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EVOBRUTINIB EXERTS A THERAPEUTIC ACTION ON EAE BY INCREASING THE PERIPHERAL AND CENTRAL CLASSICAL DENDRITIC CELL NUMBER AND MATURATION

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BACKGROUND

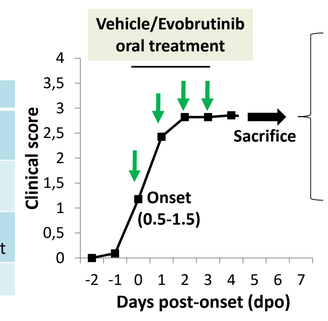
Multiple sclerosis (MS) is a chronic, inflammatory and neurodegenerative disease of the central nervous system. Currently, the search for new therapeutic strategies to control disease activity and progression is an active topic in the field**. Evobrutinib** is an oral, highly selective **covalent Bruton´s tyrosine kinase (BTK) inhibitor** with promising results in recent clinical studies. BTK is present in B cells and myeloid cells, important cell subsets with a prominent role on the pathogenesis of MS through the control of T cell activity. In the present study, we assess the **effect of evobrutinib on myeloid cells in the MOG_{35-55}-induced chronic-progressive experimental autoimmune encephalomyelitis (EAE) MS model**, a model to study the role of myeloid cells and T cells as well as and myeloid/ T cell interactions.

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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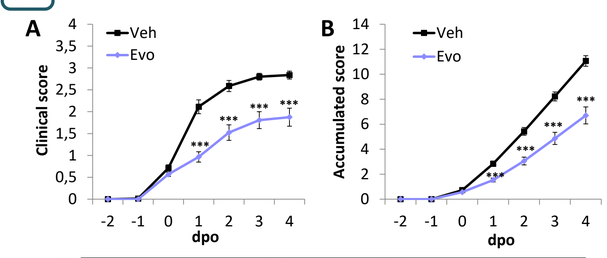

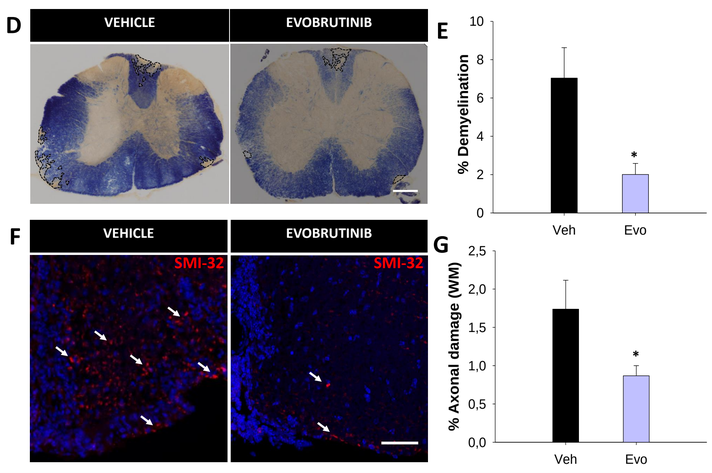

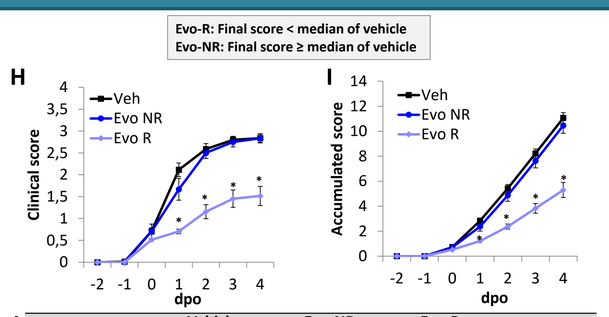

Figure 1. Evobrutinib-treated mice (EAE-Evo) showed milder EAE clinical courses and lower histopathological affectation than vehicles (EAE-Veh). A-C: EAE-Evo mice exhibited a milder EAE clinical course. **D-G:** EAE-Evo mice showed a lesser white matter demyelination and axonal damage than EAE-Veh mice 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 highly 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. Scale bar: D= 200µm, F= 50µm. H-J: n= 42, ANOVA on Ranks followed by Dunn´s *post-hoc* test.

SPLEEN

2

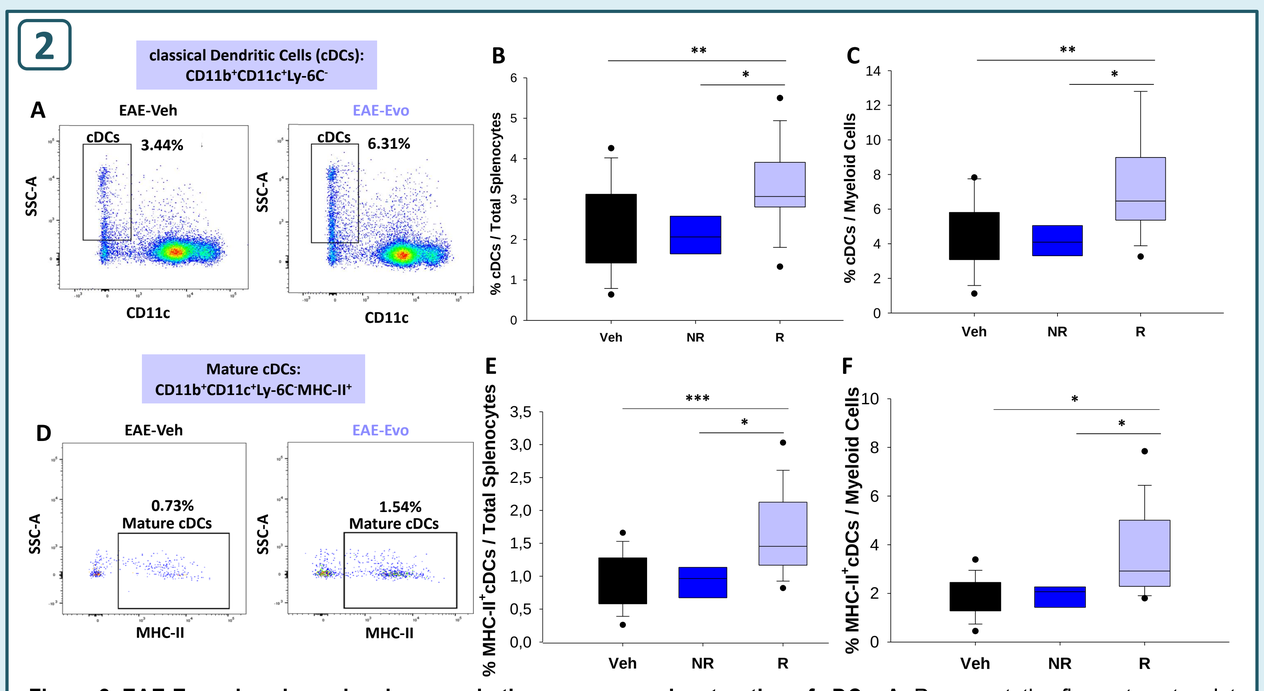

Figure 2. EAE-Evo mice showed an increase in the presence and maturation of cDCs. A: Representative flow cytometry plots comparing the percentage of cDCs in EAE-Veh *vs.* EAE-Evo mice. **B-C:** EAE-Evo mice presented a clear increase in the cDC content within total splenocytes (B) and myeloid cells (C), 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.

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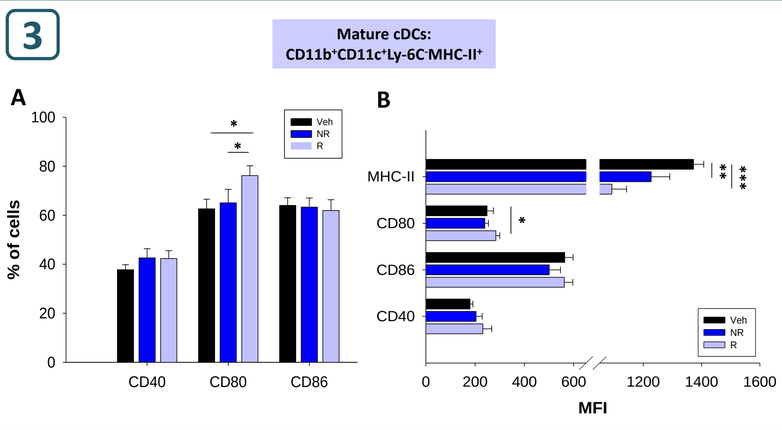

Figure 3. Evobrutinib induced the modification of the immunological synapse components in cDCs. A: EAE-R mice presented a higher percentage of the $CD80^+$ subpopulation from the whole mature cDCs than EAE-Veh and EAE-NR, while $CD40^+$ and $CD86^+$ cDCs remained stable. **B:** Evobrutinib treatment 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. n= 40; EAE-Veh: n= 18 mice, 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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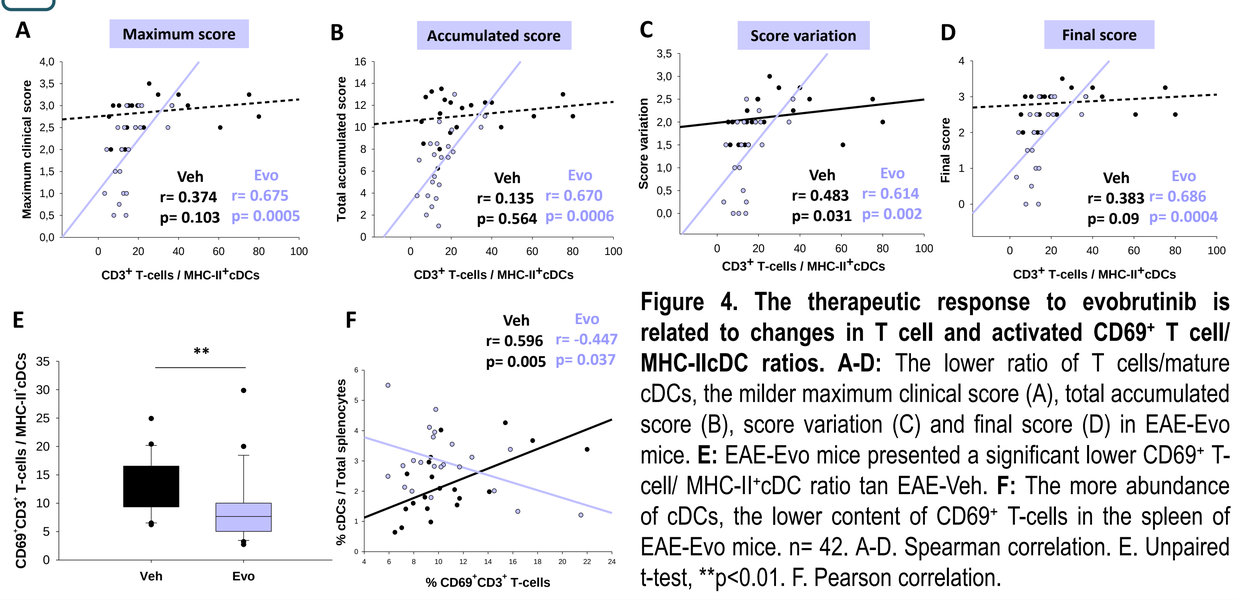

Figure 4. The therapeutic response to evobrutinib is related to changes in T cell and activated CD69⁺ T cell/ MHC-IIcDC ratios. A-D: The lower ratio of T cells/mature cDCs, the milder maximum clinical score (A), total accumulated score (B), score variation (C) and final score (D) in EAE-Evo mice. **E:**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

SPINAL CORD

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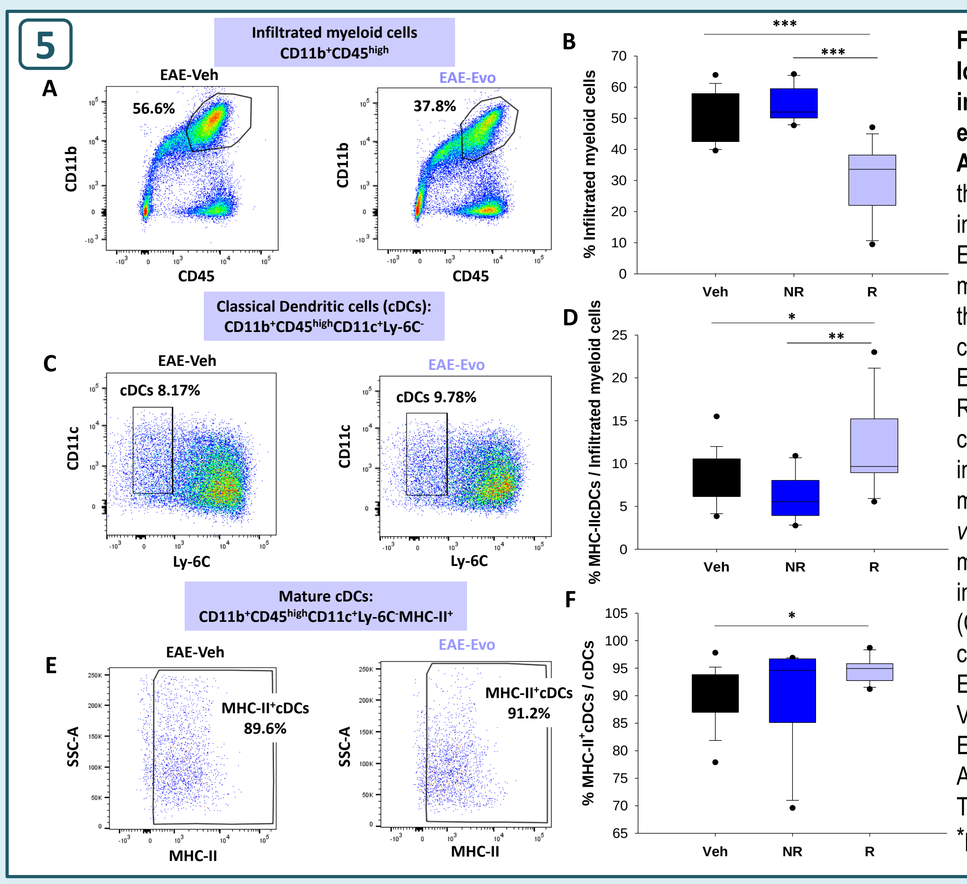

Figure 5. EAE-R exhibited a lower myeloid cell infiltrate in the spinal cord, albeit enriched in mature cDCs. A: Representative plots of the spinal cord cells present in one EAE-Veh and one EAE-Evo mouse. **B:** EAE-R mice exhibited a decrease in the myeloid cell infiltrate compared to EAE-Veh and EAE-NR 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.

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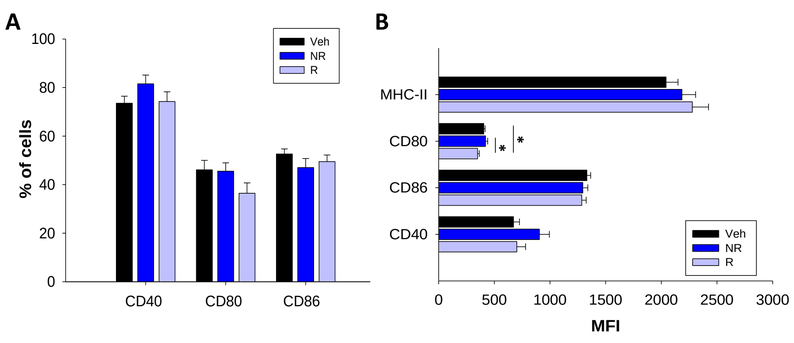

Figure 6. Analysis of the Evobrutinib-induced changes over the immunological synapse components of cDCs. A: Evobrutinib did not induce changes in the percentage of infiltrated mature cDCs presenting any of the co-stimulatory molecules. **B:** Evobrutinib treatment decreased the expression of the CD80 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.

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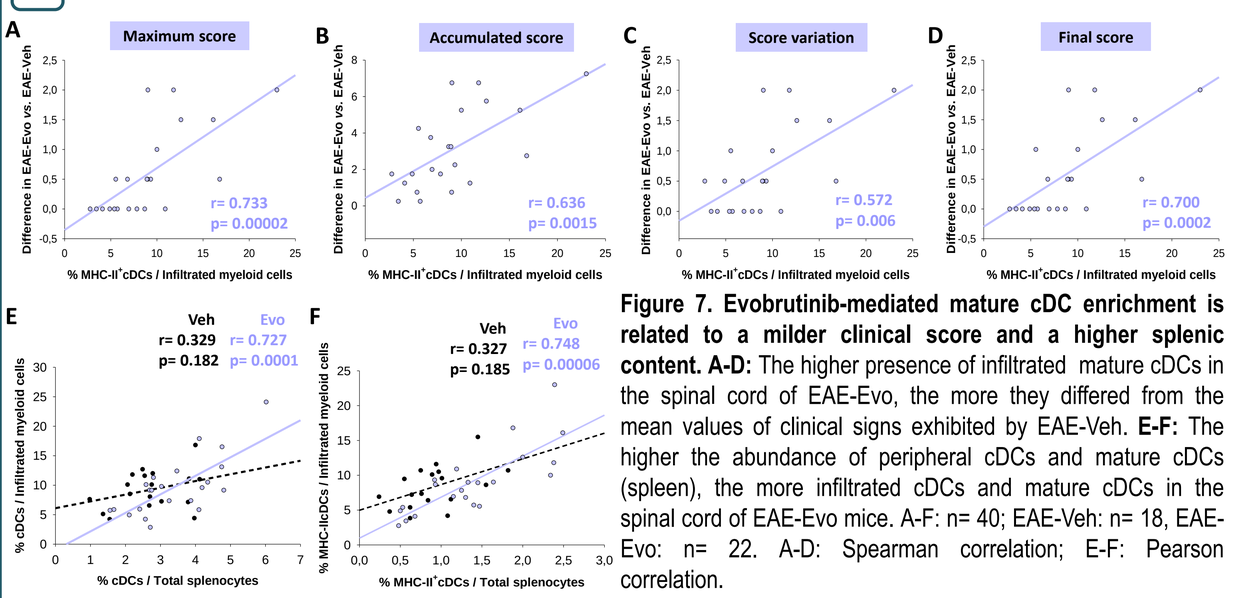

Figure 7. Evobrutinib-mediated mature cDC enrichment is related to a milder clinical score and a higher splenic content. A-D: The higher presence of infiltrated mature cDCs in the spinal cord of EAE-Evo, the more they differed from the mean values of clinical signs exhibited by EAE-Veh. **E-F:** The 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 lesser 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 to an enrichment in both peripheral and central mature cDCs together with a reduced T cell/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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