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Siponimod favours expression of less pro-inflammatory, alternatively activated microglia in a microglia repopulation model of progressive Multiple Sclerosis - implication for neuroprotection

N. Heitmann, A. Gude, H. Hendek, R. Gold, S. Faissner
Ruhr-University Bochum, Department of Neurology, St. Josef-Hospital, Bochum, Germany

RUHR UNIVERSITÄT BOCHUM RUB

Katholisches Klinikum Bochum

St. Josef-Hospital UK RUB UNIVERSITÄTSKLINIKUM DER RUHR-UNIVERSITÄT BOCHUM

Zentrum Neuroimmunologie RUB

Background

Progressive multiple sclerosis (MS) is characterized by chronic inflammation with diffuse microglial activation behind a relatively closed blood-brain-barrier, diffuse activation of T- and B-lymphocytes and alteration of the microglial phenotype to an inflammatory state.

Siponimod (BAF312) is an effective therapeutic approach in secondary-progressive MS due to its anti-inflammatory and potential neuroprotective effects with presumably modulatory properties on microglia.

Aim

We hypothesized that depleting microglia during experimental autoimmune encephalomyelitis (EAE) might lead to a renewal of neuroprotective microglia in the presence of siponimod in a mouse model of progressive MS.

Methods

Experimental autoimmune encephalomyelitis (EAE) as model for MS: 2x 50 µL s.c. of 500 µg/mL Myelin Oligodendrocyte Glycoprotein$_{35-55}$ (MOG$_{35-55}$) in Complete Freund Adjuvant containing 2,000 µg/ml *M. tuberculosis* and 200 ng Pertussis toxin in PBS on days 0 and 2 of EAE induction. 0.45 µg or 1 µg siponimod p.o. were administered daily from 1 day post immunization or vehicle. Conditional microglia (MG) depletion by using CX3CR1CreER/iDTR (MG^{ko}) mice. At the end of the experiment: flow cytometry analysis of microglia and lymphocytes and histological stainings and qRT-PCR of tissues. Authorization: LANUV 81-02.04.2019.A425.

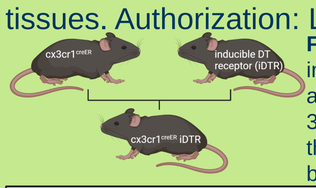
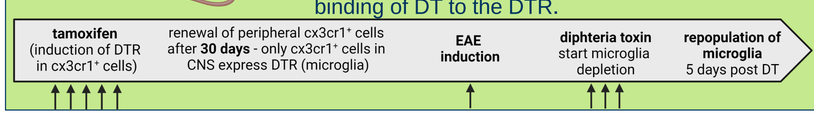


Figure 1: General method for MG^{ko} in EAE mice. The administration of 5x 1 mg Tamoxifen induces cre-recombinase expression under the CX3CR1 promoter. The cre-recombinase allows diphtheria toxin receptor (DTR) gene expression in CX3CR1 expressing cells. After 30 days CX3CR1-expressing cells in the periphery are renewed and only microglia express the DTR. When injecting 1 µg DT on three consecutive days, microglia are depleted by the binding of DT to the DTR.



Models

- EAE: Implementation of a suboptimal dosage (0.45 µg) and an effective dosage (1µg) siponimod in C57Bl/6 EAE.
- MG^{ko}-EAE: diphteria toxin administration resulted in CX3CR1-specific microglia depletion and repopulation after 7 days. Repopulating microglia displayed a pro-inflammatory phenotype during acute phase of EAE.

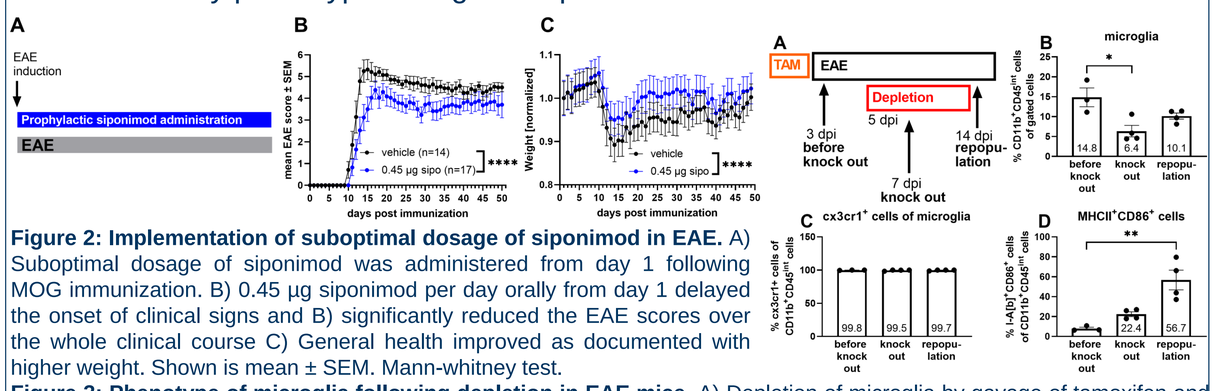


Figure 2: Implementation of suboptimal dosage of siponimod in EAE. A) Suboptimal dosage of siponimod was administered from day 1 following MOG immunization. B) 0.45 µg siponimod per day orally from day 1 delayed the onset of clinical signs and B) significantly reduced the EAE scores over the whole clinical course C) General health improved as documented with higher weight. Shown is mean ± SEM. Mann-whitney test.

Figure 3: Phenotype of microglia following depletion in EAE mice. A) Depletion of microglia by gavage of tamoxifen and diphtheria toxin. B) Microglia were significantly reduced during depletion. C) nearly all microglia cells express CX3CR1. D) Repopulating microglia exhibited a proinflammatory phenotype (CD86$^+$ and MHC II$^+$). Shown is mean ± SEM. Kruskal-Wallis with Dunn's post hoc analysis. *p < 0.05; **p < 0.01; *** p < 0.001.

Microglia phenotyping

- Repopulating microglia exhibited a proinflammatory phenotype despite 0.45 µg/day siponimod pretreatment
- 1 µg/day siponimod significantly reduced proinflammatory markers expression in repopulating MG with elevated alternative activation
- Microglia without siponimod: Steady state markers reduced, increased proinflammatory markers
- Niche left behind in brain by depleted microglia is replenished by monocytes

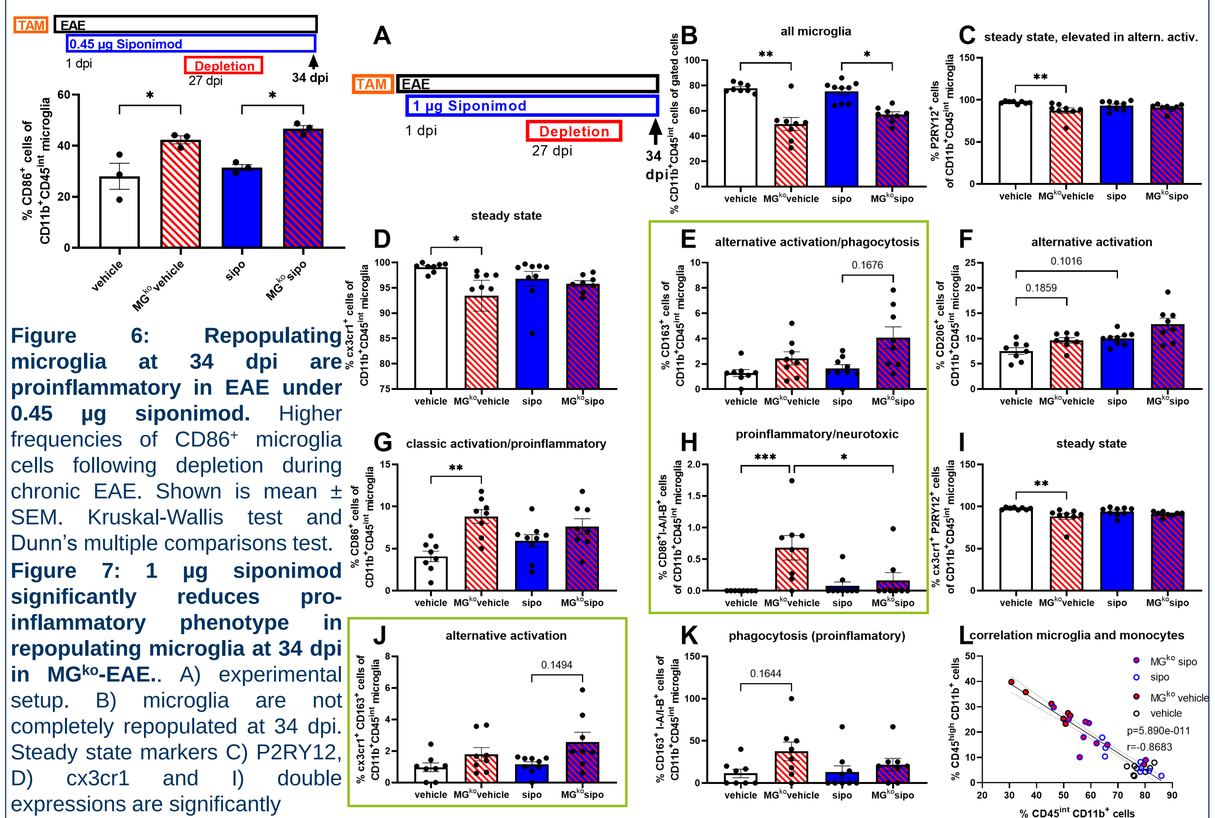


Figure 6: Repopulating microglia at 34 dpi are proinflammatory in EAE under 0.45 µg siponimod. Higher frequencies of CD86$^+$ microglia cells following depletion during chronic EAE. Shown is mean ± SEM. Kruskal-Wallis test and Dunn's multiple comparisons test.

Figure 7: 1 µg siponimod significantly reduces pro-inflammatory phenotype in repopulating microglia at 34 dpi in MG^{ko}-EAE.. A) experimental setup. B) microglia are not completely repopulated at 34 dpi. Steady state markers C) P2RY12, D) cx3cr1 and I) double expressions are significantly reduced in repopulating MG. Alternative activation is favoured by siponimod in repopulating microglia by expression of E) CD163, F) CD206 and J) CX3CR1 and CD163. Proinflammatory markers G) CD86 and H) CD86 with MHC-II are higher in repopulating microglia without siponimod treatment. There is a trend towards higher expression of phagocytosis markers E) CD163 and K) CD163 wit MHC-II in repopulating MG. L) brain monocytes increase with lowered frequency of microglia cells during repopulation phase after knock out. Shown is mean ± SEM. Kruskal-Wallis test, Dunn's multiple comparisons.

Conclusion

- Siponimod ameliorates EAE symptoms in mice when administered preventively – clinical signs, immune cell regulation in spleen, lymph nodes, and brain (microglia)
- In mice with repopulating microglia, the inflammation status is elevated (blood serum, spinal cord, microglia phenotype), but reduces infiltration and demyelination
- Repopulating microglia under 1 µg siponimod have a favourable phenotype characterized by alternative activation and phagocytosis.

This effect adds more mechanistic insight regarding microglia modulatory properties of siponimod with implications for putative neuroprotection during progression.

Contact: Neele Heitmann M.Sc., Neele.Heitmann@rub.de

Siponimod treatment in MG^{ko} EAE - early chronic phase

- Microglia depletion led to deterioration of EAE
- Demyelination and infiltration of spinal cord were altered by 0.45 µg siponimod per day and MG^{ko}

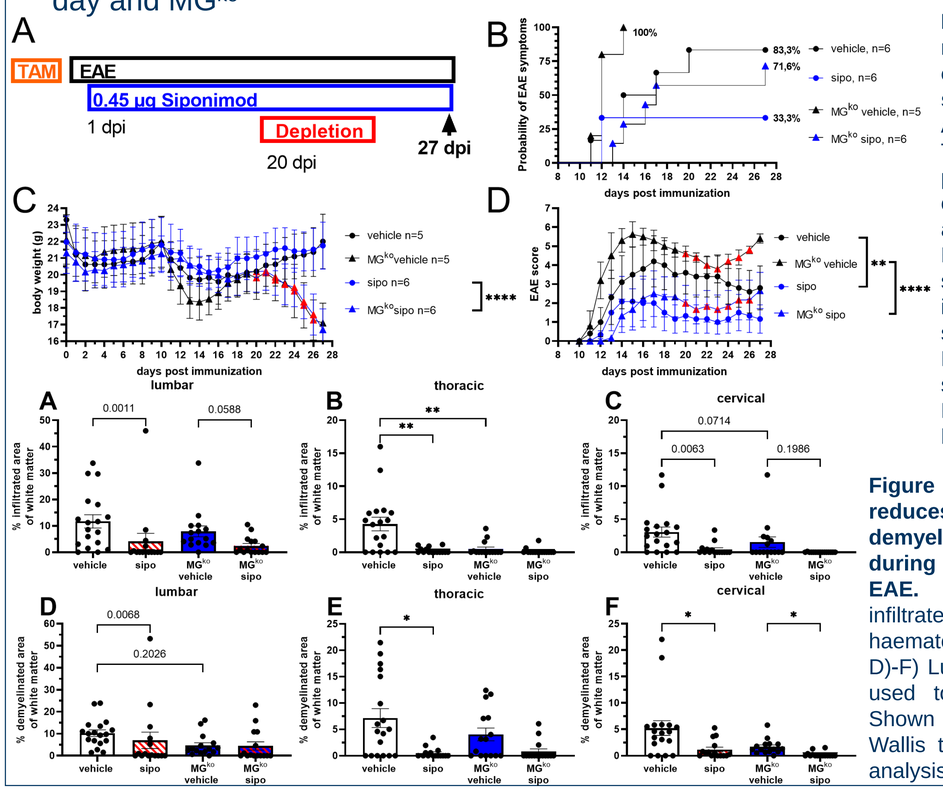


Figure 4: Depletion of microglia during early chronic phase of EAE in siponimod treated mice. A) experimental setup: TAM: tamoxifen. B) 0.45 µg siponimod per day orally delayed the onset and reduced incidence of EAE symptoms C) siponimod reduces EAE induced weight loss and D) significantly reduced the EAE scores. Data are shown as mean ± SEM. Kruskal-Wallis test with Dunn's post hoc analysis.

Figure 5: microglia knock out reduces infiltration and demyelination of spinal cord during early chronic phase of EAE. A)-C) percentage of infiltrated area was determined via haematoxylin and eosin staining. D)-F) Luxol fast blue staining was used to analyse demyelination. Shown is mean ± SEM. Kruskal-Wallis test with Dunn's post hoc analysis.

Siponimod treatment in MG^{ko} EAE - chronic phase

- 1 µg siponimod ameliorates EAE symptoms and incidence
- EAE severity depends on frequencies of b cells and different t cell subsets
- Brain immune cells express more inflammation markers after MG^{ko}

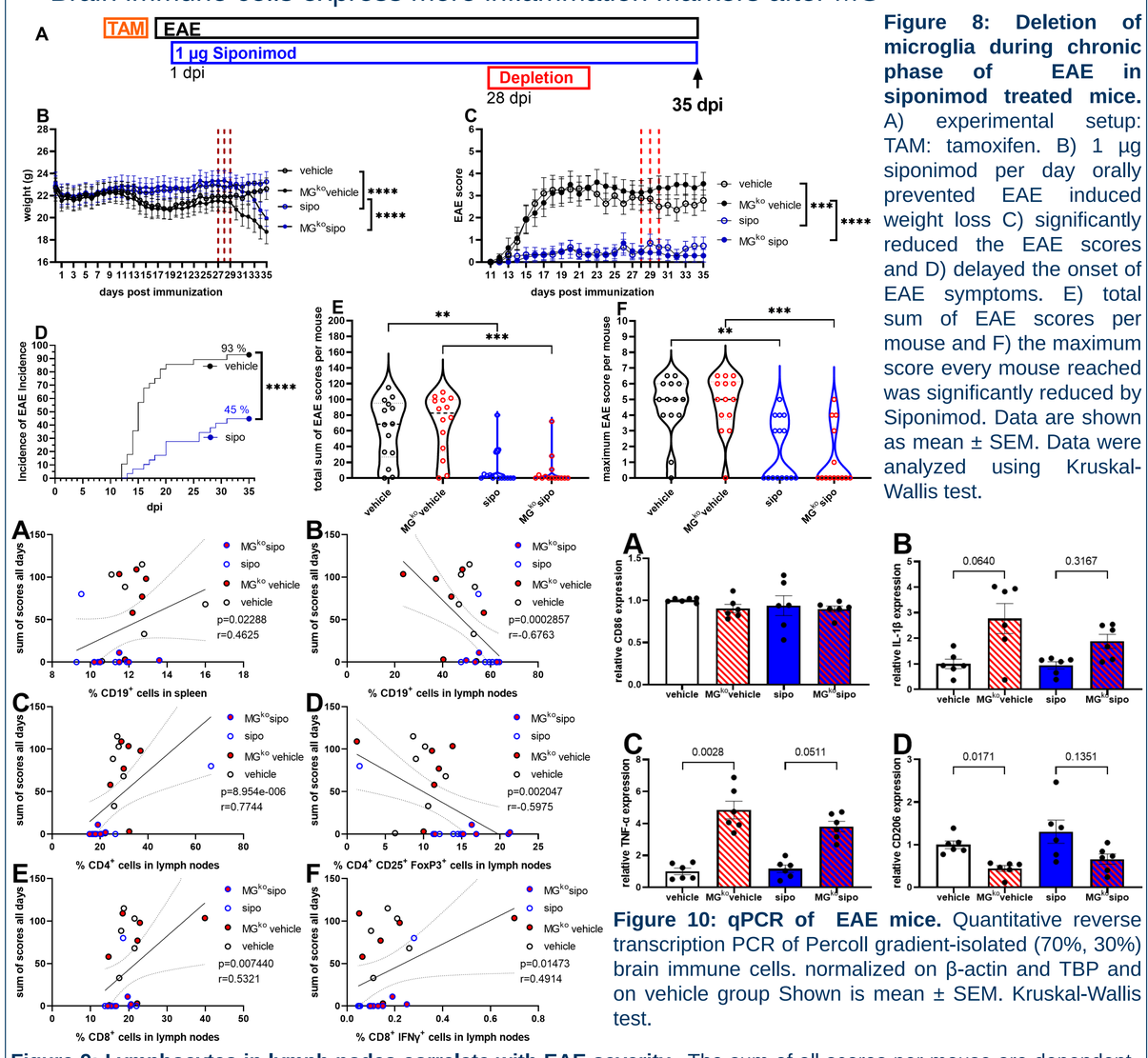


Figure 8: Deletion of microglia during chronic phase of EAE in siponimod treated mice. A) experimental setup: TAM: tamoxifen. B) 1 µg siponimod per day orally prevented EAE induced weight loss C) significantly reduced the EAE scores and D) delayed the onset of EAE symptoms. E) total sum of EAE scores per mouse and F) the maximum score every mouse reached was significantly reduced by Siponimod. Data are shown as mean ± SEM. Data were analyzed using Kruskal-Wallis test.

Figure 10: qPCR of EAE mice. Quantitative reverse transcription PCR of Percoll gradient-isolated (70%, 30%) brain immune cells. normalized on β-actin and TBP and on vehicle group Shown is mean ± SEM. Kruskal-Wallis test.

Figure 9: Lymphocytes in lymph nodes correlate with EAE severity. The sum of all scores per mouse are dependent on different b and t cell populations. Spearman correlation.

Disclosure

- This project was funded by a grant from Novartis (MBAF312A_FVTD007). NH, AG and HH have nothing to declare.
- RG serves on scientific advisory boards for Teva Pharmaceutical Industries Ltd., Biogen, Bayer Schering Pharma, and Novartis; has received speaker honoraria from Biogen, Teva Pharmaceutical Industries Ltd., Bayer Schering Pharma, and Novartis; serves as editor for Therapeutic Advances in Neurological Diseases and on the editorial boards of Experimental Neurology and the Journal of Neuroimmunology; and receives research support from Teva Pharmaceutical Industries Ltd., Biogen Idec, Bayer Schering Pharma, Genzyme, Merck Serono, and Novartis.
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