	12.5	6.0	28.5	28.5	36.3	30.0
EDSS[a]	1.6 (1.6–3.6)	2.7 (2.0–4.0)	2.2 (1.0–3.0)	1.7 (1.5–2.0)	1.9 (1.5–2.0)	2.2 (1.4–3.2)
No. of relapses[b]	5.5 (3.0)	7.8 (3.9)	2.7 (2.0)	2.6 (1.1)	3.9 (2.0)	3.6 (2.4)
No. of T2 lesions[c,d] **(n [%])**						
1	0 (0)	0 (0)	1 (14.3)	0 (0)	3 (27.3)	0 (0)
2	5 (62.5)	2 (13.3)	4 (57.1)	2 (28.6)	4 (36.4)	5 (50.0)
3	3 (37.5)	13 (86.7)	2 (28.6)	5 (71.4)	4 (36.4)	5 (50.0)
No. of Gd-enhancing lesions[c]	2.8 (3.8)	4.1 (5.2)	0.3 (0.7)	0.7 (1.9)	0.5 (1.0)	0.6 (1.3)

Abbreviation: EDSS = Expanded Disability Status Scale.
Data are expressed as mean (SD), unless otherwise stated.
[a] Data are expressed as mean (interquartile range).
[b] Refers to the number of relapses in the 2 previous years of blood extraction.
[c] Refers to the number of T2 and gadolinium-enhancing lesions at the time of blood extraction.
[d] T2 lesions were classified into 3 categories according to the number of lesions: 1 = 1–9 lesions, 2 = 10–50 lesions, and 3 = >50 lesions.

cytometry tubes (Falcon) with Opti-MEM Reduced-Serum Media. PBMCs from responders and nonresponders were analyzed at baseline before treatment and after 6 months of fingolimod treatment. After PBMC stimulation, cells were stained for the detection of an ASC oligomer by time-of-flight inflammasome evaluation[14,15] using the phycoerythrin-conjugated mouse monoclonal anti-ASC antibody (clone HASC-71, catalog 653903, BioLegend, 1:500). Monocytes were gated using the fluorescein isothiocyanate-conjugated mouse monoclonal anti-CD14 antibody (clone M5E2, catalog 557153, BD Biosciences, 1:10) and using the phycoerythrin-Cy7–conjugated mouse monoclonal anti-CD16 antibody (clone 3G8, catalog 557744, BD Biosciences, 1:10). In all cases, samples were analyzed by flow cytometry using fluorescence-activated cell sorting Canto (BD Biosciences) and the flow cytometry standard express software (De Novo Software).

Quantification of Cytokine and Galectin-3 Levels in Supernatants by ELISA

Levels of the proinflammatory cytokines IL-1β, IL-18, IL-6, and tumor necrosis factor (TNF)α, as well as galectin-3, a marker of pyroptosis,[16] were quantified by ELISA in supernatants of PBMCs from responders, nonresponders, and healthy donors both under unstimulated and stimulated conditions. IL-1β and IL-18 levels were determined at baseline (IL-1β) and after 6 months of fingolimod treatment (IL-1β and IL-18) under unstimulated conditions and after NLRP3 inflammasome activation with LPS alone or with LPS and ATP, as previously described.[15,17] Both IL-6 and TNFα levels were determined at baseline and after 6 months of fingolimod treatment under unstimulated conditions and after stimulation with LPS alone. Galectin-3 was determined after stimulation with LPS and ATP at baseline and after 6 months of treatment. Cell-free supernatants from PBMCs were collected after treatment and clarified by centrifugation. ELISA kits were acquired from Invitrogen for IL-1β and galectin-3, from MBL International for IL-18, and from R&D Systems for TNFα and IL-6. ELISAs were performed following the manufacturer's indications and read in a Synergy Mx (BioTek) plate reader at 450 nm and corrected at 570 or 620 nm.

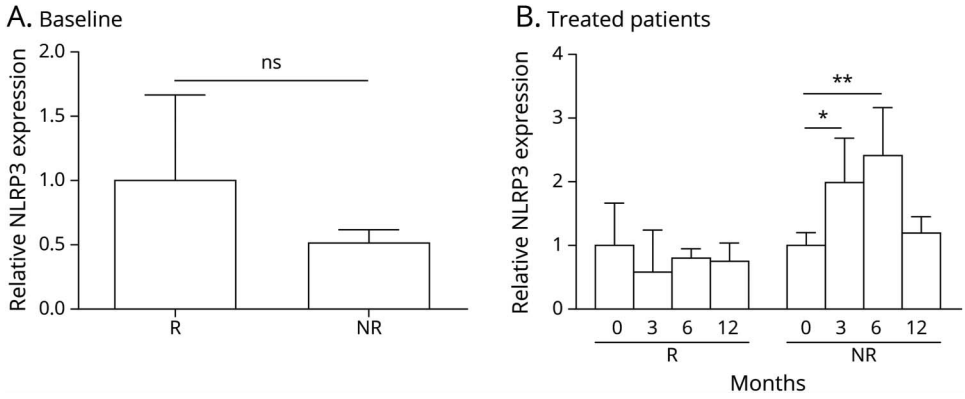
Standard Protocol Approvals, Registrations, and Patient Consents

This study was approved by the local ethics committee, and patients with MS gave their informed consent (EPA[AG]57/2013[3834]).

Statistical Analysis

Statistical analysis was performed by using the IBM SPSS Statistics for Windows version 20.0 (IBM Corp, Armonk, NY) and GraphPad Prism version 9 (GraphPad Software Inc). The normality of the values was determined with the D'Agostino and Pearson omnibus K2 normality test. Outliers were detected in the datasets by the robust regression and outlier removal method with Q = 1%. Comparisons of mRNA expression and protein levels of the markers measured in the study between responders and nonresponders and within each group at different time points were analyzed with appropriate unpaired and paired nonparametric and parametric tests. Data are shown as mean values, and error

Figure 1 *NLRP3* Gene Expression Levels in Responders and Nonresponders to Fingolimod



Messenger RNA expression levels for *NLRP3* were determined in PBMCs from responders (n = 8) and nonresponders (n = 15) to fingolimod at baseline and after 3, 6, and 12 months of treatment by real-time PCR relative quantification. Results are expressed as fold change in gene expression in nonresponders (NR) relative to responders (R) (A) and after 3, 6, and 12 months of treatment relative to the baseline (B). PBMCs = peripheral blood mononuclear cells.

bars represent standard error from the number of independent assays indicated in the figure legend. p values are indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $p > 0.05$ not significant (ns).

Data Availability

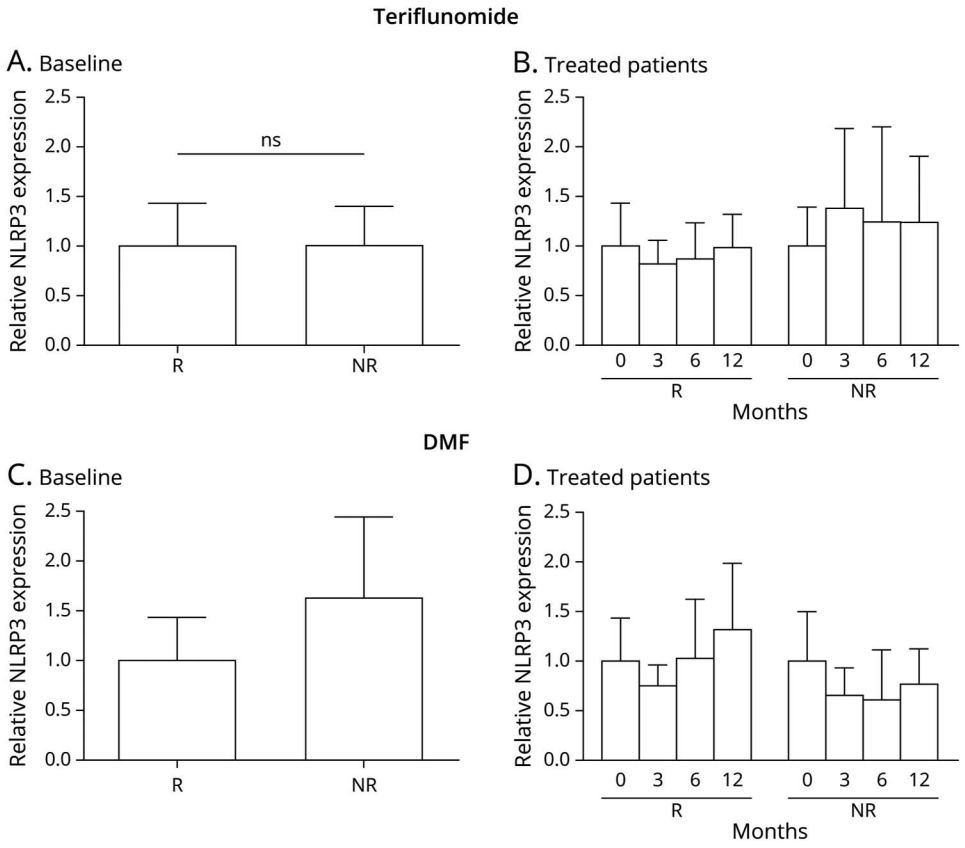
The data that support the findings of this study are available from the corresponding author on reasonable request.

Results

NLRP3 Gene Expression Levels Are Increased in Patients With MS With a Lack of Response to Fingolimod

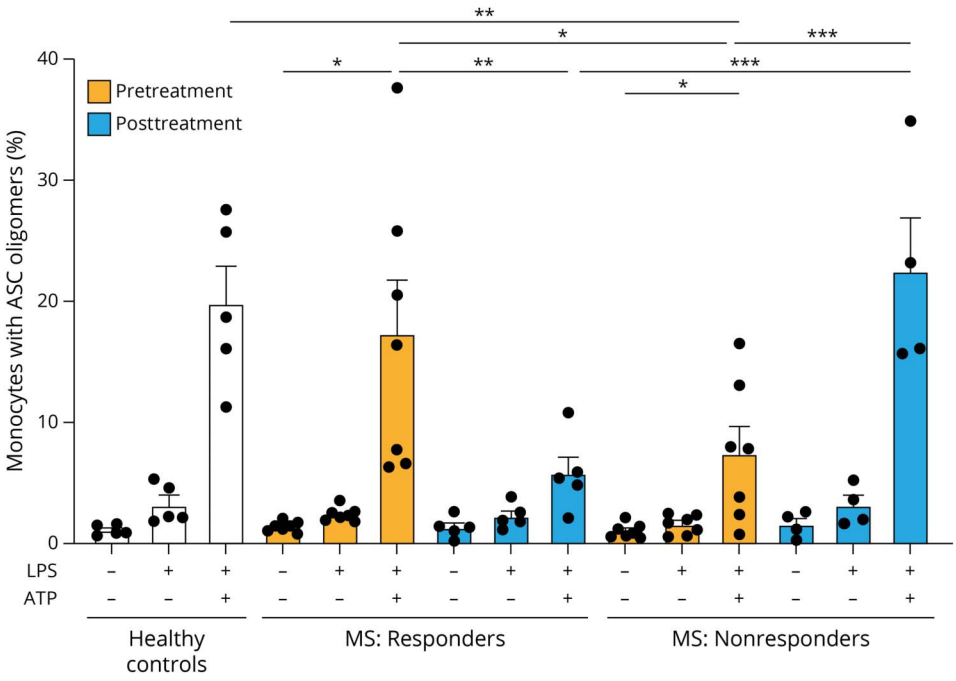
To investigate whether the NLRP3 inflammasome was involved in the response to fingolimod in patients with MS, we first measured *NLRP3* expression levels in PBMCs of

Figure 2 *NLRP3* Gene Expression Levels in Responders and Nonresponders to Other Oral Therapies



Messenger RNA expression levels for *NLRP3* were determined in PBMCs from responders (n = 11) and nonresponders (n = 10) to teriflunomide and responders (n = 7) and nonresponders (n = 7) to dimethyl fumarate (DMF) at baseline and after 3, 6, and 12 months of treatment by real-time PCR relative quantification. Results are expressed as fold change in gene expression in nonresponders (NR) relative to responders (R) (A, C) and after 3, 6, and 12 months of treatment relative to the baseline (B, D). PBMCs = peripheral blood mononuclear cells.

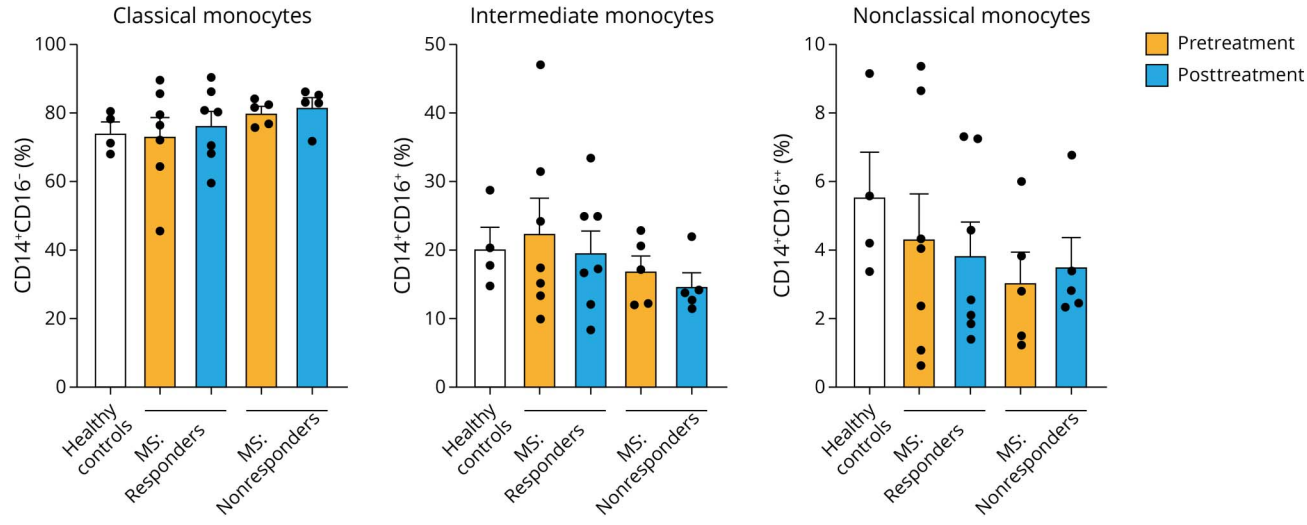
Figure 3 Percentage of Monocytes With Oligomers of ASC Before and After Fingolimod Treatment



The percentage of $CD14^+$ cells with an oligomer of ASC was determined by time-of-flight inflammasome evaluation and analyzed by flow cytometry in healthy controls and in responders and nonresponders to fingolimod at baseline (pretreatment, yellow bars) and after 6 months of treatment (posttreatment, blue bars). Dots within bars represent individuals included in each group. Experiments were conducted under unstimulated conditions, with LPS alone, and with a combination of LPS and ATP, as indicated in the graphs. ASC = apoptosis-associated speck-like protein containing a caspase recruitment domain; ATP = adenosine 5′-triphosphate; LPS = lipopolysaccharide.

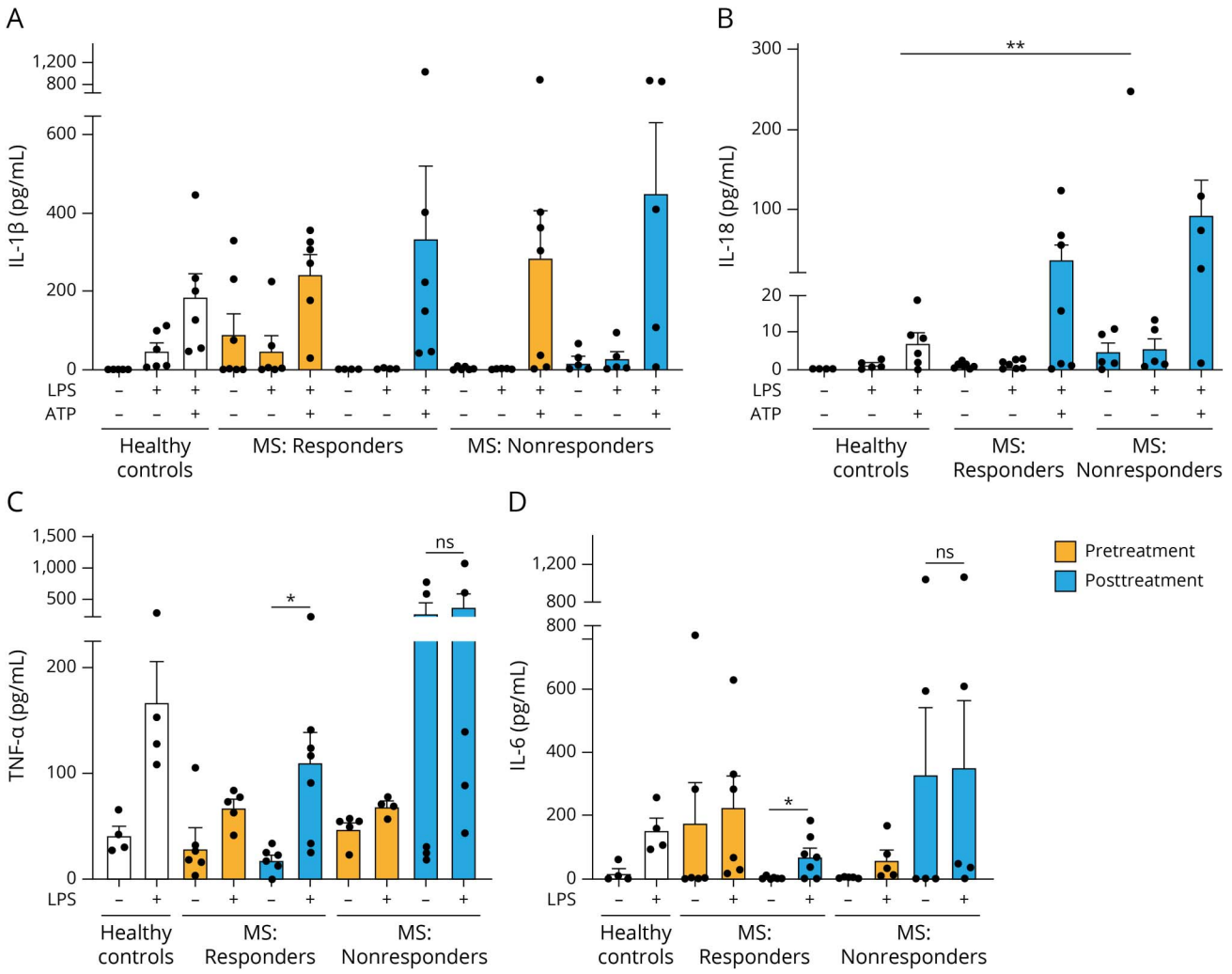
responders and nonresponders at baseline before treatment and at different time points during fingolimod treatment. As shown in Figure 1A, mRNA expression levels for *NLRP3* were comparable at baseline between responders and nonresponders to fingolimod. By contrast, in nonresponders, treatment with fingolimod was associated with a significant increase in *NLRP3* expression levels at 3 months ($p = 0.03$) and 6 months ($p = 0.008$) compared with the baseline values, whereas in fingolimod responders *NLRP3* expression levels remained similar at all time points (Figure 1B). Considering that the 6-month treated time point was associated with the highest differences in *NLRP3* expression compared with the baseline in nonresponders, this time point was selected for further NLRP3 inflammasome–related experiments.

Figure 4 Percentage of Classical, Intermediate, and Nonclassical Monocytes in Responders and Nonresponders to Fingolimod



The percentage of $CD14^+CD16^-$, $CD14^+CD16^+$, and $CD14^+CD16^{++}$ monocytes was determined by flow cytometry in healthy controls and in responders and nonresponders to fingolimod at baseline (pretreatment, yellow bars) and after 6 months of treatment (posttreatment, blue bars). Dots within bars represent individuals included in each group.

Figure 5 Release of Proinflammatory Cytokines in Responders and Nonresponders Before and After Fingolimod Treatment



The concentration of IL-1β (A), IL-18 (B), TNFα (C) and IL-6 (D) was measured by ELISA in supernatants of PBMCs from healthy donors and fingolimod responders and nonresponders under unstimulated conditions and following stimulation with LPS or LPS and ATP as indicated, both at baseline (pretreatment, yellow bars) and after 6 months of treatment with fingolimod (posttreatment, blue bars). Dots within bars represent individuals included in each group. ATP = adenosine 5′-triphosphate; LPS = lipopolysaccharide; PBMCs = peripheral blood mononuclear cells.

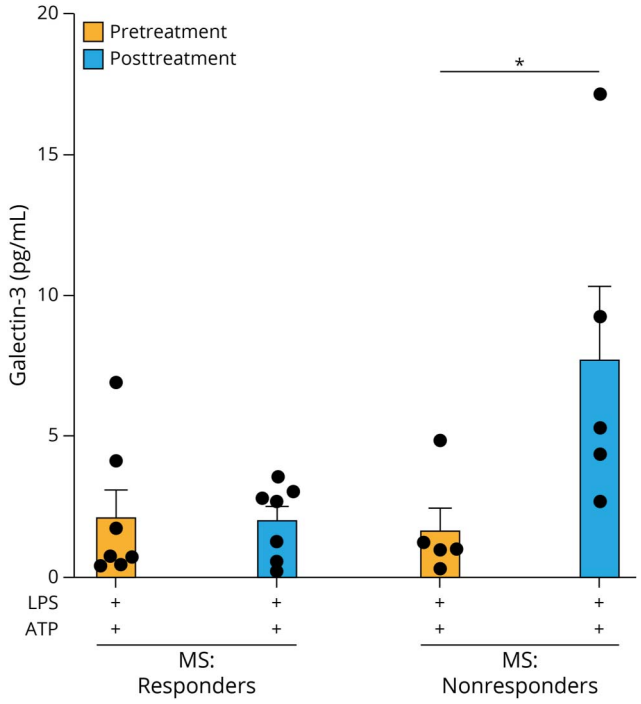
NLRP3 Gene Expression Levels Are Similar in Responders and Nonresponders to DMF and Teriflunomide

To assess whether the increase in *NLRP3* expression levels observed in nonresponders is specific for fingolimod or, on the contrary, is also observed in patients with MS receiving other oral therapies, *NLRP3* expression was also measured in PBMCs from a cohort of patients with MS treated with DMF and teriflunomide. As shown in Figure 2, *NLRP3* expression levels were comparable at baseline between responders and nonresponders and also at the different treated time points with DMF or teriflunomide.

NLRP3 Inflammasome Activation Is Inhibited in Responders but Increased in Nonresponders After 6 Months of Treatment With Fingolimod

We next measured the percentage of monocytes with an ASC oligomer from patients treated with fingolimod as a readout for inflammasome activation.[18] eFigure 1, links.lww.com/NXI/A816, depicts the gating strategy to detect monocytes with an ASC oligomer. As shown in Figure 3, no differences in the formation of an ASC oligomer were observed under unstimulated conditions between responders and nonresponders either at baseline or after 6 months of treatment. Stimulation of the alternative NLRP3 inflammasome pathway with LPS alone was associated with a minor and nonsignificant increase in the percentage of monocytes with an ASC oligomer compared with the unstimulated condition both in responders and nonresponders at baseline and at the 6-month treated point. At baseline before treatment, stimulation of the canonical NLRP3 inflammasome with a combination of LPS and ATP, 2 well-known signals involved in inflammasome activation,[19] was associated with a significant increase in the percentage of monocytes with an ASC oligomer from responders and nonresponders ($p = 0.01$ for both

Figure 6 Release of the Alarmin Galectin-3 in Responders and Nonresponders to Fingolimod Treatment



The concentration of galectin-3 was quantified by ELISA in supernatants of PBMCs from healthy controls and also fingolimod responders and nonresponders following LPS and ATP stimulation at baseline (pretreatment, yellow bars) and after 6 months of treatment (posttreatment, blue bars). Dots within bars represent individuals included in each group. ATP = adenosine 5′-triphosphate; LPS = lipopolysaccharide; PBMCs = peripheral blood mononuclear cells.

groups), although the percentage was significantly lower in nonresponders when compared with responders and healthy controls (p = 0.01 and p = 0.002, respectively) (eFigure 1, links.lww.com/NXI/A816 and Figure 3). Of interest, 6-month treatment with fingolimod was associated with an opposite effect between responders and nonresponders, whereas in responders the percentage of monocytes with an ASC oligomer formation after stimulation with LPS and ATP was significantly inhibited by treatment compared with the baseline (p = 0.006) and in nonresponders the percentage of monocytes with an ASC oligomer was increased compared with both the baseline (p = 0.0003) and the responder group (p = 0.0001) (Figure 3).

To assess whether changes in monocytes with an ASC oligomer were due to potential differences in the proportion of the different classes of monocytes, the percentage of classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$), and nonclassical monocytes ($CD14^+CD16^{++}$) was also investigated in patients with MS before and after fingolimod treatment. As shown in Figure 4, no significant differences in the percentage of classical, intermediate, and nonclassical monocytes were observed in fingolimod responders and nonresponders either before or during treatment (Figure 4).

Release of Proinflammatory Cytokines After NLRP3 Activation Is Similar Between Responders and Nonresponders to Fingolimod

Based on the increase in monocytes with an ASC oligomer observed following NLRP3 activation from nonresponders after fingolimod treatment, we next aimed to evaluate the release of the inflammasome-related cytokines IL-1β and IL-18. As shown in Figure 5, A and B, IL-1β and IL-18 levels were comparable between fingolimod responders and nonresponders at baseline (IL-1β) and after 6 months of treatment (IL-1β and IL-18) when stimulated with LPS alone or LPS and ATP. Only IL-18 levels were significantly increased in supernatants of PBMCs from fingolimod nonresponders after stimulation with LPS and ATP when compared with healthy controls (Figure 5B).

A similar picture was observed for the noninflammasome cytokines TNFα and IL-6, whose levels were comparable between responders and nonresponders to fingolimod at baseline and after treatment (Figure 5, C and D). However, although in responders treated with fingolimod LPS stimulation was able to significantly increase TNFα and IL-6 release from PBMCs (p = 0.04 and p = 0.03, respectively), whereas in nonresponders receiving fingolimod LPS failed to promote a similar cytokine increase because of the high TNFα and IL-6 levels already present in these patients under unstimulated conditions (Figure 5, C and D).

Pyroptosis Is Increased in PBMCs Following NLRP3 Activation in Fingolimod Nonresponders After 6 Months of Treatment

We finally investigated whether the increased inflammasome activation observed in fingolimod nonresponders after 6 months of treatment was associated with higher pyroptosis in this group of patients. As shown in Figure 6, the release of the alarmin galectin-3 was significantly increased in supernatants of PBMCs stimulated with LPS and ATP from nonresponders compared with responders after fingolimod treatment (p = 0.02). These data support the notion that in nonresponders, NLRP3 expression is induced after fingolimod treatment resulting in increased inflammasome activation and pyroptosis, which altogether could aggravate disease activity in these patients.

Discussion

Fingolimod was the first S1P receptor modulator approved for the treatment of patients with RRMS.[20] After binding to its target, fingolimod is known to mediate the internalization of the S1P receptor, thus preventing the egress of lymphocytes from lymph nodes and consequently the migration of autoreactive cells into the CNS.[21] Because of this mechanism of action, naive and central memory T cells are trapped in secondary lymphoid organs, leading to higher than 60% reductions in blood absolute lymphocyte counts.[22] Several studies have proposed a number of candidate treatment response biomarkers in patients with MS receiving fingolimod,

including baseline $CD4^+$ T central memory cells,[23,24] serum exosome microRNA signatures,[25] blood neurofilament light chain,[26] monocyte-derived microvesicles,[27] cerebrospinal mitochondrial DNA levels,[28] and the percentage of recent thymic emigrants.[29] Despite these studies, none of the proposed biomarkers are used routinely in MS clinical practice.

The findings reported by our group in a previous study[9] of an association between *NLRP3* expression and its associated cytokine *IL1B* in PBMCs and the therapeutic response to IFN-β, together with recent data showing an effect of fingolimod inhibiting NLRP3 inflammasome activation in different animal models,[10,11] motivated us to investigate whether the NLRP3 inflammasome could also be associated with the response to fingolimod.

In a cohort of patients with RRMS classified into responders and nonresponders after 12 months of treatment with fingolimod according to the Rio score, which takes into account both clinical and radiologic parameters,[12] patients classified as nonresponders were found to have an increase in the expression levels of the *NLRP3* gene in PBMCs after 3 and 6 months compared with fingolimod responders. These findings were more noticeable at 6 months and, of note, were specific of fingolimod treatment because similar changes were not observed in patients with RRMS treated with other oral therapies with different mechanisms of action such as DMF or teriflunomide. In fact, DMF has been found to succinate gasdermin D and block pyroptosis in a model of experimental autoimmune encephalitis and to reduce IL-1β in patients with MS.[30] Our study suggests that blocking gasdermin D–induced pyroptosis by DMF therapy does not affect *NLRP3* expression levels in patients with MS up to 12 months of therapy.

To deepen in the potential implication of the NLRP3 inflammasome in the therapeutic response to fingolimod, we investigated the NLRP3 inflammasome activation by measuring the percentage of monocytes with an oligomer of ASC. Of interest, similar to the previous findings in animal models of Parkinson disease and depression,[10,11] fingolimod inhibited NLRP3 inflammasome activation in myeloid cells after 6 months of treatment following stimulation with LPS and ATP. By contrast, in nonresponders, fingolimod treatment failed to inhibit NLRP3 inflammasome activation, and the formation of an oligomer of ASC and pyroptosis after NLRP3 activation was even further enhanced in this group of patients after 6 months of treatment compared with the pretreatment levels. Considering that fingolimod has been shown to downregulate nuclear factor kappa B (NF-κB) signaling in several models,[31,32] an explanation for these differential findings between responders and nonresponders is a lack of inhibition of the NF-κB signaling pathway by the effect of fingolimod in nonresponders. This notion is supported by the results in our study of an increase in the *NLRP3* expression levels observed in PBMCs from nonresponders after 6 months of treatment under unstimulated conditions. The NF-κB pathway is known to upregulate the transcription of inflammasome components.[33] In this context, a higher provision of the inflammasome machinery in nonresponders before stimulation would definitely result in higher formation of ASC oligomers and pyroptosis after the canonical NLRP3 inflammasome activation. This was also consistent with the fact that PBMCs from nonresponders after fingolimod treatment presented high IL-6 and TNFα levels in the absence of LPS stimulation, whereas levels for these NF-κB–dependent cytokines were lower in fingolimod responders.

Other approved S1P receptor modulators such as siponimod, ozanimod, and ponesimod may also be acting by a similar NLRP3 inflammasome–related mechanism. In fact, siponimod was shown to decrease astrocyte activation by controlling NF-kB and NLRP3 induction and preventing the increase of the proinflammatory cytokine IL-6 and the IL-8 and chemokine (C-C motif) ligand 2 chemokines.[34]

The study has a limitation of the relatively small sample size of responders and nonresponders to fingolimod. In addition, although responders and nonresponders to other oral therapies were included to assess the specificity of our findings with fingolimod, the lack of a control arm is another limitation of the study.

Our study suggests that fingolimod treatment may exert their beneficial effects by inhibiting NLRP3 inflammasome activation (patients labeled as "responders" in our study). However, in a subgroup of patients with MS, fingolimod treatment may fail to inhibit inflammasome activation, thus resulting in increased ASC-forming oligomers and pyroptosis, which could maintain disease activity (patients labeled as "nonresponders" in our study). The lack of inhibition of NLRP3 inflammasome activation in patients with MS receiving fingolimod could be used as a treatment response biomarker to identify fingolimod nonresponders in clinical practice, who may benefit of alternative MS therapies.

Acknowledgment

The authors would like to thank the patients for their participation, and the nurses and lab technicians for their involvement in sample collection.

Study Funding

MCIN/AEI/10.13039/501100011033 (grant PID2020-116709RB-I00)—Fundación Séneca (grant 20859/PI/18) and the Instituto Salud Carlos III (grant DTS21/00080). L. Hurtado-Navarro was supported by the fellowship 21214/FPI/19 (Fundación Séneca, Región de Murcia, Spain).

Disclosure

S. Malhotra reports no disclosures relevant to the manuscript; L. Hurtado-Navarro is a cofounder of Viva In Vitro Diagnostics SL but declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest; A. Pappolla, L.M. Villar, J. Río, X. Montalban, and M. Comabella report no disclosures relevant to the manuscript; P. Pelegrin is a cofounder of Viva In Vitro Diagnostics SL but declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Go to Neurology.org/NN for full disclosures.

Publication History

Previously published at bioRxiv doi: doi.org/10.21203/rs.3.rs-1831031/v1. Received by *Neurology: Neuroimmunology & Neuroinflammation* September 23, 2022. Accepted in final form January 12, 2023. Submitted and externally peer reviewed. The handling editor was Deputy Editor Scott S. Zamvil, MD, PhD, FAAN.

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