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Extended Data and Supplementary Materials for:

GM-CSF and CXCR4 Define a T Helper Cell Signature in Multiple Sclerosis

This PDF file includes:

Captions of Extended Data Figs. 1 to 10
Captions for Tables S1 to S8

Other Supplementary Materials for this manuscript includes the following:

Tables S1 to S8

Extended Data Fig.1. Single-cell t-SNE profiling of immune cells. PBMCs from all sample groups were restimulated with PMA/ionomycin and analyzed by mass cytometry. The tSNE algorithm (30,000 cells, equally selected from HD (n = 29) and NINDC patients (n = 31) and MS (n = 31) groups and from all samples) was used to depict different populations therein. (**A**) Expression of each indicated marker is overlaid. (**B**) FlowSOM-based immune cell populations are overlaid as a color dimension.

Extended Data Fig.2. FlowSOM-guided clustering of peripheral blood immune cell lineages. (**A**) PBMCs from HD (n = 29) and NINDC patients (n = 31) and MS (n = 31) were restimulated with PMA/ionomycin and analyzed by mass cytometry. Heatmap of FlowSOM-identified initial nodes and their mean surface marker expression levels, together with their lineage assignment (color-coded). (**B**) Biaxial plots showing the expression of the main lineage markers of FlowSOM-based populations (colored). The total samples from HD (n = 29) and NINDC patients (n = 31) and MS (n = 31) is shown in grey. (**C**) Data as in A was manually gated to define the same populations. Samples from 3 independent runs are analyzed. (**D**) Correlation of frequencies for the immune populations (color-coded) as defined by FlowSOM and manual gating. Each dot represents the frequency of a leukocyte population of one donor (n = 91). P-value was calculated using linear regression. (**E**) Frequencies of immune cell lineages in peripheral leukocytes between NINDC (n = 31) and RRMS patients during remission (n = 18), or during relapse (n = 12), SPMS patients (n = 5), PPMS patients (n = 3) and HD (n = 29). (**F**) Frequencies of cytokine$^+$ cells within PBMCs between NINDC (n = 31) and RRMS patients during remission (n = 18), or during relapse (n = 12), SPMS patients (n = 5), PPMS patients (n = 3) and HD (n = 29). Boxplots depict the interquartile range (IQR) with a horizontal line representing the median. Whiskers extend to the farthest data point within a maximum of 1.5x IQR. Points represent individuals.

Extended Data Fig.3. Age analysis of MS and control groups. (**A**) Boxplots depict the age of patients in HD (n = 29) and NINDC patients (n = 31) and MS (n = 39). (**B**) Age distribution among HD (n = 29) and NINDC patients (n = 31) and MS (n = 39). (**C**) Correlation between frequencies of cytokine producing PBMCs and age. Regression curve with confidence intervals are depicted in HD (n = 29), NINDC (n = 31) or MS (n = 39) groups. Boxplots depict the IQR with a white horizontal line representing the median. Whiskers extend to the farthest data point within a maximum of 1.5x IQR. P values are based on two-tailed Mann-Whitney-Wilcoxon tests between the groups. Controlling for multiple comparisons was accomplished with the Benjamini-Hochberg approach. Every point represents one individual.

Extended Data Fig.4. Leukocyte and cytokine production characterization in MS patient subgroups. (**A**) Exemplary GM-CSF production by total leukocytes with (right)

or without (left) prior PMA/ionomycin stimulation (three independent experiments). (**B**) Frequencies of immune cell lineages within GM-CSF$^+$ cells in NINDC (n = 21) and RRMS patients during remission (n = 17), or during relapse (n = 10), SPMS patients (n = 3), PPMS patients (n = 2) and HD (24). (**C**) Frequency of GM-CSF positive cells in major immune lineages in NINDC (n = 31) and RRMS patients during remission (n = 18), or during relapse (n = 12), SPMS patients (n = 5), PPMS patients (n = 3) and HD (n = 29). (**D**) Frequencies of FlowSOM-based Th memory subpopulations in total Th cells in NINDC (n = 31) and RRMS patients during remission (n = 18), or during relapse (n = 12), SPMS patients (n = 5), PPMS patients (n = 3) and HD (n = 29). (**E**) Frequencies of GM-CSF$^+$ Th cells in patients in NINDC (n = 23) and RRMS patients during remission (n = 18), or during relapse (n = 11), SPMS patients (n = 4), PPMS patients (n = 3) and HD (n = 27). (**F**) Coproduction of other cytokines by GM-CSF$^+$ Th cells in NINDC (n = 23) and RRMS patients during remission (n = 18), or during relapse (n = 11), SPMS patients (n = 4), PPMS patients (n = 3) and HD (n = 27). (**G**) Frequencies of GM-CSF production by cytokine$^+$ Th cells in NINDC (n = 13) and RRMS patients during remission (n = 14), or during relapse (n = 8), SPMS patients (n = 4), PPMS patients (n = 2) and HD (n = 22). (**H**) FlowSOM was used to identify total Th cell subsets based on their cytokine production profile (k = 17, elbow criterion). Clusters were manually annotated based on this profile. Shown are mean expressions of surface and cytokine markers by the respective Th cell subsets. (**I**) Frequencies of FlowSOM defined GM-CSF$^+$ Th cell subsets in NINDC (n = 31) and RRMS patients during remission (n = 19), or during relapse (n = 12), SPMS patients (n = 5), PPMS patients (n = 3) and HD (n = 29). (**J**) The tSNE algorithm (30,000 cytokine-expressing Th cells, equally selected from different clinical groups and from all samples) was used to depict different populations therein. FlowSOM-based Th subsets (left) and expression of each indicated marker (right) is overlaid. Representation plots from randomly selected cells from 3 independent experiments. Boxplots depict the IQR with a white horizontal line representing the median. Whiskers extend to the farthest data point within a maximum of 1.5x IQR. Every point represents one individual.

Extended Data Fig.5. GM-CSF producing CD8$^+$ T cells display largely analogous cytokine production profiles to CD4$^+$ T cells. CD8$^+$ T cells were subdivided into naïve, effector, effector memory and central memory cells based on FlowSOM-defined clusters. (**A**) Mean expression levels of the indicated surface markers in the respective subpopulation (left). Frequencies of these subpopulations in total CD8$^+$ T cells in NINDC (n = 31), RRMS patients during remission (n = 18), or during relapse (n = 12), SPMS patients (n = 5), PPMS patients (n = 3) and HD (n = 29). (**B**) Frequency of these subpopulations in GM-CSF$^+$ CD8$^+$ T cells in NINDC (n = 16), RRMS patients during remission (n = 16), or during relapse (n = 10), SPMS patients (n = 3), PPMS patients (n = 1) and HD (n = 21). (**C**) Frequency of cytokine$^+$ in GM-CSF$^+$ CD8$^+$ T cells. (**D**)

Production of GM-CSF by $CD8^+$ T cells positive for the indicated cytokine cells in NINDC (n = 11), RRMS patients during remission (n = 5), or during relapse (n = 3), SPMS patients (n = 2), PPMS patients (n = 1) and HD (n = 14). (**E**) FlowSOM was used to identify GM-CSF^+ $CD8^+$ T cell subsets based on their cytokine production profile (k = 10, elbow criterion). Clusters were manually annotated based on this production profile. Mean expression of surface and cytokine markers by the respective subsets (top). Relative fractions (bottom, left) and absolute frequencies (bottom, right) of FlowSOM defined GM-CSF^+ $CD8^+$ T cell subsets in NINDC (n = 11), RRMS patients during remission (n = 12), or during relapse (n = 6), SPMS patients (n = 1), PPMS patients (n = 1) and HD (n = 14). (**F**) Categorical tSNE analysis with heatmaps depicting mean expression levels in each bin. Boxplots depict the IQR with a white horizontal line representing the median. Whiskers extend to the farthest data point within a maximum of 1.5x IQR. Every point represents one individual.

Extended Data Fig.6. Co-production profiles of GM-CSF expressing NK and B cells. (**A**) Total NK cells were selected and the expression level of all relevant surface markers was correlated (Pearson's r) with GM-CSF expression on a single-cell level. Heatmap depicts Spearman correlation coefficients. (**B**) Frequencies (left) and example (right) of cytokine coexpression by GM-CSF^+ NK cells in NINDC (n = 31), RRMS patients during remission (n = 19), or during relapse (n = 12), SPMS patients (n = 5), PPMS patients (n = 3) and HD (n = 29). (**C**) B cells were selected and the expression level of all relevant surface markers was correlated (Pearson's r) with GM-CSF expression on a single-cell level. (**D**) Frequencies (left) and example (right) of cytokine expression by GM-CSF^+ B cells in patients as in **B**. Boxplots depict the IQR with a white horizontal line representing the median. Whiskers extend to the farthest data point within a maximum of 1.5x IQR. Every point represents one individual.

Extended Data Fig.7. Clinical correlations in the validation cohort. (**A**) Age distribution among RRMS (n = 12), HC (n = 15), NINDC (n = 14), IDC (n = 9) and CIS (n =8) patients. (**B**) Boxplots depict the age in RRMS (n = 12), HC (n = 15), NINDC (n = 14), IDC (n = 9) and CIS (n =8) patients. (**C**) Correlation between frequencies of the CellCNN-defined immune signature and age in RRMS (n = 12), HC (n = 15), NINDC (n = 14), IDC (n = 9) and CIS (n =8) patients. Regression curve with confidence intervals are depicted per each group. (**D**) Correlation of the frequency of the signature population in T-helper cells and age among RRMS (n = 12), HC (n = 15), NINDC (n = 14), IDC (n = 9) and CIS (n =8) patients. Each symbol identifies an individual patient among CIS (n = 8), RRMS in remission (n = 8) or relapsing (n = 4) groups. The regression line with confidence intervals are based on the frequency of the signature population among the other control groups. (**E**) Correlation between frequencies of the CellCNN-defined immune signature and clinical parameters in RRMS in remission (n = 8) or relapsing (n =

3) and CIS (n = 8) patients. Regression curve with confidence intervals are depicted for each parameter. Boxplots depict the IQR with a white horizontal line representing the median. Whiskers extend to the farthest data point within a maximum of 1.5x IQR. P values are based on two-tailed Mann-Whitney-Wilcoxon tests between the groups. Linear correlation equation is calculated on the pool of all analysed samples. Every point represents one individual.

Extended Data Fig.8. Immune profiling of validation cohort. (**A**) PBMCs from RRMS (n = 12), HC (n = 15), NINDC (n = 14), IDC (n = 9) and CIS (n =8) patients were restimulated with PMA/ionomycin and analyzed by mass cytometry. The tSNE algorithm (20,000 cells, randomly selected from all samples) was used to depict different populations therein. FlowSOM-based immune cell populations are overlaid as a color dimension. (**B**) Mean population expression levels of all markers used for tSNE visualization and FlowSOM clustering. (**C**) Sample specific and (**D**) frequencies of immune cell lineages in peripheral leukocytes in RRMS (n = 12), HC (n = 15), NINDC (n = 14), IDC (n = 9) and CIS (n =8) patients. (**E**) Representative plot of cytokine staining in the unstimulated control (upper) and stimulated samples (lower). Cells randomly selected from the experimental run are shown. Positivity threshold was set on the residual staining as described in the methods section. Frequencies of cytokine production by Th cells (**F**), Tc cells (**G**), NK cells (**H**) and B cells (**I**) among RRMS (n = 12), HC (n = 15), NINDC (n = 14), IDC (n = 9) and CIS (n =8) patients. Boxplots depict the IQR with a white horizontal line representing the median. Whiskers extend to the farthest data point within a maximum of 1.5x IQR. Every point represents one individual.

Extended Data Fig.9. SDF1α induces signature-cells migration toward a chemokine gradient. (**A**) Representative plots of gating strategy of immune cells populations (n = 7). (**B**) Frequency of migrating cells when SDF1α was added to the lower, upper or both chambers (n = 7). (**C**) Frequency migrating cells in the lower chamber toward an SDF1α gradient calculated as frequency of population specific input cells (n = 7). Representative plots (**D**) and quantification (**E**) of different cytokine-producing T-helper cells calculated as frequency of input cells (n = 7). Representative plots of 2 independent experiments. P values are based on two-tailed Mann-Whitney-Wilcoxon tests between the groups. Boxplots depict the IQR with a horizontal line representing the median. Columns plot represent the mean. Whiskers extend to the farthest data point within a maximum of 1.5x IQR. Every point represents one individual.

Extended Data Fig.10. CNS immune features of MS patients. Quantification (**A**) of cell viability in paired PBMC- CSF-samples (n = 9). (**B**) Frequencies of immune cell lineages in CSF between fresh (n = 3) and cryopreserved (n = 9) CSF samples. (**C**) Scaffold reference map of the Th cell compartment was constructed from mass cytometry

data. Grey bubbles represent the 100 FlowSOM nodes and colored landmarks are based on FlowSOM defined Th cell subsets. (**D**) Expression of CellCNN signature-defining cytokines and chemokine receptors within mapped FlowSOM nodes. (**E**) Immunohistochemistry of MS brain lesions depicting a demyelinated lesion (top left; myelin IHC) with KiM1P positive macrophages/activated microglial cells (top right, scale bar 200µm); CD3 positive perivenular T cell infiltration within the demyelinated lesion (bottom left) as well as in the meninges (bottom right, scale bar 30 µm). (**F**) Immunofluorescence control for secondary antibodies staining (left) and MS-brain lesion (right). Experiment repeated from brain biopsies of 3 individual MS patients as for Figure 6J. Scale bars = 30 µm. P values are based on two-tailed Mann-Whitney-Wilcoxon tests between the groups. Correlation coefficients (r) were calculated from the z-statistic of the Wilcoxon-Mann-Whitney test. Boxplots depict the IQR with a white horizontal line representing the median. Whiskers extend to the farthest data point within a maximum of 1.5x IQR. Every point represents one individual.

Table S1.

Clinical and demographic characteristics of all patients and controls used for mass cytometry study. M = male, F = female, HD = healthy donor, RRMS = relapsing-remitting MS, NINDC = non-inflammatory neurological disease control, CIS = clinical isolated syndrome, DMT = disease modifying treatment (including steroids) within the last six months, EDSS = expanded disability status scale, MSSS = multiple sclerosis severity score, OCB = oligoclonal bands, CSF = cerebrospinal fluid, MMP9 = matrix metalloproteinase 9, OPN = osteopontin, NFL = neurofilament light chain.

Table S2.

Heavy-metal labeled antibody panel for mass cytometry.

Table S3.

Population frequencies of all clinical groups. s.e.m = standard error of median, n = number of samples in group.

Table S4.

Statistical parameters of comparisons between clinical groups.

Table S5.

Clinical and demographic characteristics of all patients and controls used for validation cohort analysis. M = male, F = female, HD = healthy donor, RRMS = relapsing-remitting MS, NINDC = non-inflammatory neurological disease control, INDC = inflammatory neurological disease control, CIS = clinical isolated syndrome, DMT = disease modifying treatment, EDSS = expanded disability status scale, MSSS = multiple sclerosis severity score, OCB = oligoclonal bands, CSF = cerebrospinal fluid, MMP9 = matrix metalloproteinase 9, OPN = osteopontin, NFL = neurofilament light chain.

Table S6.

Clinical and demographic characteristics of all patients used for dimethyl fumarate cohort analysis. M = male, F = female, MS = multiple sclerosis, BL = baseline, EDSS = expanded disability status scale, MSSS = multiple sclerosis severity score, OCB = oligoclonal bands, CSF = cerebrospinal fluid.

Table S7.

Fluorescence antibody panel for flow cytometry.

Table S8.

Clinical and demographic characteristics of all patients and controls used for CSF flow cytometry analysis. M = male, F = female, HD = healthy donor, RRMS = relapsing-remitting MS, NINDC = non-inflammatory neurological disease control, DMT = disease modifying treatment , EDSS = expanded disability status scale, MSSS = multiple sclerosis severity score, OCB = oligoclonal bands, CSF = cerebrospinal fluid, MMP9 = matrix metalloproteinase 9, OPN = osteopontin, NFL = neurofilament light chain.