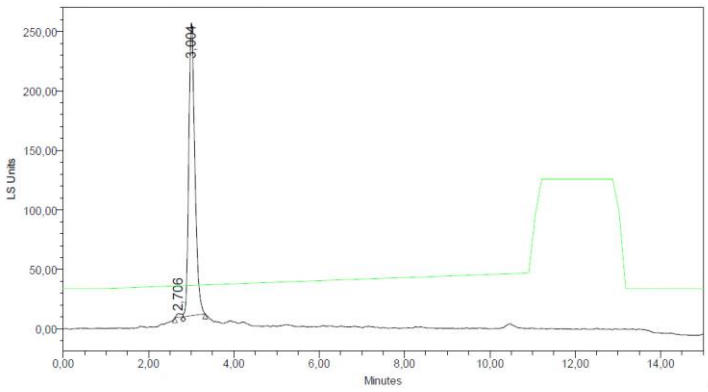
Supplementary Material

Supplementary Methods:

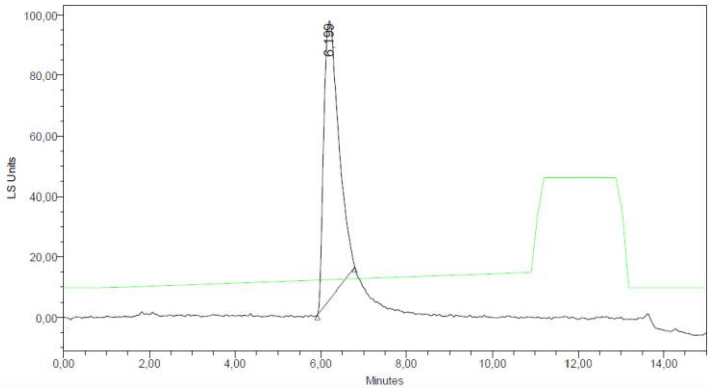
A) RT-HPLC and MALDI-ToF-MS analyses of synthesized pMOG-PEG20

RT-HPLC before coupling:

MOG-Cys-35-55 in water

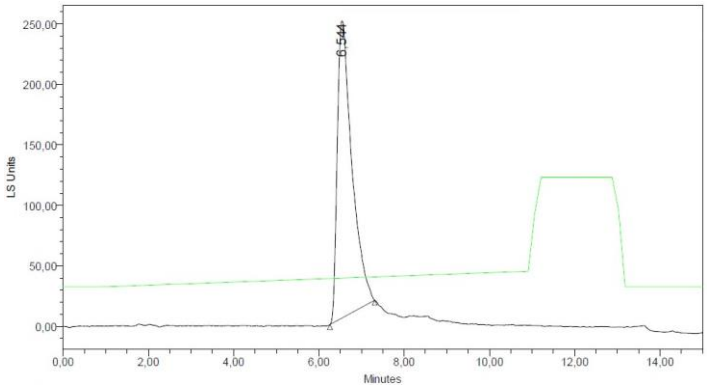


20kDa mPEG-maleimid (Sunbright ME-200MA0B)

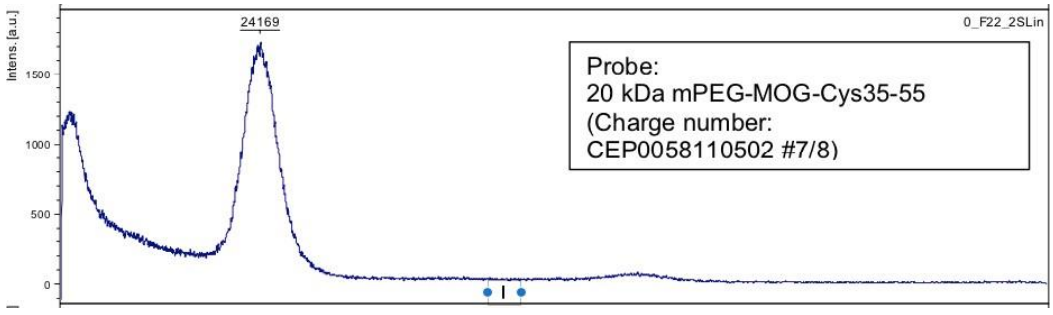


RT-HPLC after coupling and purification:

20 kDa mPEG-MOG-Cys35-55:



MALDI-ToF-MS **of** 20 kDa mPEG-MOG-Cys35-55 after coupling and purification



B) The SJLxB10.PL model.

Mice

SJLxB10.PL F1 mice were crossed in the breeding facility of the Deutsches Rheuma-Forschungszentrum Berlin (DRFZ). Mice were maintained under specific pathogen-free conditions according to national and institutional guidelines. All experiments were approved by the Landesamt für Gesundheit und Soziales (LAGeSo).

Peptides

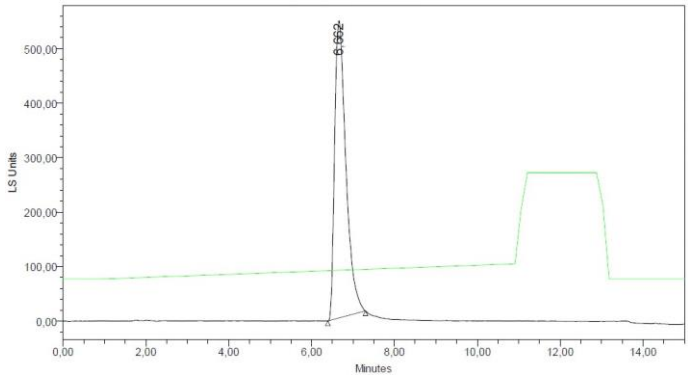
The peptides MBP_{Ac1-9} (Ac-ASQKRPSQR) and $pPLP_{139-154}$ (HCLGKWLGHPDKFVGI) were synthesized in house (Institute for Medical Immunology, Charité Universitätsmedizin Berlin, Germany).

Synthesis of $pPLP_{139-154}$-PEG20

PEG20 was coupled to C_{140} of pPLP139-154. All other procedures were as described for pMOG-PEG20 in the main text.

RP-HPLC

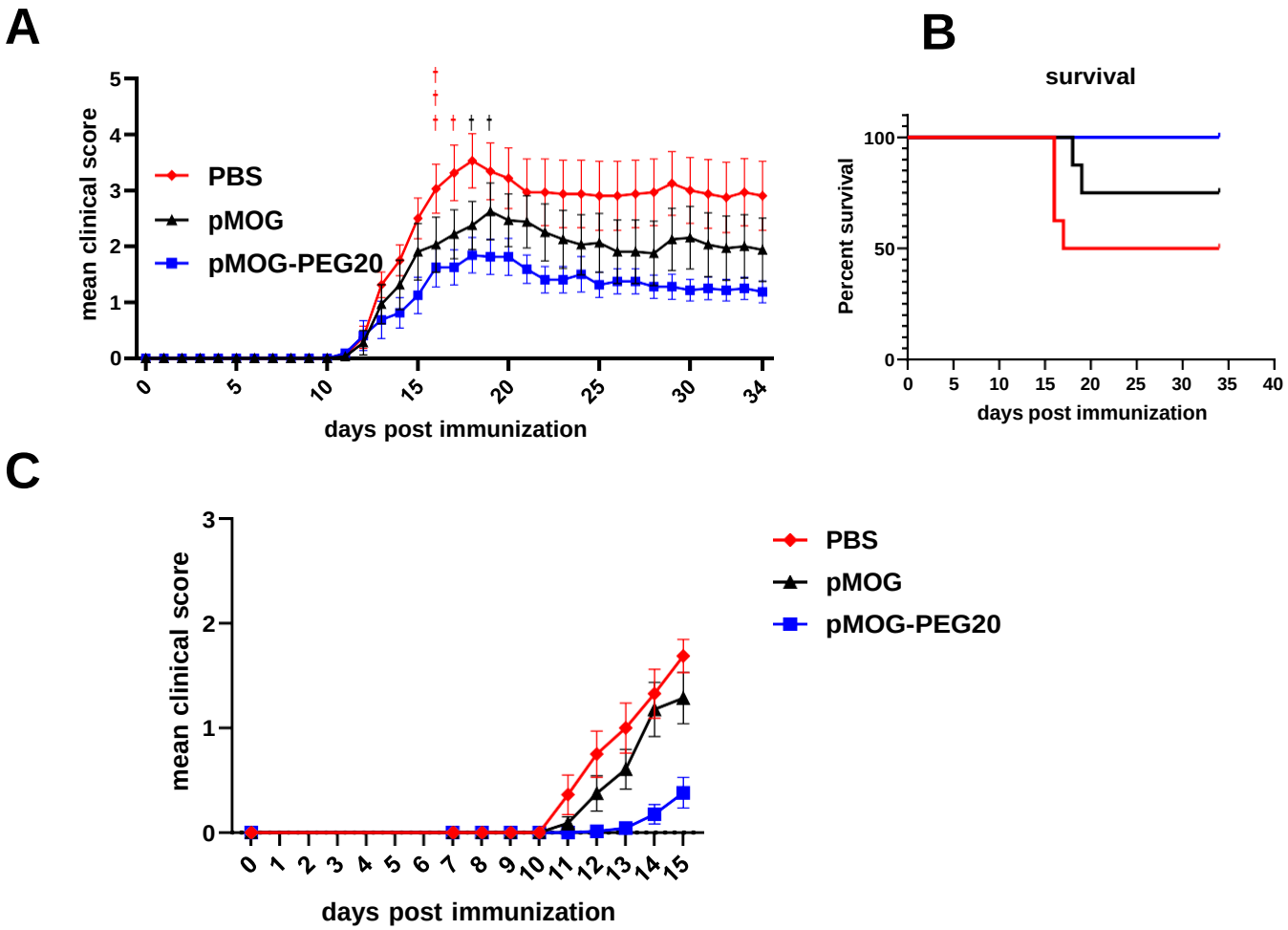
20 kDa mPEG-PLP139-154, after coupling and purification



Induction of relapsing-remitting EAE in SJLxB10.PL mice and treatment protocol

RR-EAE was induced according to Miller et al., 2010 by *s.c.* immunization of SJLxB10.PL mice with 100 µg MBP_{Ac1-9}-peptide in CFA (Difco, Heidelberg, Germany) supplemented with *Mycobacterium tuberculosis* 0.8 mg H37Ra on day 0**.** In addition, mice received 200 ng *Bordetella pertussis* (List Biological Laboratories, Campbell, USA) *i.p.* on day 0 and day 2, respectively.

Supplementary Figures:



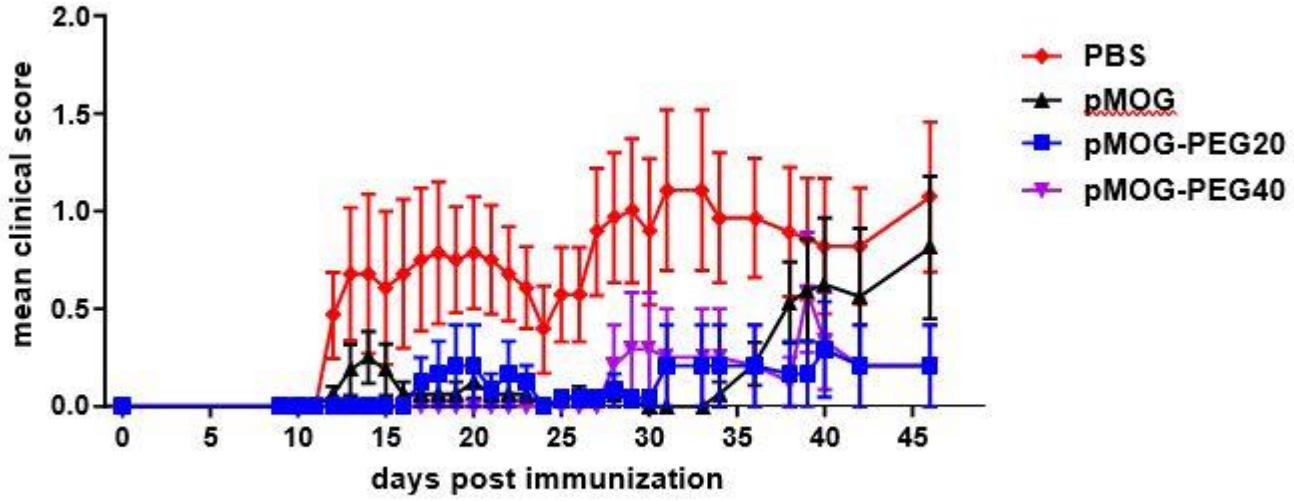
Supplementary Figure S1. Improved tolerogenicity of pMOG-PEG20 compared to unconjugated peptide in EAE. **Repetitions of experiments, relating to Fig. 1 of the paper.** EAE was induced by *s.c.* immunization of C57BL/6 mice with $pMOG_{35-55}$ in complete Freund´s adjuvans (CFA) containing *Mycobacterium tuberculosis* H37Ra on day 0**.** In addition, mice received *Bordetella pertussis i.p.* on day 0 and day 2. 7 days prior to EAE induction mice were tolerized with PBS (control), 7.6 µg pMOG or equimolar amounts (based on peptide amount) of pMOG-PEG20. Individual animals were observed every day, and clinical scores were assessed as an accumulative score. Mean clinical score per group ± SEM.

A) 8 animals /group. In this experiment, the clinical course was unusual severe, and several deaths were encountered. Died animals or animals removed for euthanasia because of animal protection rules were continuingly considered by their last score (5 for dead, 4 for euthanized animals).

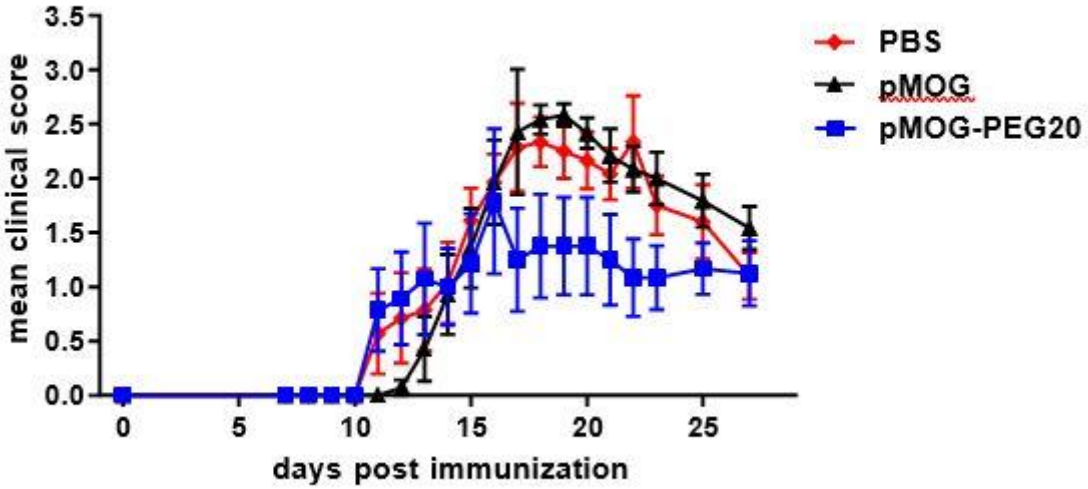
B) Effect of tolerization on survival (death or human endpoint) in the above experiment. 4 of 8 animals died in the PBS control group, 2 animals died upon tolerization with pMOG, and none upon tolerization with pMOG.PEG20. All groups are significantly different with p=0.04 (Gehan-Breslow-Wilcoxon test; Prism 8).

C) Further short-term experiments (used for cellular analyses at peak of disease). Pooled data of 3 independent experiments. In total n= 16 (PBS), 14 (pMOG), 17 (pMOG-PEG20) animals.

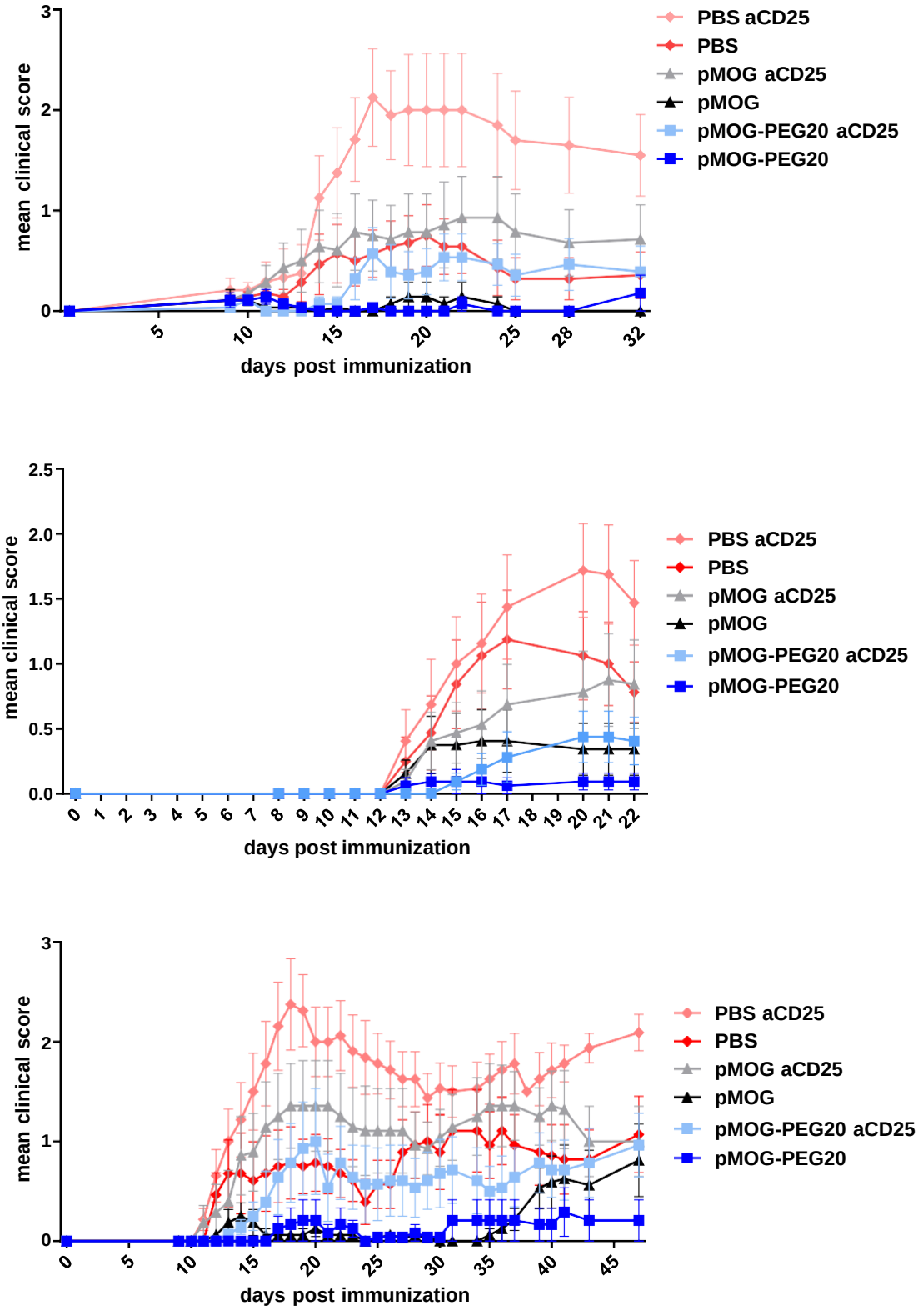
Further analogous data (only difference: tolerization at day -14 instead day -7) **can be found as part of Fig. 2 main paper, and Fig. S4 in this supplement.**



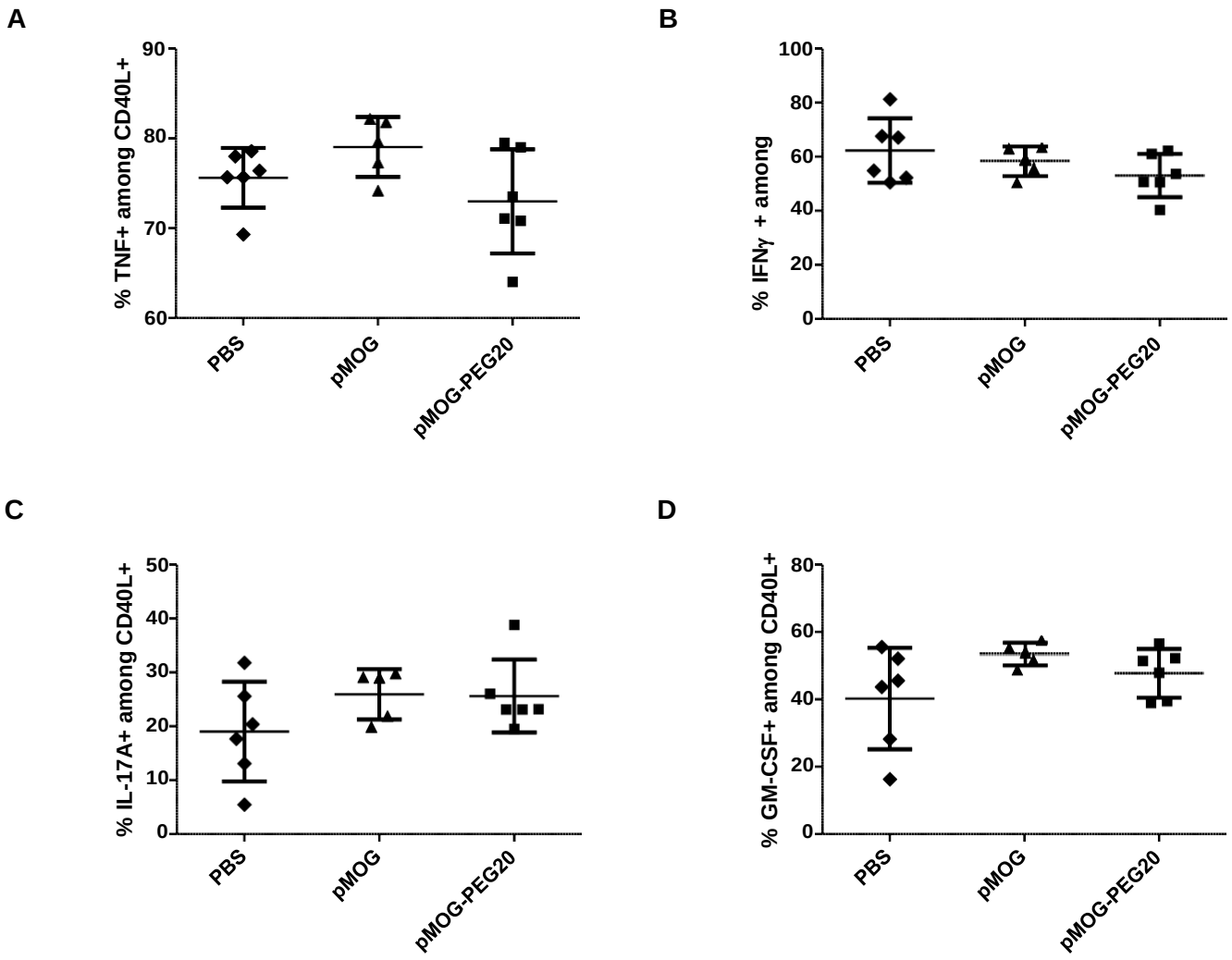
Supplementary Figure S2. pMOG-PEG40 also ameliorates EAE symptoms. 14 days prior to EAE induction mice were tolerized with PBS (control), 7.6 µg pMOG, equimolar amounts of pMOG-PEG20 or equimolar amounts of pMOG-PEG40. Mean clinical score per group ± SEM is shown (n = 6-8 per group). One representative of 2 independent experiments is shown.



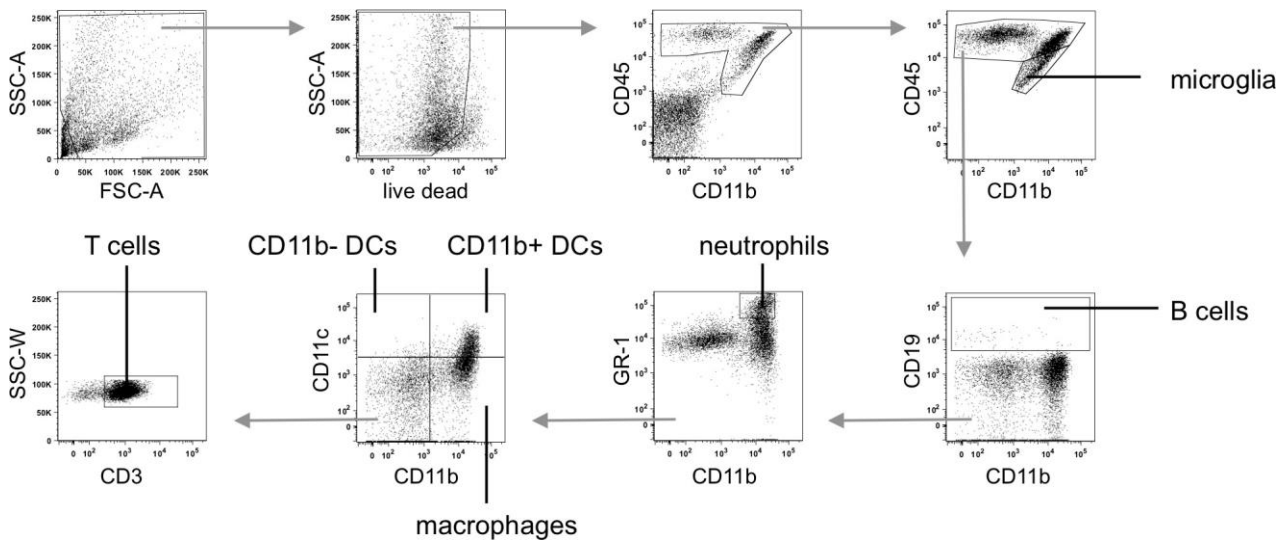
Supplementary Figure S3. Reduced protection by pMOG-PEG20 upon administration 4 weeks prior to EAE induction. 28 days prior to EAE induction, C57BL/6 mice were tolerized *i.v.* with PBS (control), 7.6 μg pMOG or equimolar amounts of pMOG-PEG20. Mean clinical score per group ± SEM is shown (n = 7 animals). One representative of 2 independent experiments is shown.



Supplementary Figure S4. Tregs are involved in protective tolerance induced by pMOG-PEG20. **Repetitions of experiments, relating to Fig. 2 of the paper.** 14 days prior to EAE induction, C57BL/6 mice were tolerized with PBS (control), 7.6 µg pMOG or equimolar amounts of pMOG-PEG20. After 7 days mice received either 500µg anti-CD25 (PC61) antibody or PBS (control) *i.p.*. EAE was induced as described above. Mean clinical score per group ± SEM, n= 6-8 animals per group.

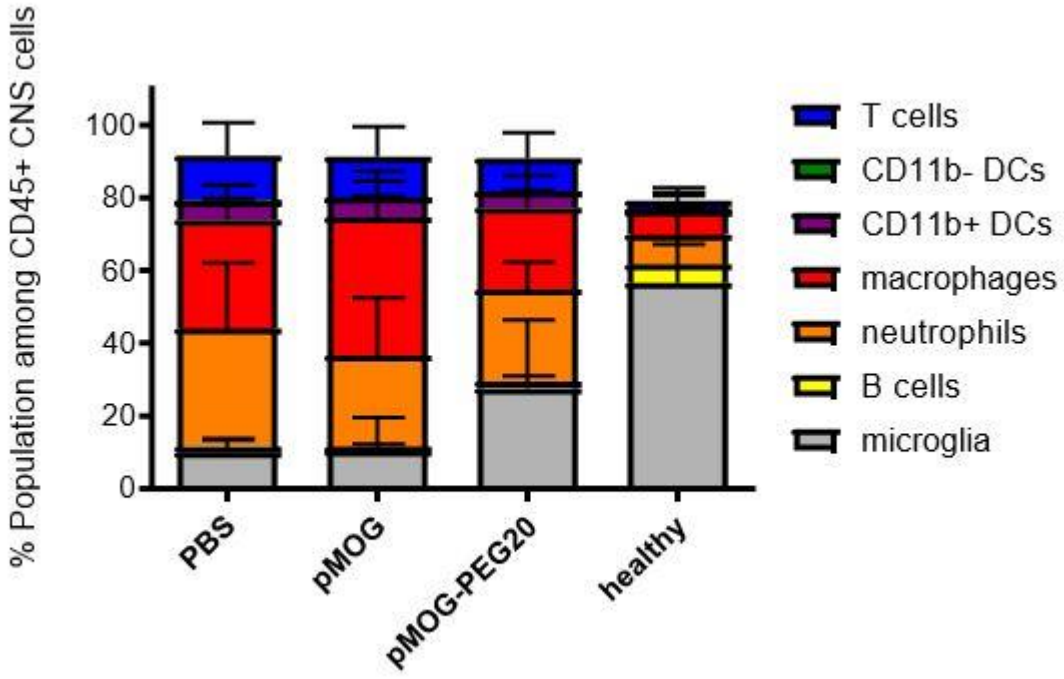


Supplementary Figure S5. At the peak of disease, frequencies of proinflammatory cytokine producing CD4$^+$ cells are not affected by pMOG-PEG20. C57BL/6 mice were tolerized *i.v.* with PBS (control), 7.6 μg pMOG or an equimolar amount of pMOG-PEG20 7 days prior to EAE induction. Animals were sacrificed at the peak of disease (d15 post EAE induction). Splenocytes were re-stimulated overnight with pMOG and analyzed by flow cytometry. Data represent mean of (**A**) % TNF$^+$, (**B**) % IFN-γ$^+$, (**C**) % IL-17A$^+$ and (**D**) % GM-CSF$^+$ splenocytes among CD4$^+$ CD40L$^+$ (MOG-specific) cells ± SD (n = 5–6 animals per group). None of the differences are statistically significant (Mann-Whitney U test). One representative of 2 independent experiments is shown.



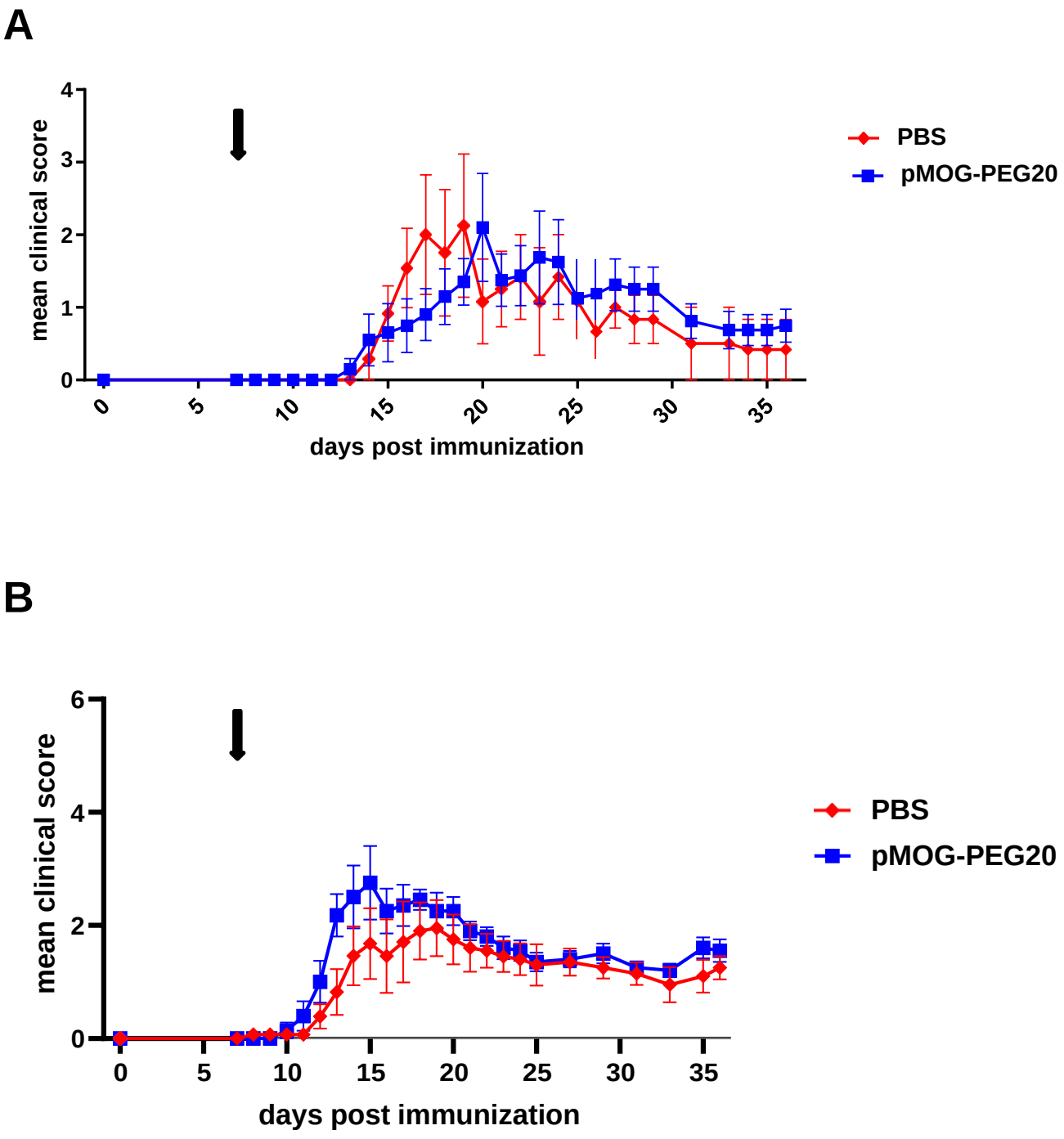
Supplementary Figure S6. Sequential gating strategy used for the identification of leukocyte subsets in the CNS: microglia ($CD45^{int}$ $CD11b^{+}$), neutrophils ($CD45^{high}$ $GR1^{high}$ $CD19^{-}$), $CD11b^{-}$ DCs ($CD45^{high}$ $GR1^{-}$ $CD11c^{+}$ $CD11b^{-}$ $CD19^{-}$), $CD11b^{+}$ DCs ($CD45^{high}$ $GR1^{-}$ $CD11c^{+}$ $CD11b^{+}$ $CD19^{-}$), macrophages ($CD45^{high}$ $GR1^{-}$ $CD11c^{-}$ $CD11b^{+}$ $CD19^{-}$), B cells ($CD45^{high}$ $CD19^{+}$), and T cells ($CD45^{high}$ $GR1^{-}$ $CD11c^{-}$ $CD11b^{-}$ $CD19^{-}$ $CD3^{+}$).

A

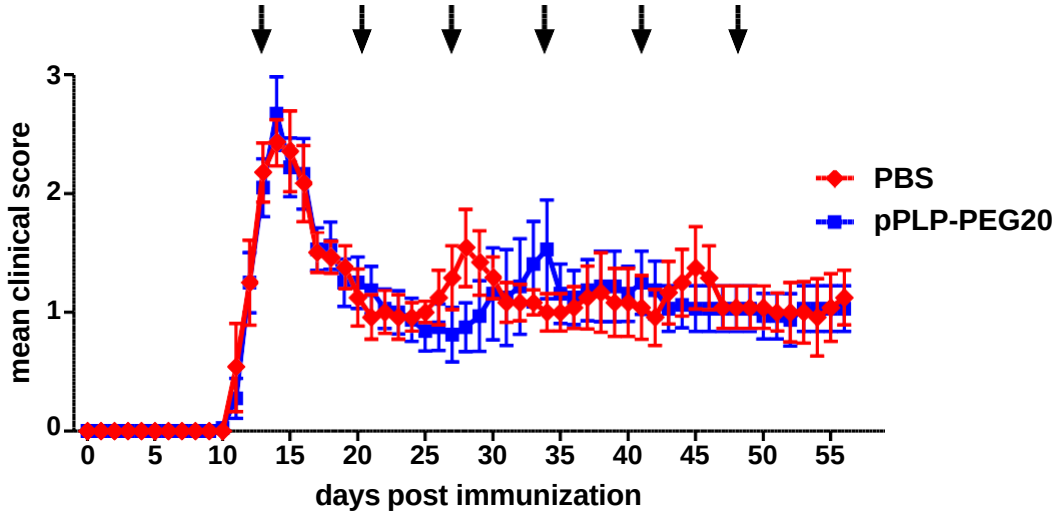


Supplementary Figure S7. Frequency of cell populations under various conditions. These data complement those of Figure 4 in main text.

Relative proportion of the analyzed subsets within $CD45^+$ cells isolated from the CNS. Frequencies at the peak of the disease (d15) are depicted as mean ± SD (n = 6-12 animals). Data is summarized from 2 independent experiments. For statistical analysis see Table 2 below.



Supplementary Figure S8. Therapeutic administration of pMOG-PEG20 does not prevent EAE development, but also does not lead to a significant exacerbation of the disease. **Repetitions of experiments, relating to Fig. 5 of the paper.** 7 days post EAE induction, C57BL/6 mice received PBS (control) or pMOG-PEG20 (equivalent to 7.6 µg pMOG). Mean clinical score per group ± SEM (A: PBS n = 6; pMOG-PEG20 n = 5; B: n= 7).



Supplementary Figure S9. Repetitive treatment with pPLP$_{139\text{-}154}$-PEG20 slightly delays, but does not prevent the pPLP$_{139\text{-}154}$-specific relapse. RR-EAE is induced by *s.c.* immunization of SJLxB10.PL mice with MBP$_{Ac1\text{-}9}$-peptide. Accordingly, in this model the first peak is MBP-specific while secondary peaks in the chronic phase result from epitope spreading involving PLP epitopes to which tolerization was intended. MBP$_{Ac1\text{-}9}$-peptide in CFA is injected on day 0**.** In addition, mice received *Bordetella pertussis i.p.* on day 0 and day 2, respectively. Mice were repetitively treated with PBS (control) or 50 µg (based on peptide amount) pPLP-PEG20 weekly from day 13 (arrows). Mean clinical score per group ± SEM (PBS n = 7; pPLP-PEG20 n = 10). Data shown are from one experiment.

Supplementary Tables:

Comparison	adj. p.Value
p (PBS vs. PBS-aCD25)	0.013
p (PBS vs. pMOG)	0.001
p (PBS vs. pMOG-aCD25)	<0.001
p (PBS vs. pMOG-PEG20)	<0.001
p (PBS vs. pMOG-PEG20-aCD25)	0.001
p (PBS-aCD25 vs. pMOG)	<0.001
p (PBS-aCD25 vs. pMOG-aCD25)	0.222
p (PBS-aCD25 vs. pMOG-PEG20)	<0.001
p (PBS-aCD25 vs. pMOG-PEG20-aCD25)	1.000
p (pMOG vs. pMOG-aCD25)	<0.001
p (pMOG vs. pMOG-PEG20)	<0.001
p (pMOG vs. pMOG-PEG20-aCD25)	<0.001
p (pMOG-aCD25 vs. pMOG-PEG20)	<0.001
p (pMOG-aCD25 vs. pMOG-PEG20-aCD25)	0.348

Supplementary Table 1. Statistical analysis of differences between various treatments in Figure 2. Adjusted p-values were determined by nonparametric comparison of relative contrast effects.

population	PBS vs pMOG	PBS vs pMOG PEG	PBS vs healthy	pMOG vs pMOG PEG	pMOG vs healthy	pMOG PEG vs healthy
# CD45+	ns	*	**	*	**	ns
# microglia	ns	ns	ns	ns	ns	ns
# B cells	ns	ns	ns	ns	ns	ns
# neutrophils	ns	**	**	ns	*	*
# macrophages	ns	**	**	*	***	*
# CD11b+ DCs	ns	*	***	*	***	**
# CD11- DCs	ns	*	ns	ns	ns	ns
# T cells	ns	ns	**	ns	**	*
% CD45+	ns	ns	ns	ns	ns	ns
% microglia	ns	*	**	*	**	*
% B cells	ns	ns	ns	ns	*	ns
% neutrophils	ns	ns	**	ns	ns	**
% macrophages	ns	ns	**	**	**	*
% CD11b+ DCs	ns	ns	**	*	***	**
% CD11b- DCs	ns	ns	ns	ns	ns	ns
% T cells	ns	ns	*	ns	*	*

Supplementary Table2. Statistical analysis of cell count data (Figure 5 main text and S7 in Supplement). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. P values were determined by unpaired non-parametric Mann-Whitney U test.