EVOBRUTINIB THERAPEUTIC RESPONSE IS ASSOCIATED WITH AN INCREASE IN THE NUMBER AND MATURATION OF PERIPHERAL AND CENTRAL CLASSICAL DENDRITIC CELLS

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BACKGROUND

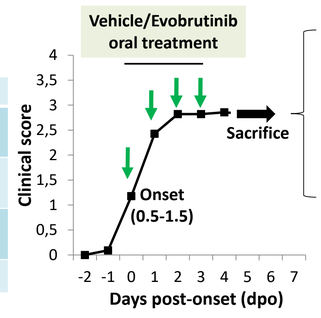
Multiple sclerosis (MS) is a chronic, inflammatory and neurodegenerative disease of the central nervous system. Currently, the search for new therapeutic strategies to control disease activity and progression is an active topic in the field**. Evobrutinib** is an oral, highly selective **covalent Bruton´s tyrosine kinase (BTK) inhibitor** with promising results in a Phase II trial for relapsing MS. Lately, the role of BTK in myeloid cell activity, including classical dendritic cells (cDCs), has been gaining importance in MS pathogenesis. In the present study, we assess the impact of **evobrutinib on classical dendritic cells (cDCs) in the MOG_{35-55}-induced chronic-progressive experimental autoimmune encephalomyelitis (EAE) MS model** (with limited B cell contribution).

METHODS

EAE (MOG_{35-55}) Individualized follow-up

Clinical signs

- **Onset**: the first day with clinical symptoms
- **Maximum clinical score**: clinical score with the maximum affectation
- **Total accumulated score**: the sum of all clinical scores from disease onset to sacrifice
- **Score variation**: the substraction of the score at the moment of sacrifice minus the score at onset
- **Final score**: score at the moment of sacrifice



RESULTS

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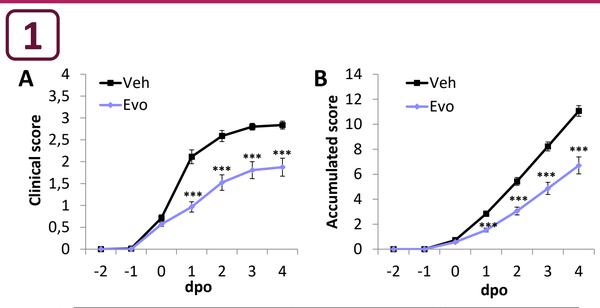
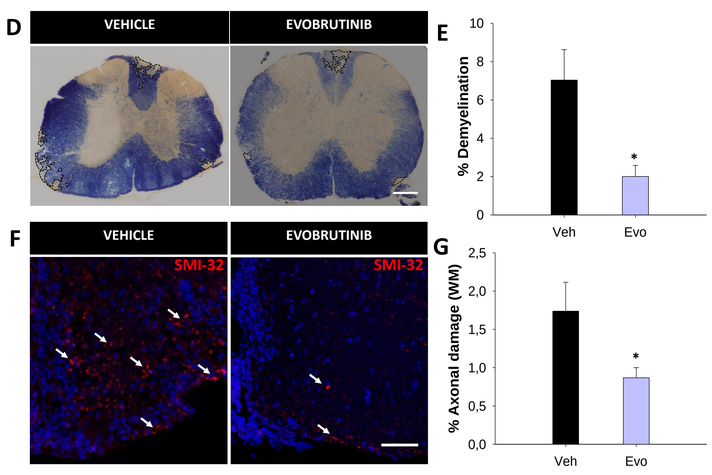
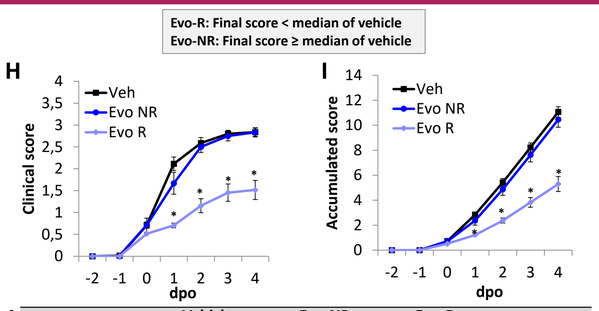


C	Vehicle n= 20	Evobrutinib n= 22	Statistics
Maximum score	3.0 (2.63 – 3.0)	2.0 (1.0 – 2.5)	***p<0.001***
Total accum. score	11.5 (10.0 – 12.38)	7.25 (3.75 – 8.5)	***p<0.001***
Score variation (4 dpo-0 dpo)	2.0 (1.75 – 2.5)	1.5 (0.5 – 2.0)	***p<0.01***
Final score	3.0 (2.5 – 3.0)	2.0 (1.0 – 2.5)	***p<0.001***





J	Vehicle n= 20	Evo-NR n= 6	Evo-R n= 16	Statistics
Maximum score	3.0 (2.63 – 3.0)	3.0 (2.50 – 3.0)	1.75 (1.0 – 2.25)	***p<0.001 (*R)***
Total accum. score	11.5 (10.0 – 12.38)	10.25 (9.75 – 11.0)	5.25 (3.63 – 7.38)	***p<0.001 (*R)***
Score variation (4 dpo-0 dpo)	2.0 (1.75 – 2.5)	2.13 (2.00– 2.5)	1.25 (0.25 – 1.75)	***p<0.001 (*R)***
Final score	3.0 (2.5 – 3.0)	3.0 (2.5-3.0)	1.75 (0.88-2.25)	***p<0.001 (*R)***

Figure 1. Evobrutinib-treated mice (EAE-Evo) showed milder EAE clinical courses and lower histopathological affectation than vehicles (EAE-Veh). A-C: EAE-Evo mice exhibited a milder EAE clinical course. **D-G:** EAE-Evo mice showed a lesser white matter demyelination and axonal damage than EAE-Veh mice within the inflammed spinal cord. **H-J:** EAE-responder mice (EAE-R) showed a milder EAE clinical course than both EAE-Veh and EAE-non responder (EAE-NR) mice. Evo-NR animals presented a very similar clinical affectation than EAE-Veh mice. A-C: n= 42, Mann-Whitney Rank Sum test. D-G: n= 6, Unpaired Student´s *t*-test and Mann-Whitney Rank Sum test. Scale bar: D= 200µm, F= 50µm. H-J: n= 42, ANOVA on Ranks followed by Dunn´s *post-hoc* test.

SPLEEN

2

classical Dendritic Cells (cDCs): $CD11b^+CD11c^+Ly\text{-}6C^-$

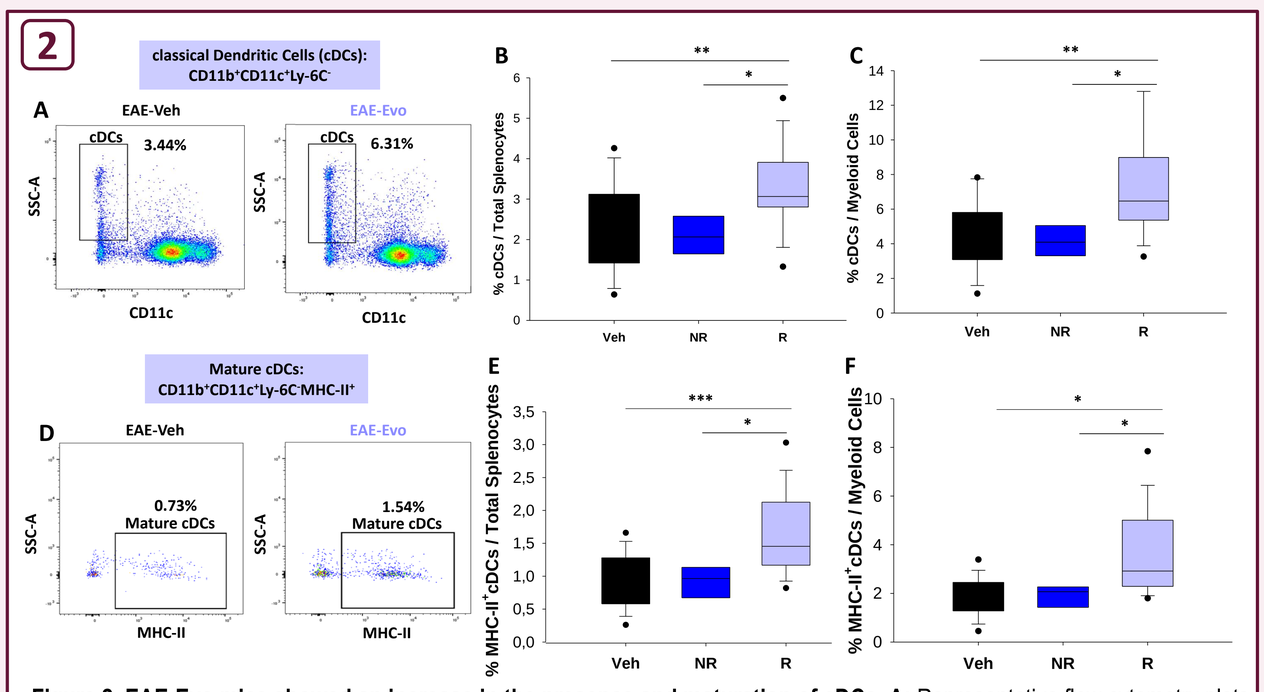


Figure 2. EAE-Evo mice showed an increase in the presence and maturation of cDCs. A: Representative flow cytometry plots comparing the percentage of cDCs in EAE-Veh *vs.* EAE-Evo mice. **B-C:** EAE-Evo mice presented a clear increase in the cDC content within total splenocytes (B) and myeloid cells (C), exclusively restricted to EAE-R mice. **D:** Flow cytometry plots comparing the percentage of mature cDCs in EAE-Veh *vs.* EAE-Evo mice. **E-F:** EAE-R mice showed a significant increase in the mature cDC content from total splenocytes (B) and from total myeloid cells (C) compared to EAE-Veh and EAE-NR mice. n= 42; EAE-Veh: n= 20, EAE-NR: n= 6, EAE-R: n= 16. One way ANOVA followed by Tukey *post-hoc* test, *p<0.05, **p<0.01, ***p<0.001.

3

Mature cDCs: $CD11b^+CD11c^+Ly\text{-}6C^-MHC\text{-}II^+$

Figure 3. Evobrutinib induced the modification of the immunological synapse components in cDCs. A: EAE-R mice presented a higher percentage of the $CD80^+$ subpopulation from the whole mature cDCs than EAE-Veh and EAE-NR, while $CD40^+$ and $CD86^+$ cDCs remained stable. **B:** Evobrutinib treatment induced a reduction of MHC-II MFI in both groups of EAE-Evo mice, together with an increase in CD80 but restricted to EAE-R mice. n= 40; EAE-Veh: n= 18, EAE-NR: n= 10, EAE-R: n= 12. One-way ANOVA followed by Tukey or Dunn´s *post-hoc* tests. *p<0.05, **p<0.01, ***p<0.001.

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Figure 4. The therapeutic response to evobrutinib is related to changes in T cell and activated $CD69^+$ T cell/ MHC-IIcDC ratios. A-D: The lower ratio of T cells/mature cDCs, the milder maximum clinical score (A), total accumulated score (B), score variation (C) and final score (D) in EAE-Evo mice. **E:** EAE-Evo presented a significant lower $CD69^+$ T-cell/ MHC-II$^+$cDC ratio than EAE-Veh mice. **F:** The more abundance of cDCs, the lower content of $CD69^+$ T-cells in the spleen of EAE-Evo mice. n= 42; EAE-Veh: n= 20, EAE-Evo: n= 22. . A-D: Spearman correlation. E: Mann-Whitney Rank Sum test, **p<0.01. F: Pearson correlation.

SPINAL CORD

5

Infiltrated myeloid cells $CD11b^+CD45^{high}$

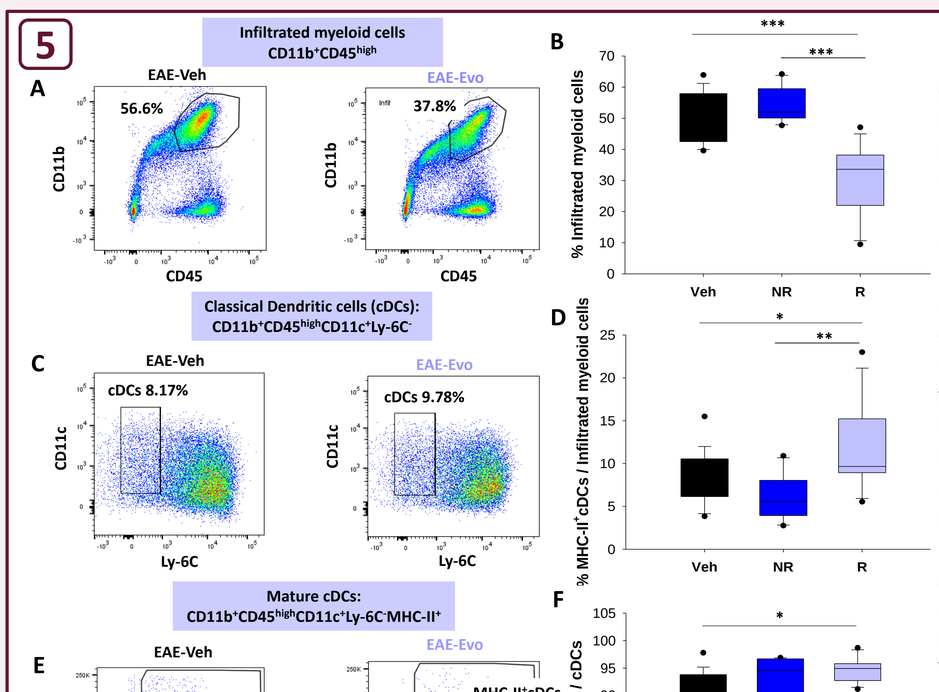

Figure 5. EAE-R mice exhibited a lower myeloid cell infiltrate in the spinal cord, albeit enriched in mature cDCs. A: Representative plots of the spinal cord cells present in one EAE-Veh and one EAE-Evo mouse. **B:** EAE-R mice exhibited a decrease in the myeloid cell infiltrate compared to EAE-Veh and EAE-NR mice. **C-F.** Representative plots comparing the percentage of infiltrated cDCs (C) and mature cDCs (E) in EAE-Veh *vs.* EAE-Evo mice. EAE-R mice showed a significant increase in infiltrated cDCs (C) and mature cDCs (D,F) compared to EAE-Veh and EAE-NR mice. n= 40; EAE-Veh: n= 18, EAE-NR: n= 10, EAE-R: n= 12. One way ANOVA followed by Tukey/Dunn´s *post-hoc* tests, *p<0.05,**p<0.01,***p<0.001.

6

Mature cDCs: $CD11b^+CD45^{high}CD11c^+Ly\text{-}6C^-MHC\text{-}II^+$

Figure 6. Analysis of the Evobrutinib-induced changes over the immunological synapse components of cDCs. A: Evobrutinib did not induce changes in the percentage of infiltrated mature cDCs presenting any of the co-stimulatory molecules. **B:** Evobrutinib treatment decreased the expression of the CD80 marker within mature cDCs exclusively in EAE-R mice. n= 40; EAE-Veh: n= 18, EAE-NR: n= 10, EAE-R: n= 12. One-way ANOVA followed by Tukey *post-hoc* test. *p<0.05.

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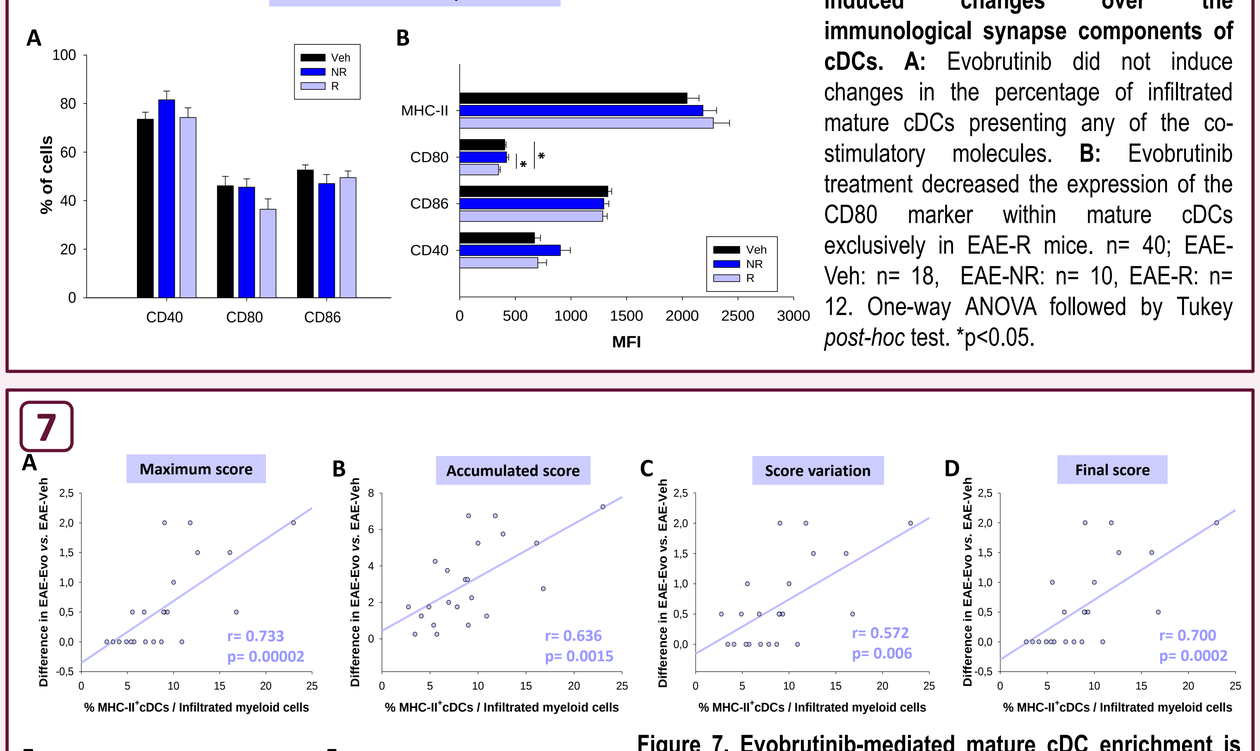
Figure 7. Evobrutinib-mediated mature cDC enrichment is related to a milder clinical score and a higher splenic content. A-D: The higher presence of infiltrated mature cDCs in the spinal cord of EAE-Evo, the more they differed from the mean values of clinical signs exhibited by EAE-Veh. **E-F:** The higher the abundance of peripheral cDCs and mature cDCs (spleen), the more infiltrated cDCs and mature cDCs in the spinal cord of EAE-Evo mice. A-F: n= 40; EAE-Veh: n= 18, EAE-Evo: n= 22. A-D: Spearman correlation; E-F: Pearson correlation.

CONCLUSIONS

1. Evobrutinib treatment induced a milder EAE clinical course and a less demyelination and axonal damage.
2. The individualized follow-up of EAE mice allowed us to distinguish between evobrutinib responder (EAE-R) and non-responder mice (EAE-NR).
3. The clinical response to evobrutinb was associated with an enrichment in both peripheral and central mature cDCs together with a reduced T cell/mature cDC ratio in the spleen.
4. In EAE Veh mice, cDCs and $CD69^+$ activated T cells increased together, while in EAE-Evo the enrichment in cDCs was related to a decrease in $CD69^+$ T cells.
5. In EAE-R mice, evobrutinib treatment induced a decrease in MHC-II and an increase in CD80 in splenic cDCs, whereas a decrease in CD80 was observed in spinal cord cDCs, whose immunological implications should be addressed in future functional *in vitro* assays.



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