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

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DISCLOSURES

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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. **Classical dendritic cells (cDCs)** are professional antigen-presenting cells known to be involved in the development of inflammation. However, cDCs can also induce immune tolerance, leading to control of the immune response. Lately, the role of BTK in myeloid cell activity, including cDCs, has been gaining importance in understanding 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).

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METHODS

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

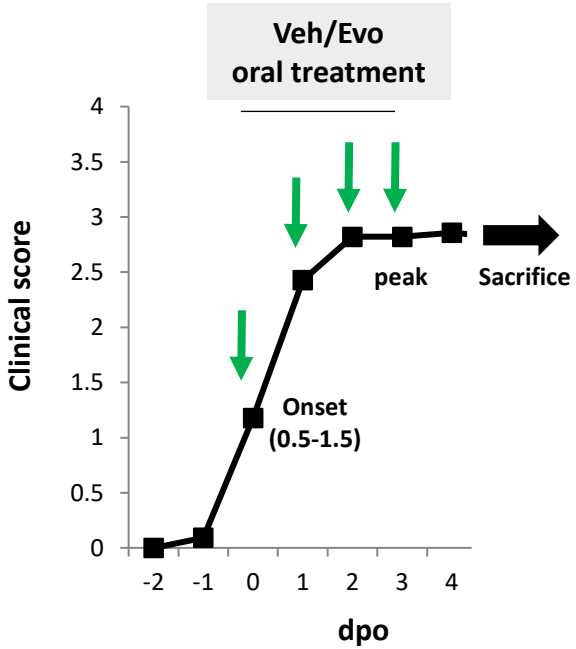
Clinical signs

Maximum clinical score: the highest clinical score from onset to the last day of the follow up

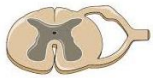
Total accumulated score: the sum of all clinical scores from disease onset to sacrifice

Score variation: the score at the moment of sacrifice minus the score at onset

Final score: score at the moment of sacrifice



Histopathology

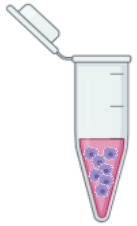


Spinal cord

Demyelination (eriochrome cyanine)

Axonal damage (SMI-32)

Spleen



Markers:
CD40
CD80
CD86
MHC-II

Flow cytometry

Classical Dendritic cells
($CD11b^{+}CD11c^{+}Ly\text{-}6C^{-}$)

MARKER	CELL TYPE
CD11b	Pan-myeloid marker
CD11c	Dendritic cell marker
Ly-6C	Monocytic marker
MHC-II	Major histocompatibility complex-II (Antigen presenting cell marker)
CD40, CD80, CD86	Costimulatory molecules

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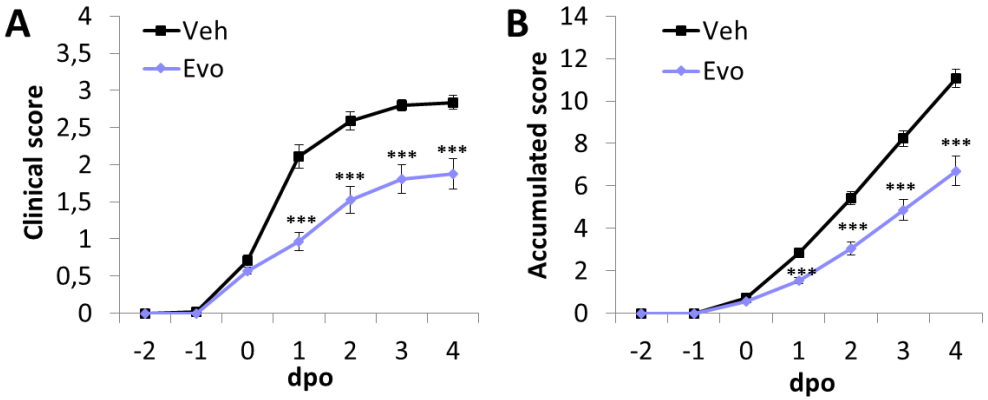
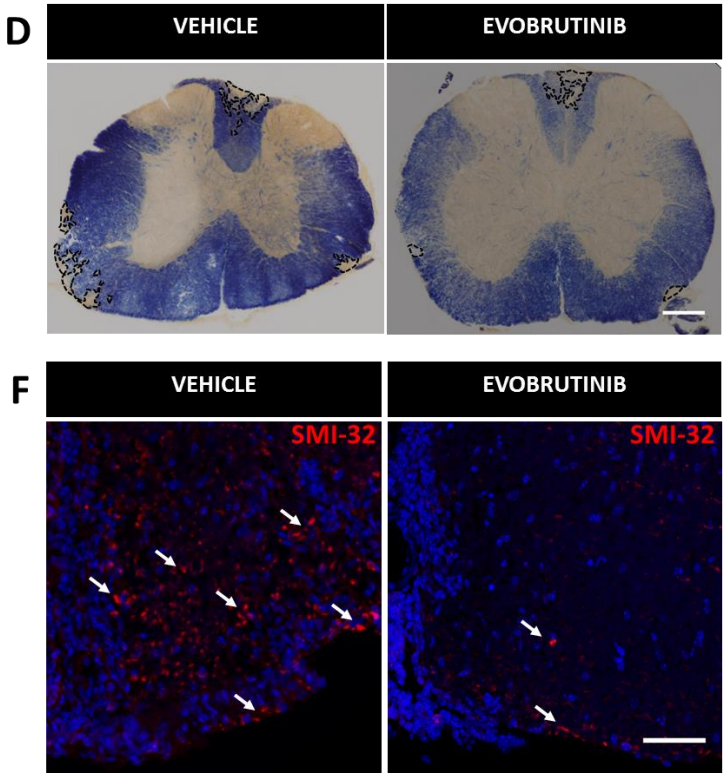
RESULTS-Figure 1a

EAE clinical course and histopathology under evobrutinib treatment



C

Clinical sign	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***



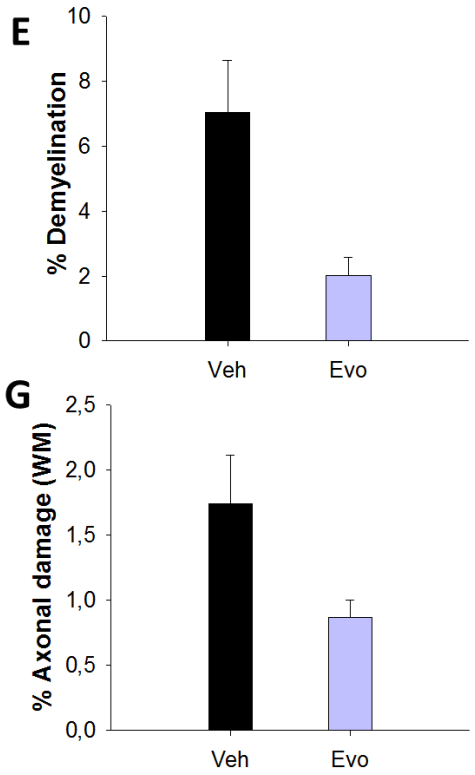


Figure 1a. Evobrutinib-treated mice (EAE-Evo) showed milder EAE clinical courses and lower histopathological alteration than vehicle-treated mice (EAE-Veh). A-C: EAE-Evo mice exhibited a milder EAE clinical course. **D-G:** EAE-Evo mice showed less white matter demyelination and axonal damage than EAE-Veh mice within the inflammed spinal cord. 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= 50 µm.

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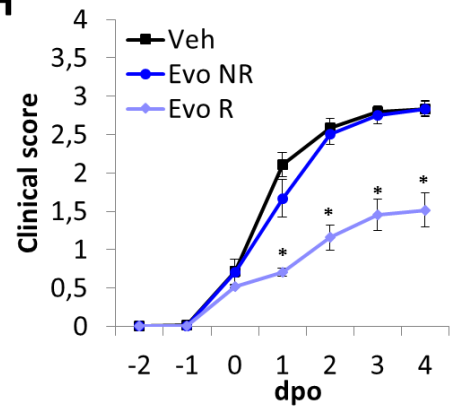
RESULTS-Figure 1b

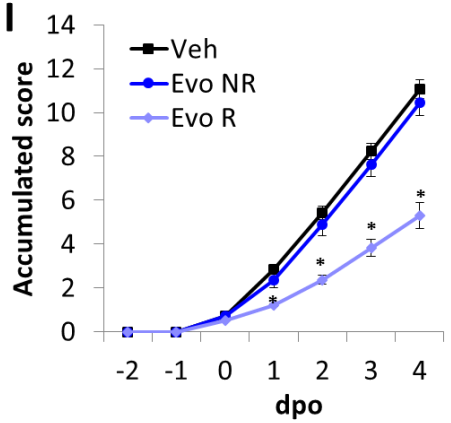
EAE clinical course in responder and non-responder mice

Evo-R: Final score < median of vehicle
Evo-NR: Final score ≥ median of vehicle

H



I



J

Clinical sign	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 1b. Evobrutinib-treated mice (EAE-Evo) showed milder EAE clinical courses and lower histopathological alteration than vehicle-treated mice (EAE-Veh). 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 course than EAE-Veh mice. H-J: n= 42, ANOVA on Ranks followed by Dunn´s *post-hoc* test.

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RESULTS-Figure 2

Evobrutinib effect on the cDC number and maturation in the periphery

Spleen

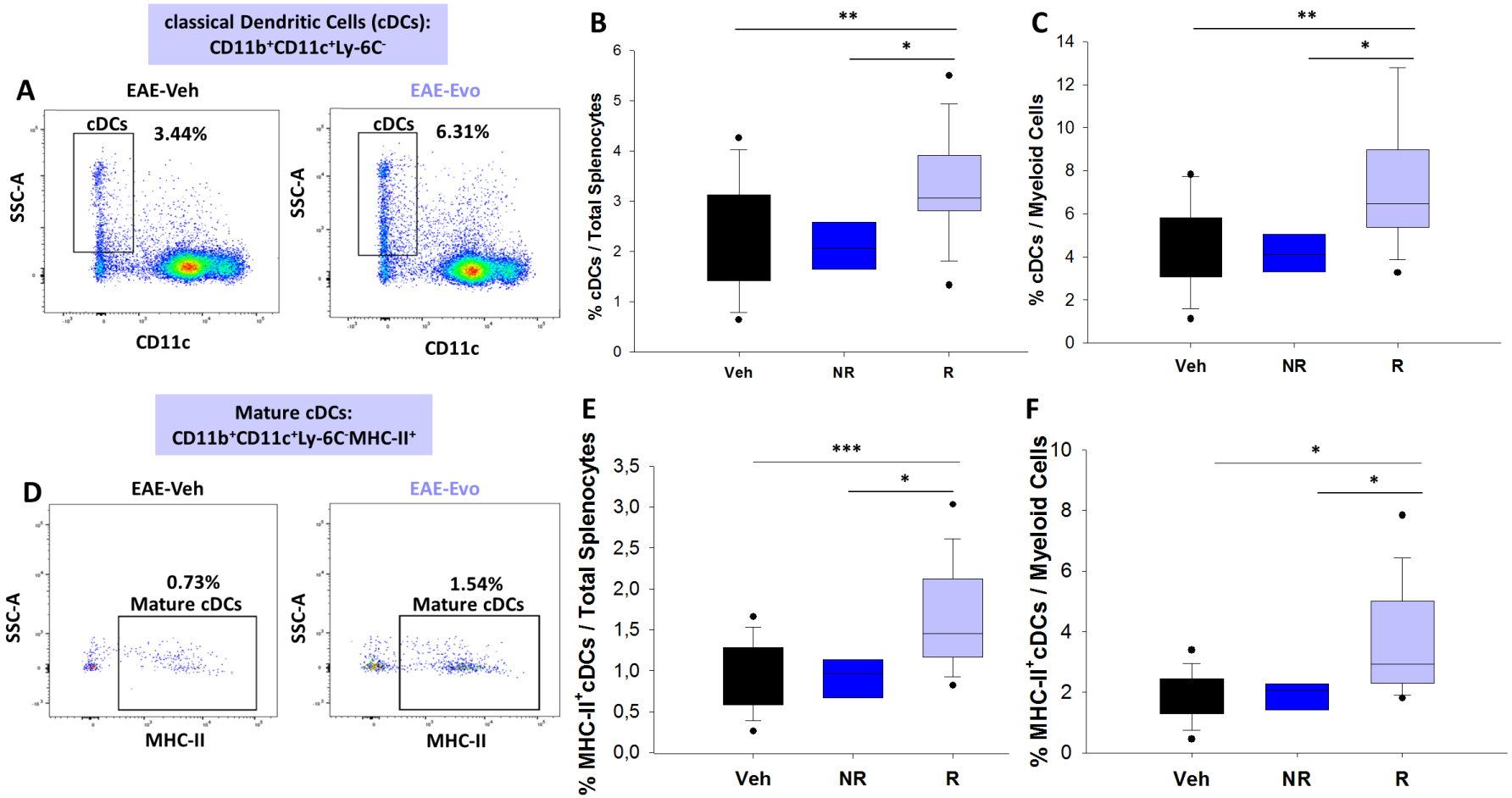

Figure 2. EAE-Evo mice showed an increase in the presence and maturation of cDCs. A: Representative flow cytometry plots comparing the percentage of cDCs in EAE-Veh vs. EAE-Evo mice**. B-C:** EAE-Evo mice presented a clear increase in the cDC content within total splenocytes (B) and myeloid cells (C), 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 (E) and from total myeloid cells (F) 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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RESULTS-Figure 3

The effect of evobrutinib on the immunological synapse in cDCs in the periphery

Spleen

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

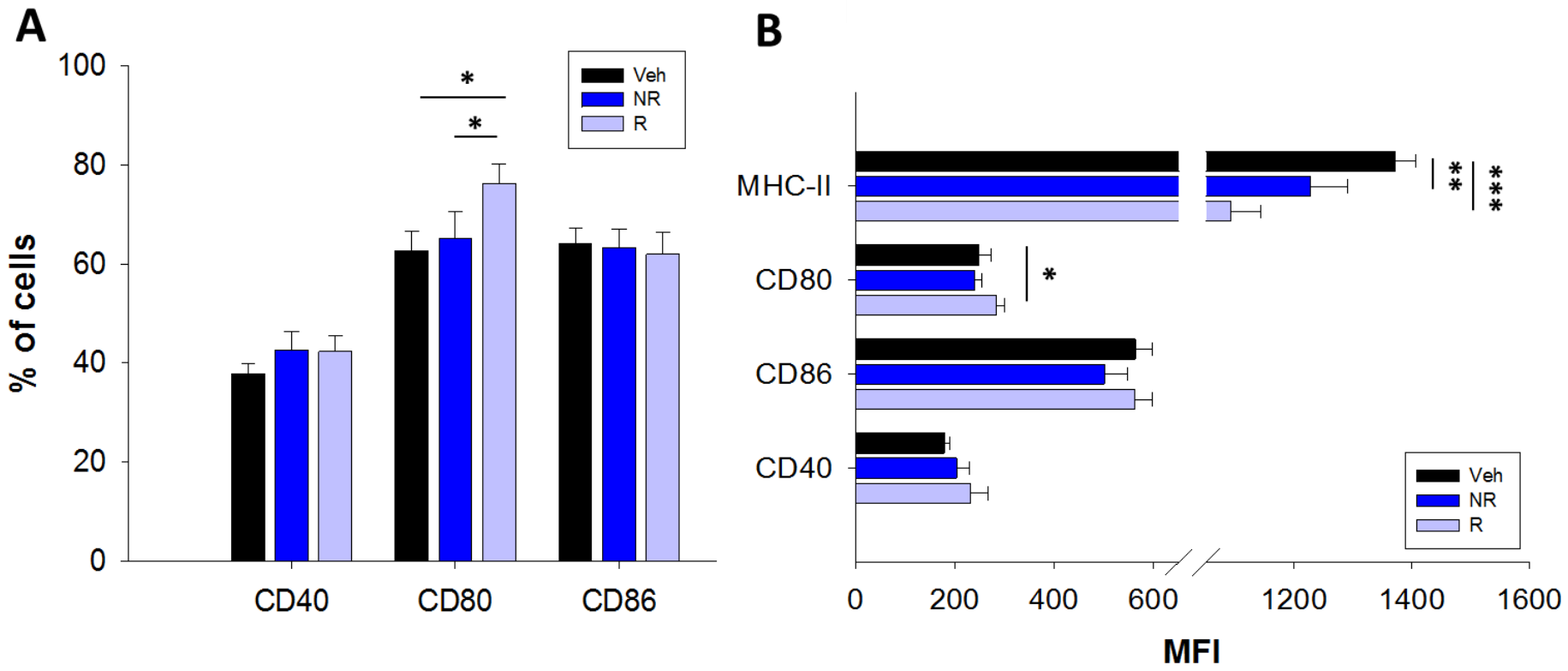

Figure 3. Evobrutinib induced the modification of the immunological synapse components in cDCs. A: EAE-R mice presented a higher percentage of the $CD80^{+}$ subpopulation from the whole mature cDCs than EAE-Veh and EAE-NR, while $CD40^{+}$ and $CD86^{+}$ cDCs remained stable. **B:** Evobrutinib treatment induced a reduction of MHC-II MFI in both groups of EAE-Evo mice. An increase in CD80 was only seen in 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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RESULTS-Figure 4

Therapeutic response to evobrutinib and changes in T cell activation in the periphery

Spleen

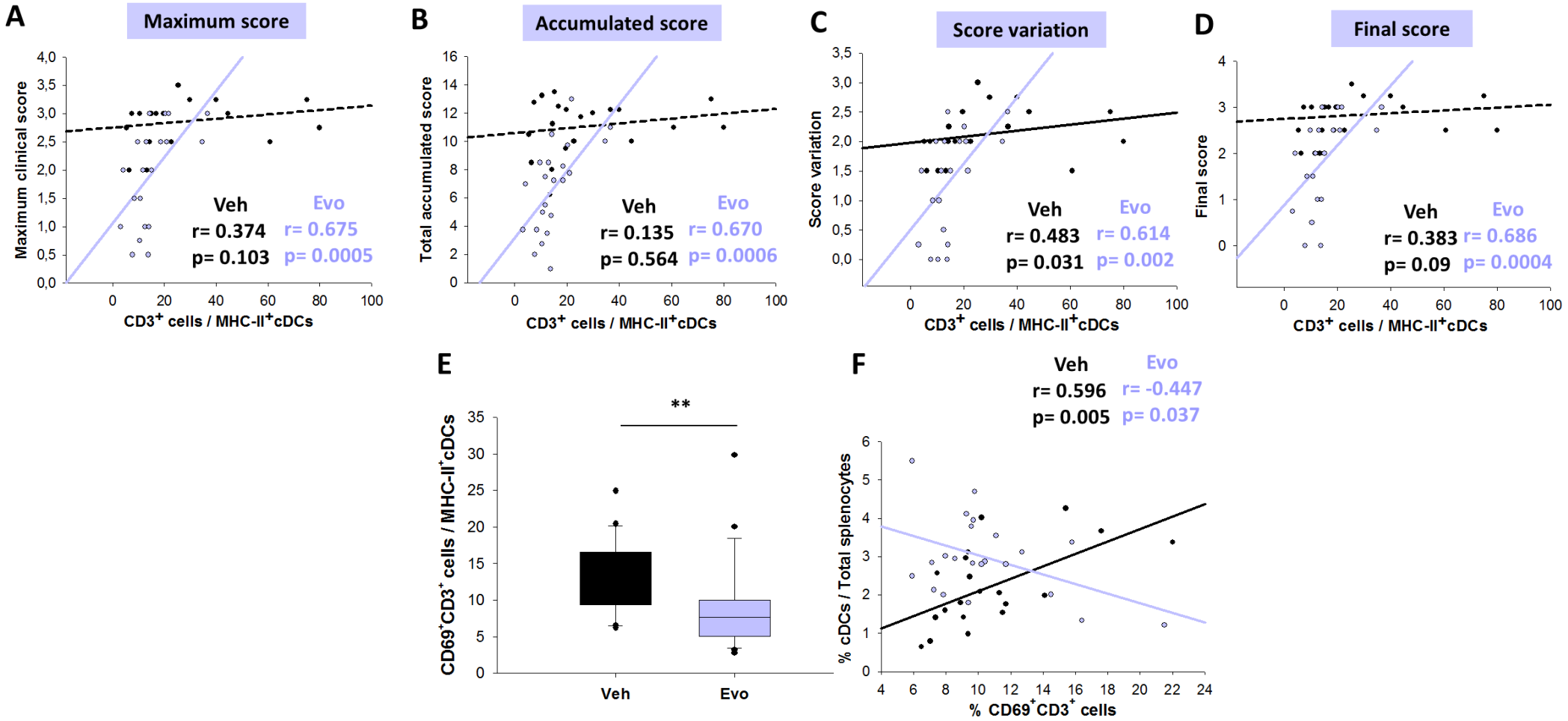

Figure 4. The therapeutic response to evobrutinib is related to changes in T cell and activated CD69⁺ T cell/ MHC-IIcDC ratios. A-D: The lower ratio of T cells/mature cDCs, the milder maximum clinical score (A), total accumulated score (B), score variation (C) and final score (D) in EAE-Evo mice. **E:** EAE-Evo presented a significantly lower CD69⁺ T-cell/ MHC-II+cDC ratio than EAE-Veh mice. **F:** The higher 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.

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RESULTS-Figure 5

Evobrutinib effect on the cDC number and maturation in the CNS

Spinal cord

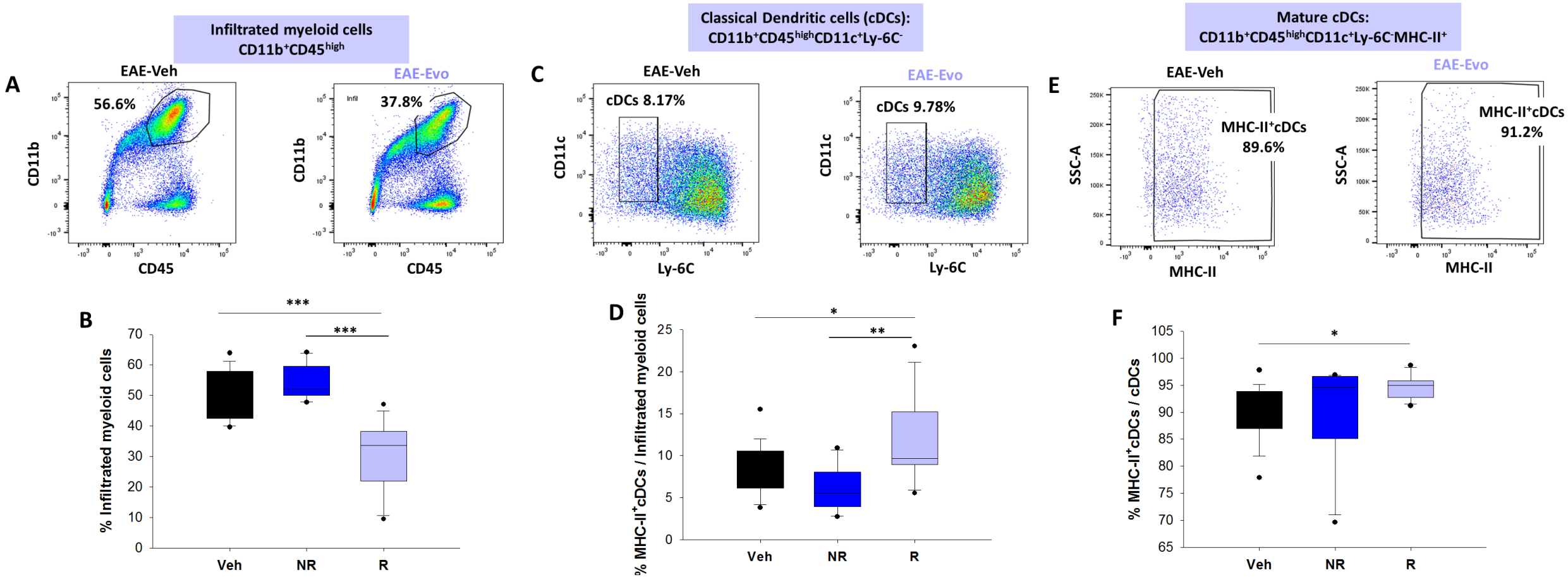

Figure 5. EAE-R mice exhibited less myeloid cell infiltrate in the spinal cord, albeit enriched in mature cDCs. A: Representative plots of the spinal cord cells present in one EAE-Veh and one EAE-Evo mouse. **B:** EAE-R mice exhibited a decrease in the myeloid cell infiltrate compared to EAE-Veh and 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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RESULTS-Figure 6

Evobrutinib impact on the immunological synapse in cDCs in the CNS

Spinal cord

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

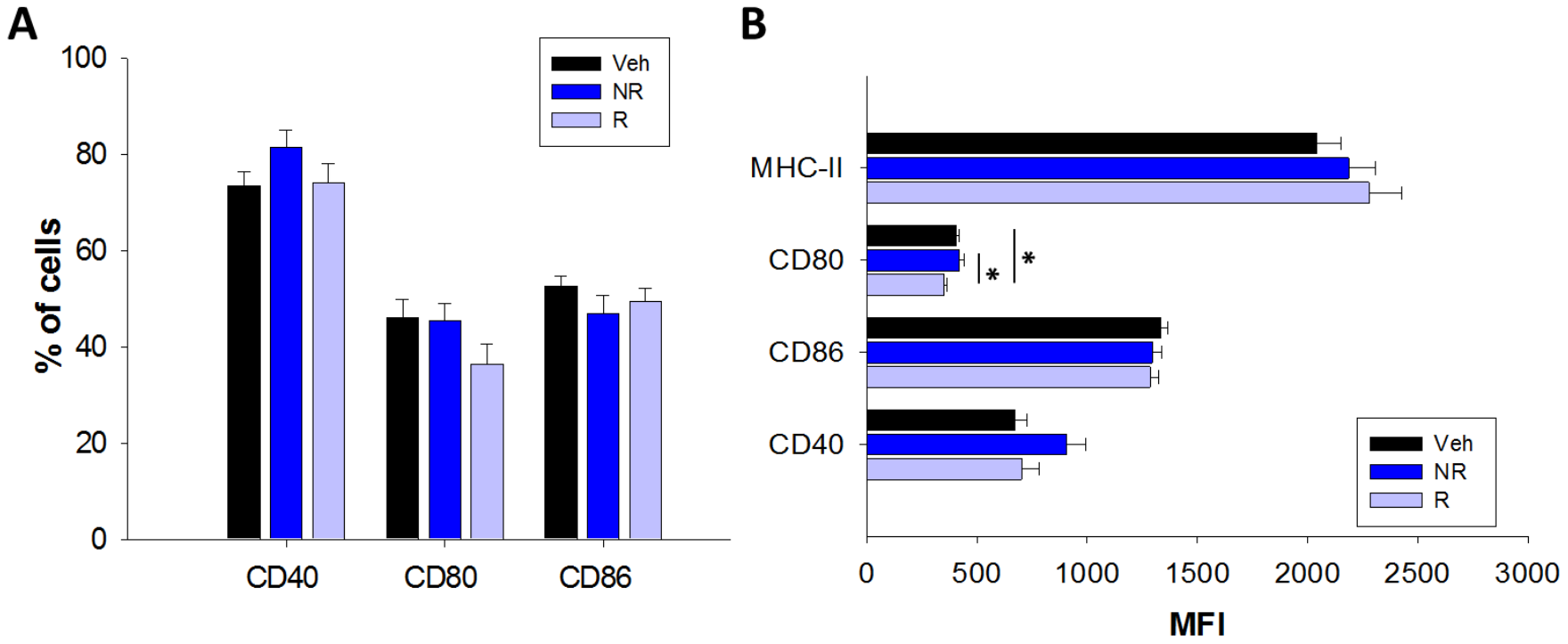

Figure 6. Analysis of the Evobrutinib-induced changes over the immunological synapse components of cDCs. A: Evobrutinib did not induce changes in the proportion of infiltrated mature cDCs presenting any of the co-stimulatory molecules. **B:** Evobrutinib treatment decreased the expression of the CD80 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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RESULTS-Figure 7 Therapeutic response to evobrutinib and changes in cDC abundance in periphery/CNS

Spinal cord/Spleen

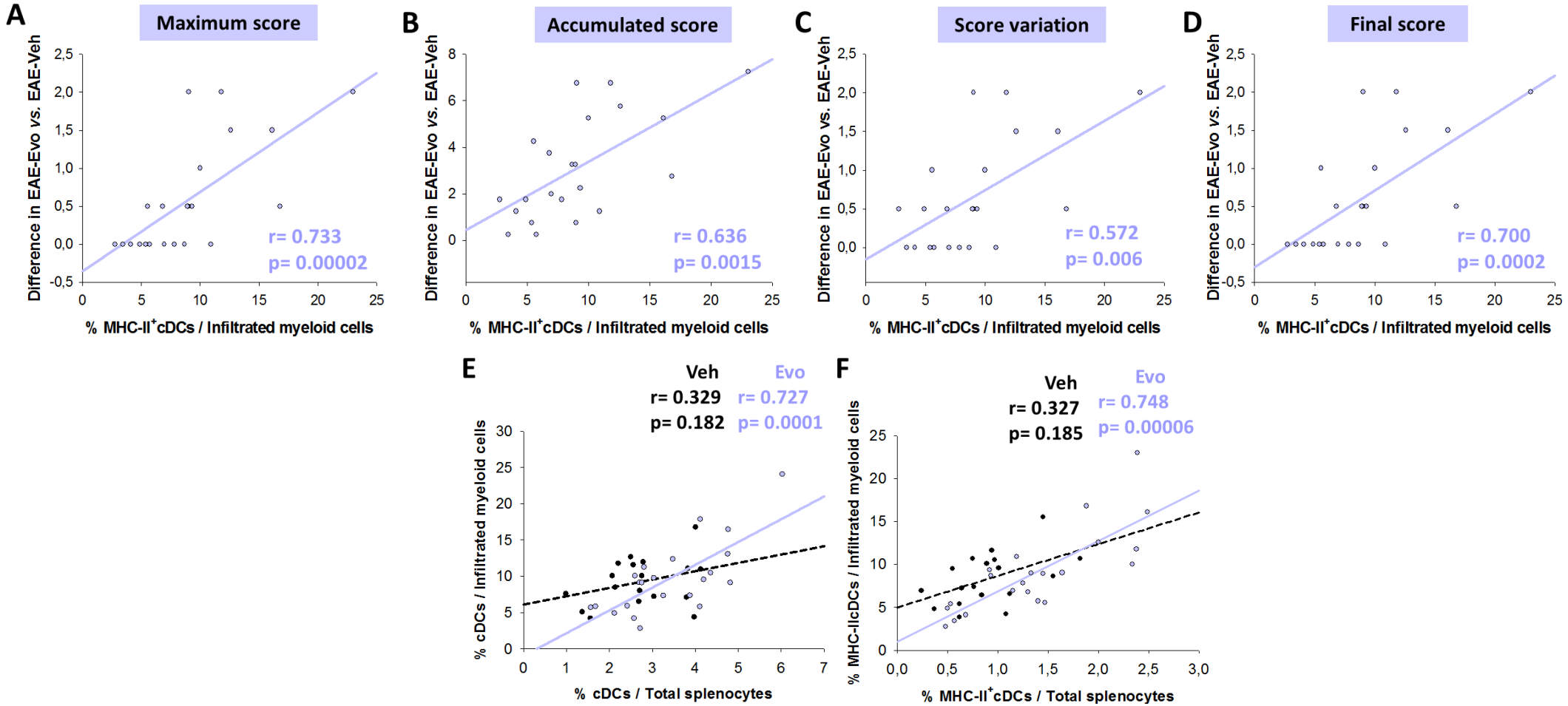

Figure 7. Evobrutinib-mediated mature cDC enrichment is related to a milder clinical score and a higher splenic content. A-D: The higher presence of infiltrated mature cDCs in the spinal cord of EAE-Evo mice, the more they differed from the mean values of clinical signs exhibited by EAE-Veh mice. **E-F:**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

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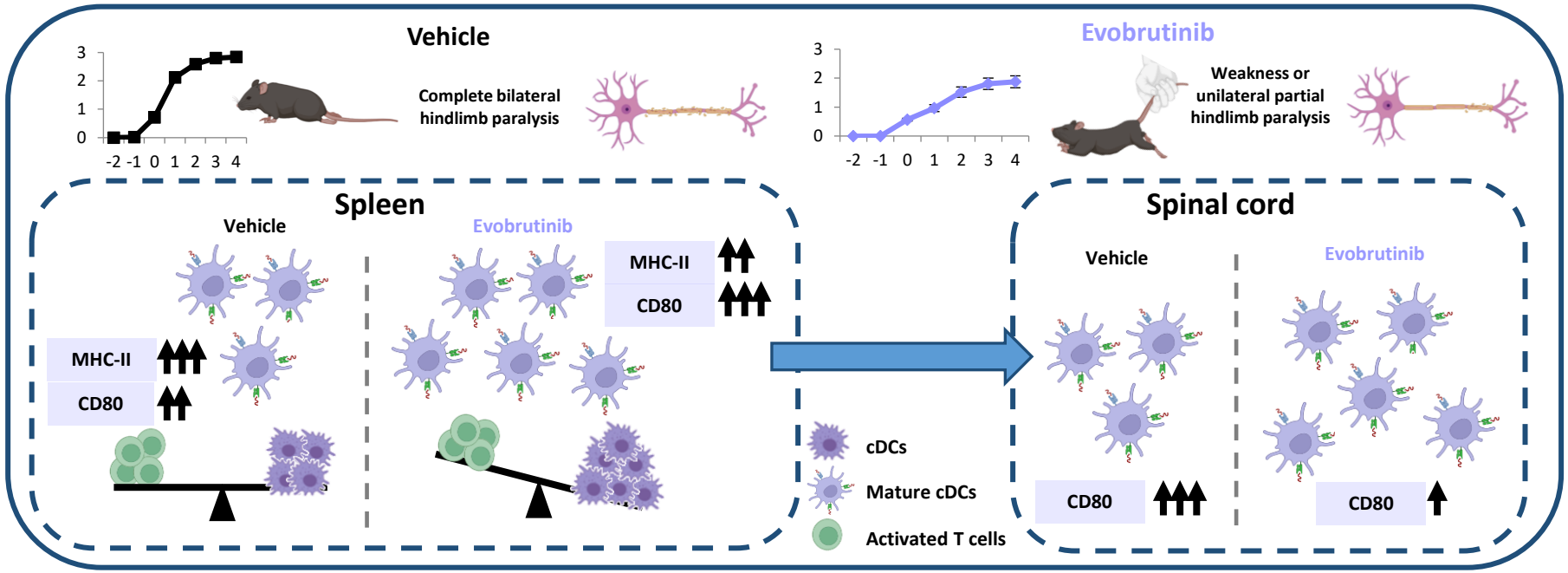
CONCLUSIONS

1. Evobrutinib treatment induced a milder EAE clinical course and 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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