

Supplementary Materials for

Identification of four novel T cell autoantigens and personal autoreactive profiles in multiple sclerosis

Mattias Bronge*, Klara Asplund Högelin, Olivia G. Thomas, Sabrina Ruhrmann, Claudia Carvalho-Queiroz, Ola B. Nilsson, Andreas Kaiser, Manuel Zeitelhofer, Erik Holmgren, Mathias Linnerbauer, Milena Z. Adzemovic, Cecilia Hellström, Ivan Jelcic, Hao Liu, Peter Nilsson, Jan Hillert, Lou Brundin, Katharina Fink, Ingrid Kockum, Katarina Tengvall, Roland Martin, Hanna Tegel, Torbjörn Gräslund, Faiez Al Nimer, André Ortlieb Guerreiro-Cacais, Mohsen Khademi, Guro Gafvelin, Tomas Olsson, Hans Grönlund

*Corresponding author. Email: mattias.bronge@ki.se

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Other Supplementary Material for this manuscript includes the following:

Data S1 to S8

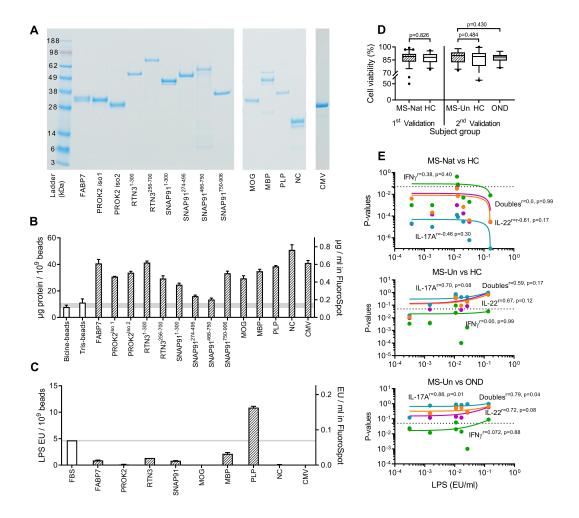


Fig. S1. Full-length antigen panel quality control. A, SDS-page gels of the full-length antigens used in the FluoroSpot-assay validations. Molecular size ladder with size indicators in the leftmost lane. Picture derived from three different gels, each containing a lane with the same ladder (cropped out) and the bands' locations in relation to the ladder are consistent. Brightness, contrast, and colour temperature corrections have been made. B, The protein content of the final, endotoxin-washed antigen beads compared to beads coupled with bicine and tris. Bars represent the mean and staples the SD of the intra-assay replicates. Bars are plotted on both the left and right y-axis (resulting protein concentration in the FluoroSpot-assay). The horizontal gray line represents the negative control results. C, LPS concentration of the finished, endotoxin-washed, and pooled antigen-beads and the fetal bovine serum (FBS) used in the cell media. Bars represent mean and staples SD of the intra-assay replicates. LPS content in antigen-bead preparations is plotted on both the left (endotoxin units (EU) per 10⁹ beads) and the right y-axis (resulting EU per ml in the FluoroSpot assay). FBS is only plotted on the right y-axis. The horizontal gray line represents the LPS (EU/ml) of the FBS. **D**, Comparison of the different cohorts' PBMC viability after thawing. Box represents median and IQR and brackets the 5-95 percentile. P-values calculated using a two-tailed Mann-Whitney U-test. E, Correlation of the p-values of the difference in autoantigen responses for the 1st (top graph) and 2nd (bottom graphs) validation runs and autoantigen LPS content. IFNy in green, IL-22 in orange, IL-17A in blue, and doublecytokines in magenta. Lines represent linear regression slopes. Dotted line at p=0.05. P- and rvalues were calculated using two-tailed non-parametric Spearman-correlation.

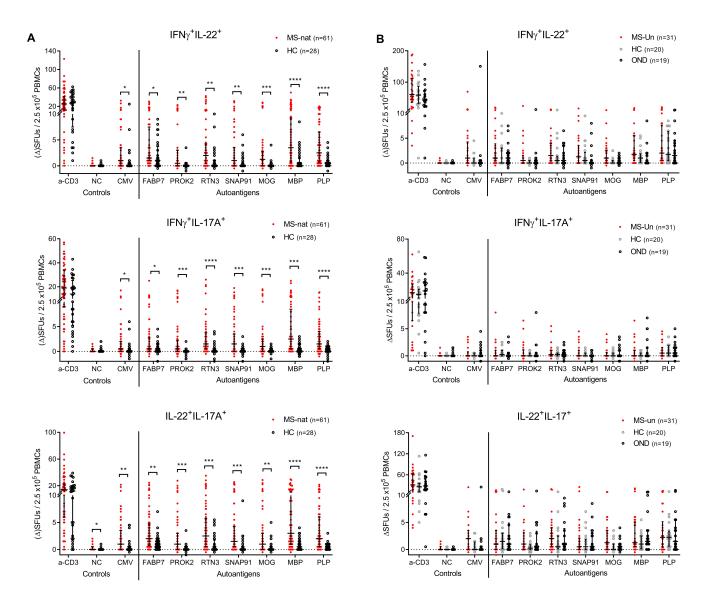


Fig. S2. Double cytokine-producing cells. Double cytokine-producing cells in the IFN γ /IL-22/IL-17A FluoroSpot assay. **A**, Results from the 1st validation cohort, using natalizumab-treated persons with MS. Each dot represents one individual with brackets representing median and IQR. The results from the anti-CD3 positive control stimulation are plotted as ΔSFUs / 1.25 x10⁵ PBMCs and NC are plotted as raw SFUs / 2.5 x10⁵ PBMCs. P-values were calculated with a two-tailed Mann-Whitney U-test with Bonferroni correction for multiple comparisons (n=10). *p<0.05, **p<0.01, ***p<0.001. **B**, Results from the 2nd validation cohort, using untreated persons with MS, presented as in (**A**).

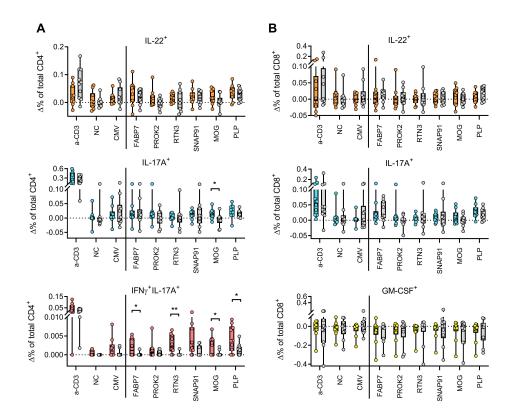


Fig. S3. Intracellular cytokine staining after autoantigen stimulation. Flow cytometry analysis of autoreactive T cells using intracellular staining after autoantigen stimulation. **A**, IL-22, IL-17A, and double-positive IFN γ ⁺IL-17A⁺ responses in CD4⁺ T cells after stimulation with autoantigens or control antigens. **B**, IL-22, IL-17A, and GM-CSF responses in CD8⁺ T cells. Plotted as % of the total population after subtracting background responses (no stimuli, Δ%). Boxes represent median and IQR. MS (n=10) in color, HC (n=10) in gray open circles. P-values calculated using a two-tailed Mann-Whitney U-test and written when significant. *p<0.05, **p<0.01.

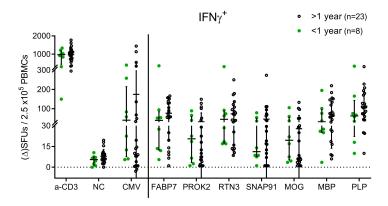


Fig. S4. Short versus long disease duration. IFN γ responses of untreated MS persons, stratified based on first known symptoms within one year of sampling (<1 year, mean and SD 0.49 \pm 0.18 years) or longer disease duration (>1 year, mean and SD 4.9 \pm 3.3 years). P-values were calculated using two-tailed Mann-Whitney U-tests and none were significant (p=0.22-0.94 in all cases).

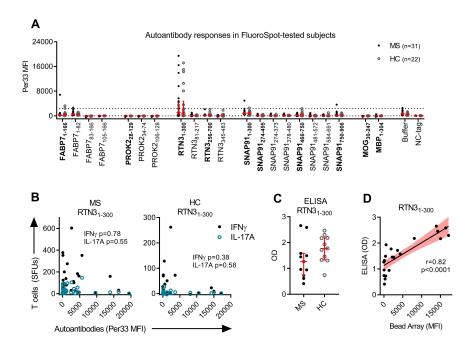


Fig. S5. Autoantibody and T cell correlation, and RTN3 validation. A, Results from the suspension bead array autoantibody detection in pwMS (n=31) and HC (n=22) which had also been tested for T cell reactivity using the FluoroSpot assay in the 1st validation cohort. Both full length antigens used in the FluoroSpot assay (x-axis, bold) and PrESTs (x-axis, not bold) were tested. The number denotes the amino acids covered in the protein. Dotted line at the threshold for positivity (MFI=2350). Brackets denote median and IQR. **B**, Correlation analysis between the suspension bead array (**A**) and FluoroSpot IFNγ (black filled circles) and IL-17A (blue open circles) responses to RTN3₁₋₃₀₀ for pwMS (left graph) and HCs (right graph). P-values calculated using a two-tailed non-parametric Spearman correlation. **C**, Enzyme-linked immunosorbent assay (ELISA) detecting autoantibodies to a second version of RTN3₁₋₃₀₀ which did not contain the NC-tag contained in the previous RTN3₁₋₃₀₀ version, testing both pwMS and HC (n=11 in each group) also tested in (**A**). **D**, Correlation of the results from ELISA (OD, y-axis) and values were calculated using a two-tailed non-parametric Spearman correlation. MFIs: Median fluorescent intensity.

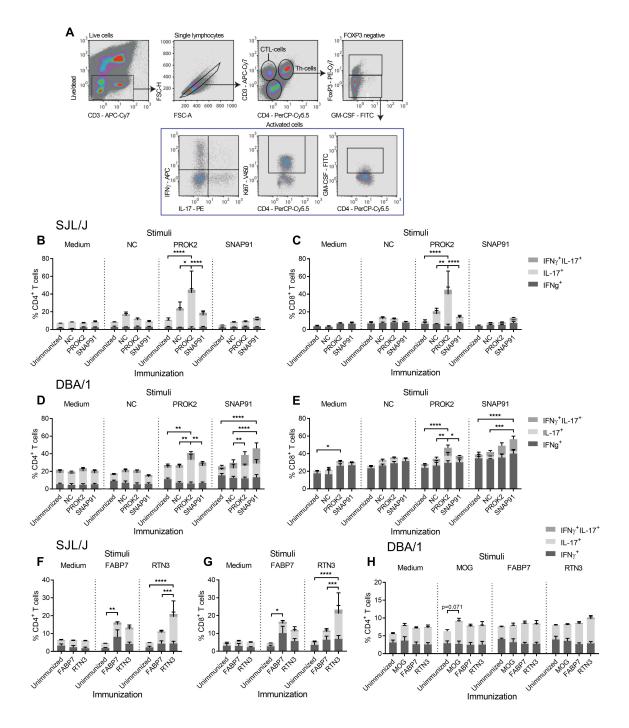


Fig. S6. Immune responses in autoantigen-immunized mice. Recall responses to the novel autoantigens in immunized mice. **A**, Gating strategy of cytokine responses. **B** and **C**, CD4⁺ and CD8⁺ T cell responses to medium only, NC, PROK2 and SNAP91 in unimmunized (n=3), NC (n=3), PROK2 (n=6) and SNAP91 (n=6) immunized SJL/J mice. **D** and **E**, Responses to medium only, NC, PROK2 and SNAP91 in unimmunized (n=3), NC- (n=3), PROK2- (n=6) and SNAP91- (n=6) immunized DBA/1 mice. **F** and **G**, Responses to medium only, FABP7 and RTN3 in unimmunized (n=3), FABP7 (n=7), and RTN3 (n=7) immunized SJL/J mice. NC was omitted as versions of FABP7 and RTN3 without the NC-tag were used for both immunisation and stimuli. **H**, CD4⁺ responses to medium only, MOG, FABP7 and RTN3 in unimmunized

(n=3), MOG (n=3), FABP7 (n=8) and RTN3 (n=8) immunized DBA/1 mice. Stacked bar graphs represent the mean ±SD cytokine positive cells as % of the total CD4⁺ or CD8⁺ cell population. The different cytokine responses are stacked on top of each other. X-axes denote the immunisation and dotted lines separate the different stimuli used ex vivo. P-values calculated using a two-tailed ANOVA with Tukey's correction for multiple comparisons and written when significant. ANOVA test based on the total % of cytokine-producing cells, not individual cytokines. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

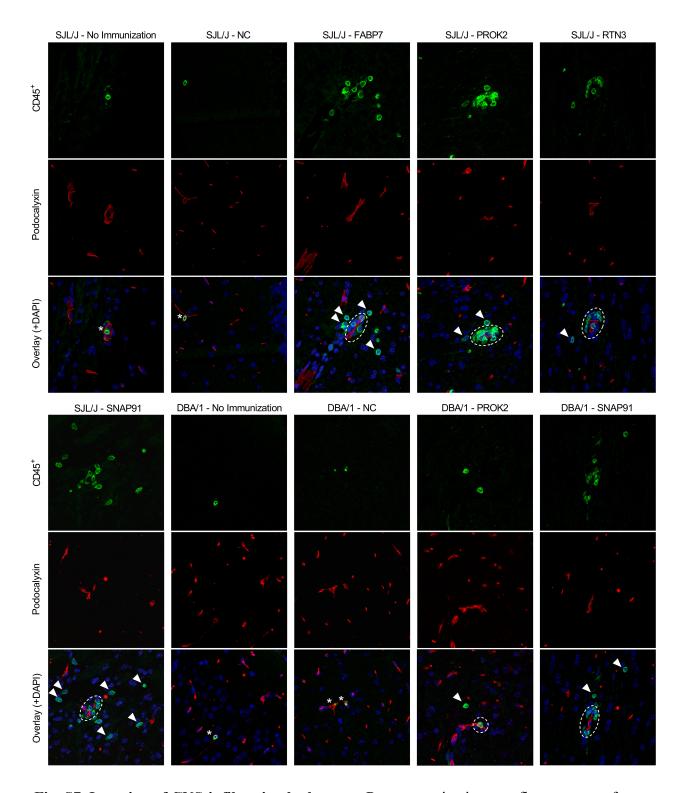


Fig. S7. Location of CNS-infiltrating leukocytes. Representative immunofluorescence of infiltrating leukocytes in the brain of control or autoantigen immunized mice. Top image shows leukocytes (CD45⁺, green), middle image blood vessels (Podocalyxin, red) and the bottom image the overlaid image together with cell nuclei (DAPI, blue). Arrowheads indicate intraparenchymal leukocytes, dotted white circles indicate perivascular leukocytes and asterixis indicate intravascular leukocytes.

Table S1. Autoantigen screening panel

MBP	Protein	PrEST	Pool#	•	Protein	PrEST	Pool#
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Table S2. Cohort characteristics

	Screening	Screening cohort		1 st Validation		2 nd Validation	
	MS-Nat (RRMS) (n=16)	HCs (n=9)	MS-Nat (RRMS) (n=61)	HCs (n=28)	MS-Un (RRMS) (n=31)	HCs (n=20)	OND (n=19)
Age (years)	42.1 ± 5.8 (32-53)	40.7 ± 11.1 (24-57)	36.3 ± 9.7 (17-61)	33.9 ± 8.2 (20-49)	34.2 ± 9.7 (19-58)	33.3 ± 6.8 (25-49)	29.3 ± 10.5 (18-57)
Female sex (n)	7 (38.9%)	5 (55.6%)	44 (72.1%)	21 (75.0%)	23 (74.2%)	15 (75.0%)	9 (47.4%)
HLA- DRB1*15:01 (n)	10 (62.5%)	Missing	28 / 56 (50.0%)	7 / 22 (31.8%)	11 / 23 (47.8%)	3 / 12 (25%)	10 / 13* (76.9 %)
HLA- A*02:01 (n)	8 (50.0%)	Missing	24 / 56 (42.9%)	14 / 22 (63.6%)	10 / 23 (43.5%)	7 / 12 (58.3%)	Missing
EDSS (score)	2.5 ± 1.6 (1–5.5)	-	2.1 ± 1.5 (0.0–7.5)	-	1.5 ± 1.3 (0.0–6.5)	-	-
Disease Duration (years)	11.1 ± 5.2 (2.5–20.7)	-	8.9 ± 5.4 (1-20)	-	2.1 ± 3.1 (0.0–12.7)	-	-

 $[\]pm$ denotes SD. Range or percentage of whole in brackets. n / N denotes the number of individuals for which data was available if it was not the whole cohort. *DR15-status in two cases based on the high linkage disequilibrium with DQB1*06:02. RRMS: Relapsing-remitting MS. HCs: Healthy Controls. Nat: Natalizumab-treated. Un: Untreated. EDSS: Expanded Disability Status Scale.

Table S3. Full-length autoantigen panel

Antigen	Function	Protein description	UniProt ID	Endotoxin wash
Anti-CD3	Positive Control – Polyclonal T cell activation	Monoclonal CD3-2 antibody, Mouse IgG2a isotype.	N/A	N/A
NC	Negative Control – Tag for production	Albumin binding domain – Derived from Strep. Protein G. (69)	N/A	0.1 M NaOH
CMV	Positive Control – Cytomegalovirus epitopes	Fusion protein of 5 epitopes from PP65. (70)	N/A	0.75 M NaOH
FABP7	Novel Candidate Autoantigen	Fatty Acid Binding Protein 7 – Isoform 2 ¹⁻¹⁶⁶	O15540-2	0.5 M NaOH
PROK2	Novel Candidate Autoantigen	Prokineticin-2 – Isoform 1 ²⁸⁻¹²⁹	Q9HC23-1	1 M NaOH
		Prokineticin-2 – Isoform 2 ²⁸⁻¹⁰⁸	Q9HC23-2	2 M NaOH + 1% Triton X-100
RTN3	Novel Candidate Autoantigen	Reticulon-3 ¹⁻³⁰⁰	O95197	0.5 M NaOH
		Reticulon-3 ²⁵⁶⁻⁷⁰⁰	O95197	2 M NaOH
SNAP91	Novel Candidate Autoantigen	Synaptosome Associated Protein 91 ¹⁻³⁰⁰	O60641	3 M NaOH + 1% Triton X-100
		Synaptosome Associated Protein 91 ²⁷⁴⁻⁴⁹⁵	O60641	0.1 M NaOH
		Synaptosome Associated Protein 91 ⁴⁶⁶⁻⁷⁵⁰	O60641	1 M NaOH
		Synaptosome Associated Protein 91 ⁷⁵⁰⁻⁹⁰⁶	O60641	0.1 M NaOH
MOG	Established MS Autoantigen	Myelin Oligodendrocyte Glycoprotein ³⁰⁻²⁴⁷	Q16653	2 M NaOH + 1% Triton X-100
MBP	Established MS Autoantigen	Myelin Basic Protein ¹⁻³⁰⁴	P02686-1	1 M NaOH
PLP	Established MS Autoantigen	Proteolipid Protein ³⁷⁻⁶³⁺⁸⁹⁻ 151+178-233+261-277	P60201	2 M NaOH + 1% Triton X-100

Data S1.

Source and complete statistical data for Fig. 1

Data S2.

Source and complete statistical data for Fig. 2

Data S3.

Source and complete statistical data for Fig. 3

Data S4.

Source and complete statistical data for Fig. 4

Data S5.

Source and complete statistical data for Fig. 5

Data S6.

Source and complete statistical data for Fig. 6

Data S7.

Source and complete statistical data for Fig. 7

Data S8.

Source data for Fig. S1-S6