Supplementary Materials

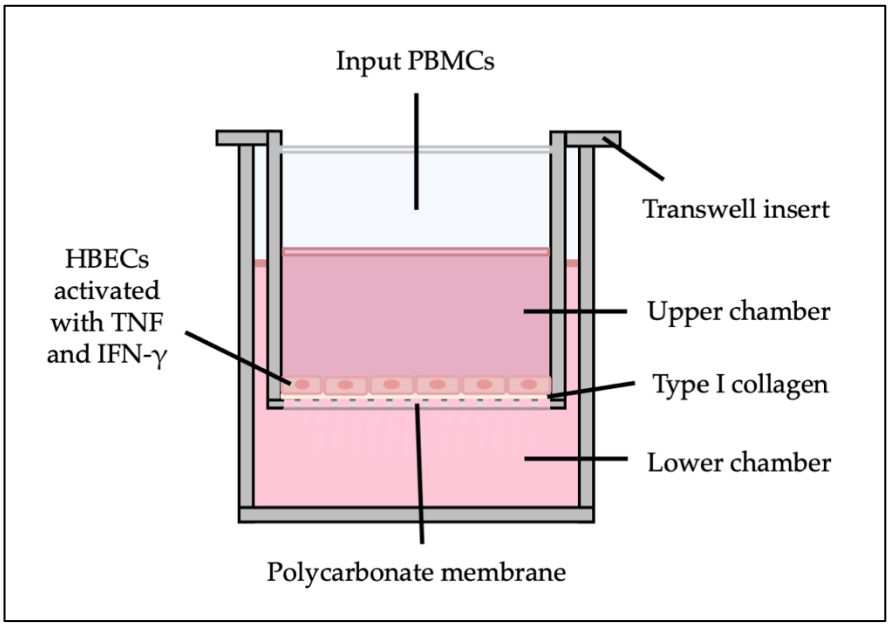

Figure S1. Experimental set-up of in vitro BBB model using a transmigration assay. The two-compartment system was separated by a porous membrane to replicate the peripheral blood circulation (upper chamber) and the CNS parenchyma (lower chamber). The polycarbonate membrane had 3 μm pores that were coated in collagen to mimic the basement membrane within the BBB. Collagen allowed HBECs, derived from the hCMEC/D3 cell line, to grow as a continuous monolayer. 5 ng/mL TNF and 10 ng/mL IFN-γ stimulated the endothelial cell layer to mimic an inflamed BBB. After cytokines were washed away, PBMCs that were isolated from healthy controls, untreated RRMS patients or alemtuzumab-treated RRMS patients were then added to the upper chamber and left overnight to migrate. HBEC, human brain endothelial cells; IFN-γ, interferon-gamma; PBMC, peripheral blood mononuclear cells; TNF, tumour necrosis factor.

Table S1. The inclusion and exclusion criteria of the study participants.

Type of Study Participant	Inclusion Criteria	Exclusion Criteria
Healthy controls	• MS patients refer a friend or relative of a similar age • People from the general population can make a voluntary contribution	• Methylprednisolone within the last 6 months • Pregnancy
Untreated RRMS patients	• Either recruited from the MS Clinic at Sydney Neurology at the Brain and Mind Centre (BMC), Sydney, and from Central West Neurology and Neurosurgery in Orange, Central Western NSW • At least yearly MRI on high resolution 3T magnets • Have serial volumetric assessments performed at the Sydney Neuroimaging Analysis Centre (SNAC) • Early non-treated patients with longstanding (>10 years) or benign RRMS	• Methylprednisolone within the last 6 months • Pregnancy

Alemtuzumab-treated patients	• Either recruited from the MS Clinic at Sydney Neurology at the Brain and Mind Centre (BMC), Sydney, and from Central West Neurology and Neurosurgery in Orange, Central Western NSW • At least yearly MRI on high resolution 3T magnets • Have serial volumetric assessments performed at the Sydney Neuroimaging Analysis Centre (SNAC) • Received first dose of alemtuzumab treatment with additional follow-ups at 6 and 12 months	• Methylprednisolone within the last 6 months • Pregnancy

Table S2. List of fluorochrome-conjugated antibodies and clones for flow cytometric analysis.

Cell Marker	Fluorochrome	Clone	Company
CD3	Alexa Fluor 532	UCHT1	eBioscience, ThermoFisher Scientific, Waltham, MA, USA
CD4	Alexa Fluor 700	RPA-T4	BioLegend, San Diego, CA, USA
CD8	BV480	RPA-T8	BD Biosciences, Franklin Lakes, NJ, USA
CD14	Pacific Blue	M5E2	BioLegend
CD16	FITC	3G8	Beckman Coulter, Lane Cove West, N.S.W., Australia
CD19	BV570	HIB19	BioLegend
CD20	Super Bright 436	2H7	eBioscience, ThermoFisher Scientific
CD24	PerCP	ML5	BioLegend
CD27	BV650	O323	BioLegend
CD28	BV510	CD28.2	BioLegend
CD38	BV785	HIT2	BioLegend
CD45RA	PerCP-Cy5	HI100	BioLegend
CD49d	PE-Cy5.5	9F10	BioLegend
CD56	BV750	5.1H11	BioLegend
CD62L	BV711	DREG-56	BioLegend
CD69	BV421	FN50	BioLegend
CD161	PE-Dazzle 594	HP-3G10	BioLegend
CD196	BV605	GO34E3	BioLegend
CD197	PE	G043H7	Beckman Coulter
CD274	PECy7	29E.2A3	BioLegend
GPR56	APC	4C3	BioLegend

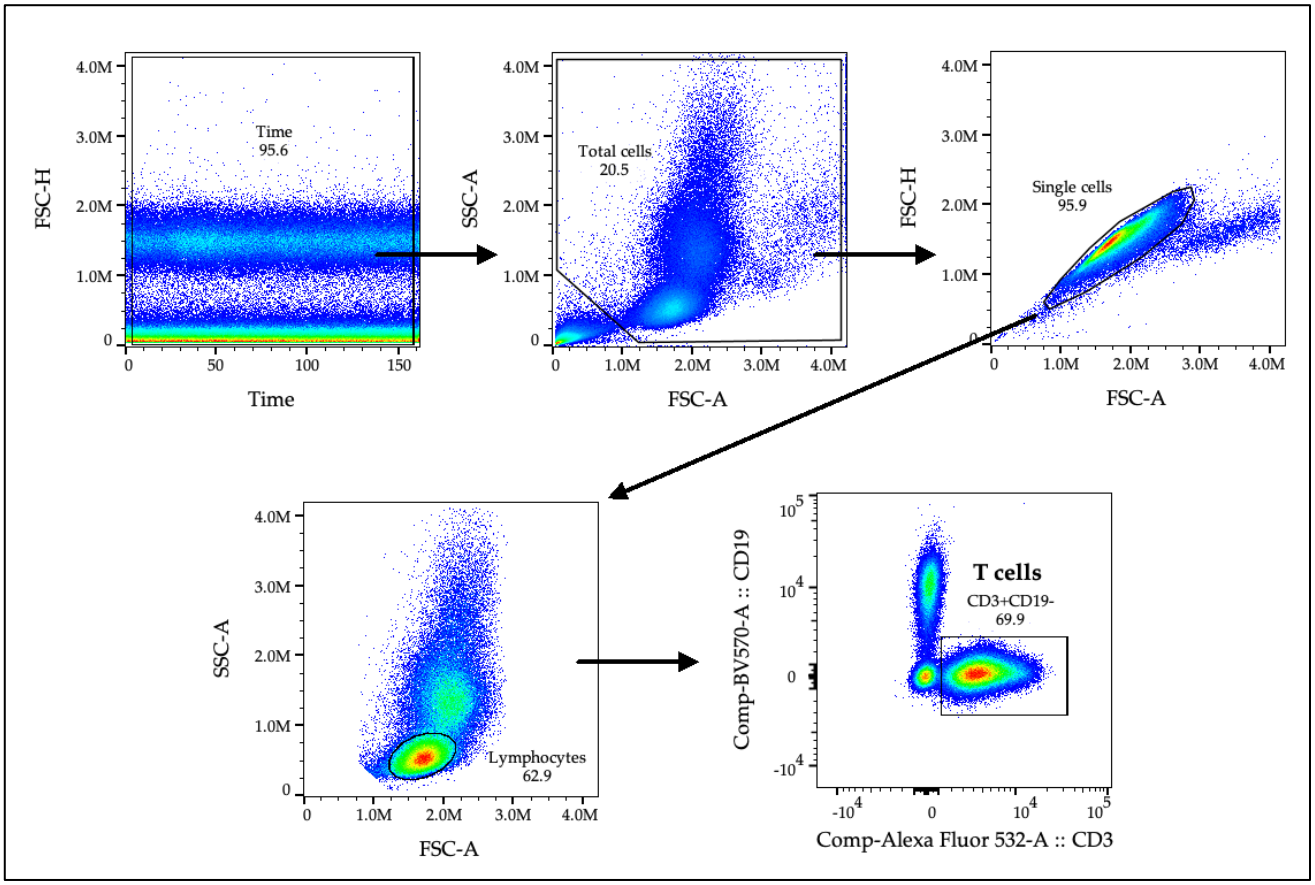

Figure S2. Preliminary gating strategy. Samples were first gated based on their forward-scatter-height (FSC-H) and time to obtain a consistent representative sample. Total PBMCs were gated based on their forward-scatter-area (FSC-A) and side-scatter-area (SSC-A). Single cells were identified by the exclusion of doublets and triplets using FSC-A and FSC-H. Lymphocytes were gated on using FSC-A and SSC-A. Major lymphocyte populations were identified and $CD3^+$ T cells were categorised based on $CD4^+$ and $CD8^+$ expression. PBMC, peripheral blood mononuclear cells.

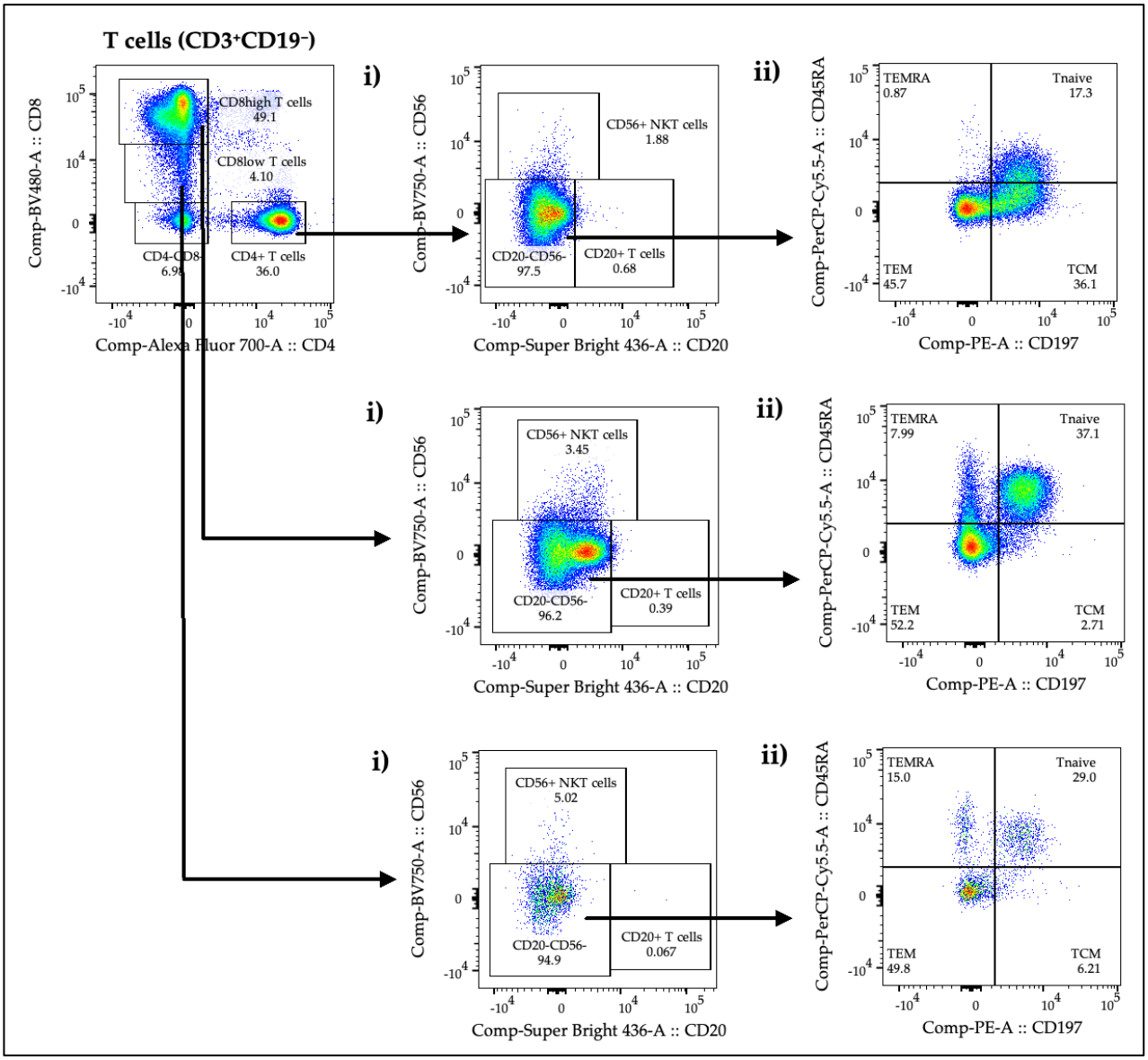

Figure S3. Gating strategy of T cell subsets. T cells were identified as $CD3^{+}CD19^{-}$; then divided into $CD4^{+}$, $CD8^{high}$ and $CD8^{low}$ T cell populations. The sequence of gating events is indicated by arrows to identify **(i)** NKT ($CD20^{-}CD56^{+}$), $CD20^{+}$ T cells ($CD20^{+}CD56^{-}$) and **(ii)** T^{EMRA} ($CD45RA^{+}CD197^{-}$), $T^{naïve}$ ($CD45RA^{+}CD197^{+}$), T^{CM} ($CD45RA^{-}CD197^{+}$) and T^{EM} ($CD45RA^{-}CD197^{-}$) cells. NKT, natural killer T cells; T^{EM}, effector memory T cells; T^{EMRA}, terminally differentiated effector memory cells re-expressing CD45RA T cells; $T^{naïve}$, naïve T cells; T^{CM}, central memory T cells.

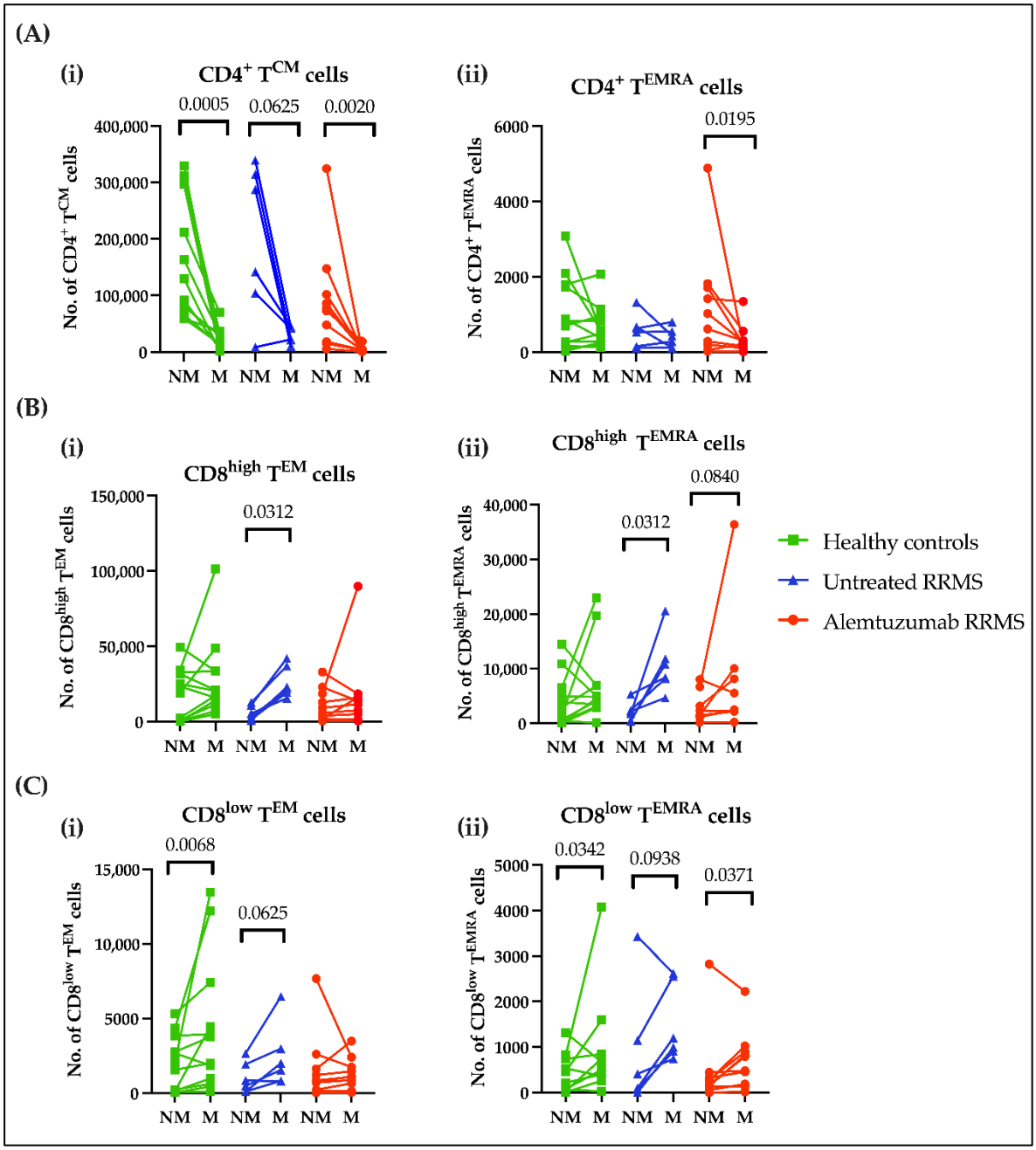

Figure S4. The trans-endothelial migration of other CD4⁺ and CD8⁺ T cells subsets. PBMCs were isolated from healthy controls (n = 12, green squares), untreated RRMS (n = 6, blue triangles) and alemtuzumab-treated RRMS patients (n = 10, red circles). After stimulation of the HBEC monolayer, PBMCs were added to the transwell assay and were left overnight to migrate. **(A)** The number of **(i)** $CD4^+$ T^{CM} cells and **(ii)** $CD4^+$ T^{EMRA}. **(B)** The number of **(i)** $CD8^{high}$ T^{EM} and **(ii)** $CD8^{high}$ T^{EMRA} cells. **(C)** The number of **(i)** $CD8^{low}$ T^{EM} cells and **(ii)** $CD8^{low}$ T^{EMRA} cells. Wilcoxon matched-pairs signed-rank test. $p \leq 0.1$ are shown. NM, non-migrated cells; M, migrated cells; RRMS, relapsing-remitting multiple sclerosis; PBMC, peripheral blood mononuclear cell; HC, healthy control; HBEC, human brain endothelial cell. T^{CM}, central memory T cells ($CD45RA^-CD197^+$); T^{EM}, effector memory T cells ($CD45RA^-CD197^-$); T^{EMRA}, terminally differentiated effector memory T cells re-expressing expressing CD45RA ($CD45RA^+CD197^-$); $T^{naïve}$, naïve T cells ($CD45RA^+CD197^+$).

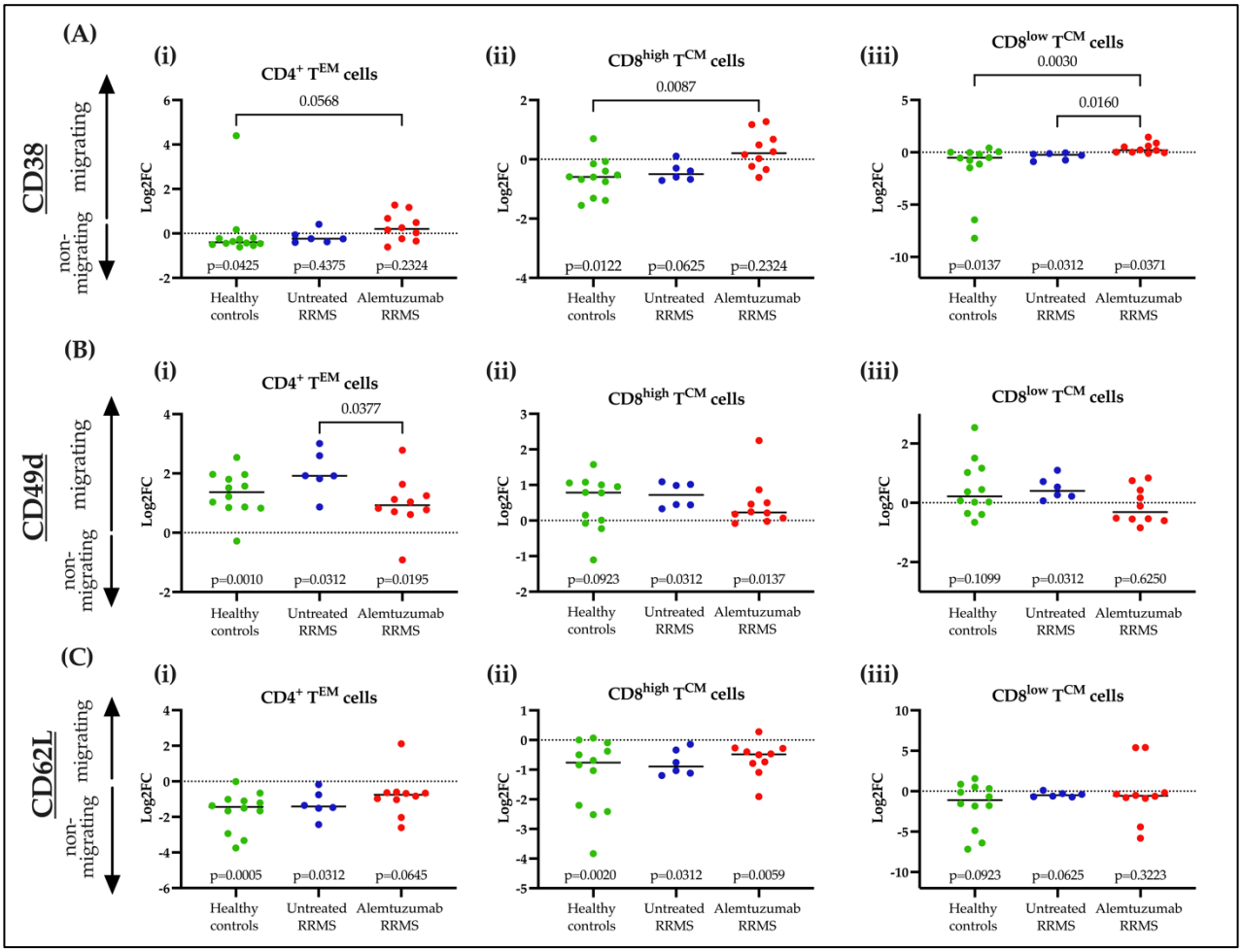

Figure S5. The logarithm of 2-fold change (Log2FC) ratios of cell surface markers on CD4⁺ and CD8⁺ memory T cells. The expression of cell surface markers was calculated in healthy controls (n = 12, green squares), untreated RRMS (n = 6, blue triangles) and alemtuzumab-treated RRMS patients (n = 10, red circles). **(A)** CD38 expression on **(i)** $CD4^+$ T^{EM} cells, **(ii)** $CD8^{high}$ T^{CM} cells and **(iii)** $CD8^{low}$ T^{CM} cells. **(B)** CD49d expression on **(i)** $CD4^+$ T^{EM} cells, **(ii)** $CD8^{high}$ T^{CM} cells and **(iii)** $CD8^{low}$ T^{CM} cells. **(C)** CD62L expression on **(i)** $CD4^+$ T^{EM} cells, **(ii)** $CD8^{high}$ T^{CM} cells and **(iii)** $CD8^{low}$ T^{CM} cells. Kruskal-Wallis nonparametric one-way ANOVA with Dunn's multiple comparisons test was done between groups, with p-values shown above data. A one sample nonparametric Wilcoxon signed-rank test with Pratt method was done to compare individual values, with p-values shown below data. Wilcoxon signed-rank test with method of Pratt, where above 0 indicates higher expression in migrating cells and below 0 indicates higher expression in non-migrating cells. Median and $p \leq 0.1$ are shown. T^{CM}, central memory T cell; T^{EM}, effector memory T cell; RRMS, relapsing-remitting multiple sclerosis.

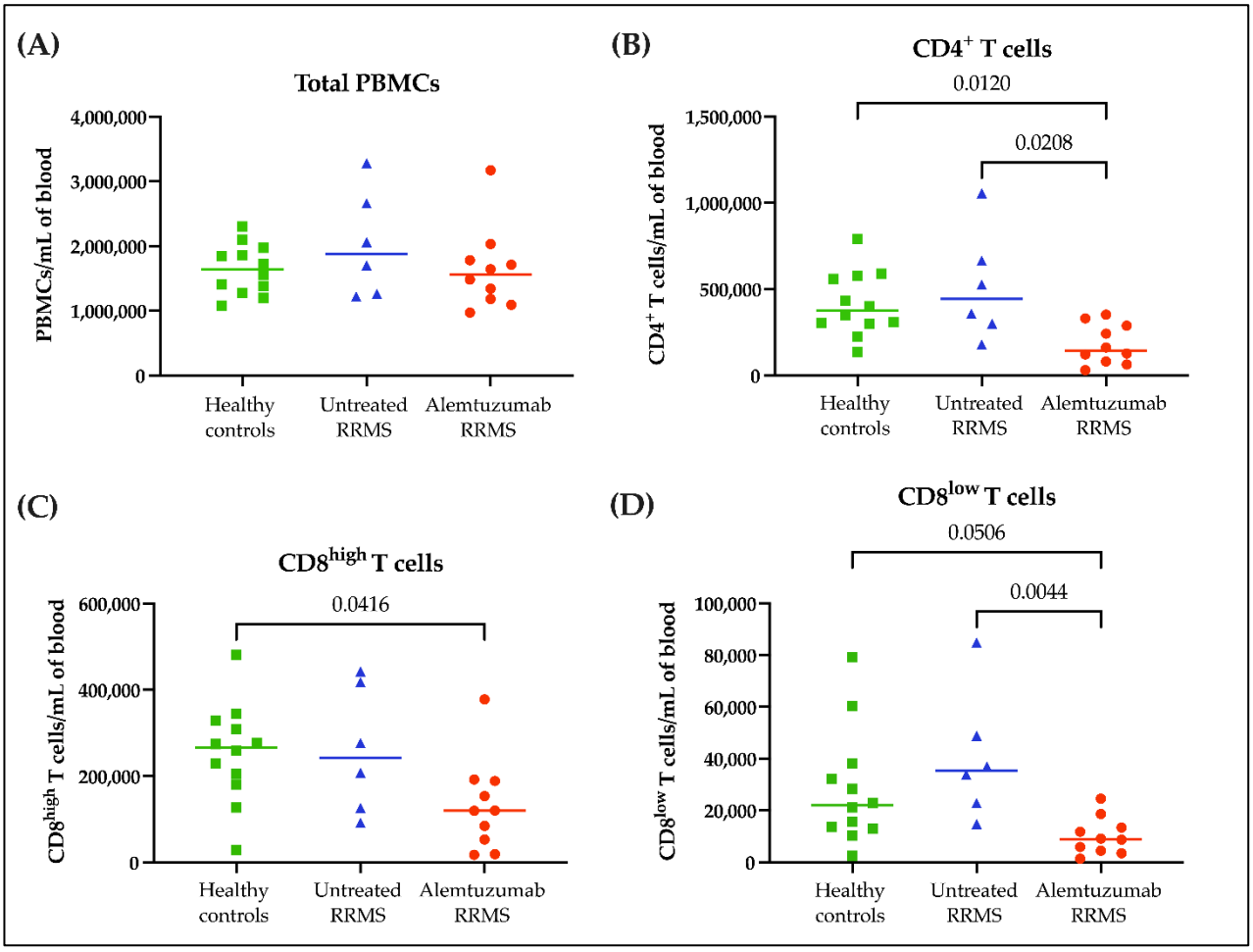

Figure S6. Fresh cell counts of total PBMCs and major lymphocyte populations. Blood was sampled from healthy controls (n = 12, green squares), untreated RRMS (n = 6, blue triangles) and alemtuzumab-treated RRMS patients (n = 10, red circles). Upon sample collection, fresh PBMCs were immediately isolated and phenotyped. **(A)** The total number of PBMCs per mL of blood. **(B)** The total number of $CD4^+$ T cells per mL of blood. **(C)** The total number of $CD8^{high}$ T cells per mL of blood. **(D)** The total number of $CD8^{low}$ T cells per mL of blood. Kruskal-Wallis nonparametric one-way ANOVA with Dunn's multiple comparisons test. Median and $p \leq 0.1$ are shown. PBMC, peripheral blood mononuclear cells; RRMS, relapsing-remitting multiple sclerosis.

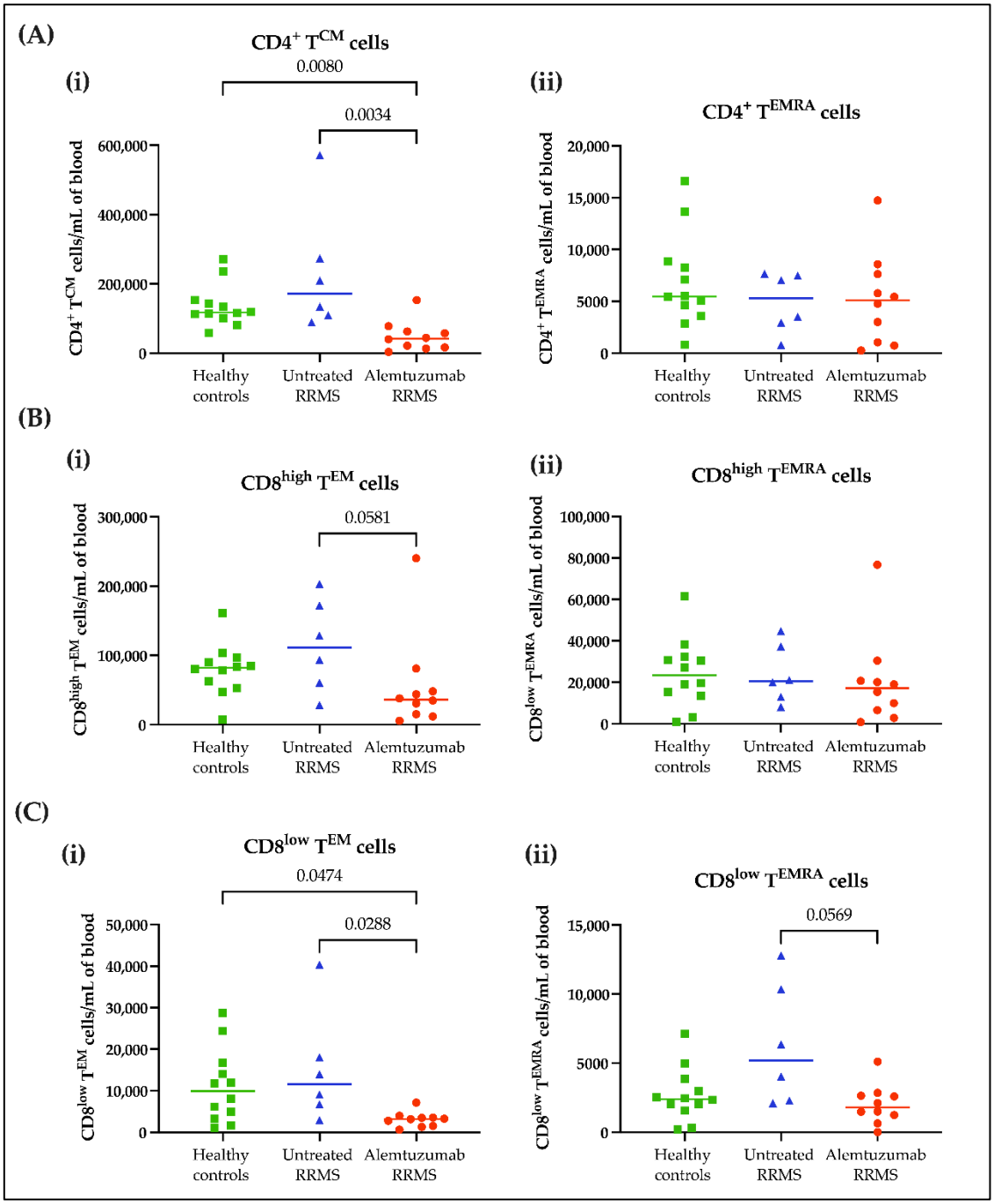

Figure S7. Fresh cell counts of CD4⁺ and CD8⁺ T cell subsets. Blood was sampled from healthy controls (n = 12, green squares), untreated RRMS (n = 6, blue triangles) and alemtuzumab-treated RRMS patients (n = 10, red circles). Upon sample collection, fresh PBMCs were immediately isolated and phenotyped. **(A)** The total number of **(i)** $CD4^+$ T^{CM} cells and **(ii)** $CD4^+$ T^{EMRA} cells per mL of blood. **(B)** The total number of **(i)** $CD8^{high}$ T^{EM} cells and **(i)** $CD8^{high}$ T^{EMRA} cells per mL of blood. **(C)** The total number of **(i)** $CD8^{low}$ T^{EM} cells and **(i)** $CD8^{low}$ T^{EMRA} cells per mL of blood. Kruskal-Wallis nonparametric one-way ANOVA with Dunn's multiple comparisons test. Median and $p \leq 0.1$ are shown. PBMC, peripheral blood mononuclear cells; RRMS, relapsing-remitting multiple sclerosis; T^{CM}, central memory T cell; T^{EM}, effector memory T cell; T^{EMRA}, terminally differentiated effector memory T cells re-expressing expressing CD45RA ($CD45RA^+CD197^-$).