BRAIN
ORIGINAL ARTICLE

Alemtuzumab-induced immune phenotype and repertoire changes: implications for secondary autoimmunity

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See Coles (https://doi.org/10.1093/brain/awac162) for a scientific commentary on this article.

Alemtuzumab is a monoclonal antibody that causes rapid depletion of CD52-expressing immune cells. It has proven to be highly efficacious in active relapsing–remitting multiple sclerosis; however, the high risk of secondary autoimmune disorders has greatly complicated its use. Thus, deeper insight into the pathophysiology of secondary autoimmunity and potential biomarkers is urgently needed. The most critical time points in the decision-making process for alemtuzumab therapy are before or at Month 12, where the ability to identify secondary autoimmunity risk would be instrumental. Therefore, we investigated components of blood and CSF of up to 106 multiple sclerosis patients before and after alemtuzumab treatment focusing on those critical time points.

Consistent with previous reports, deep flow cytometric immune-cell profiling ($n = 30$) demonstrated major effects on adaptive rather than innate immunity, which favoured regulatory immune cell subsets within the repopulation. The longitudinally studied CSF compartment ($n = 18$) mainly mirrored the immunological effects observed in the periphery. Alemtuzumab-induced changes including increased numbers of naïve $CD4^+$ T cells and B cells as well as a clonal renewal of $CD4^+$ T- and B-cell repertoires were partly reminiscent of haematopoietic stem cell transplantation; in contrast, thymopoiesis was reduced and clonal renewal of T-cell repertoires after alemtuzumab was incomplete. Stratification for secondary autoimmunity did not show clear immununological cellular or proteomic traits or signatures associated with secondary autoimmunity. However, a restricted T-cell repertoire with hyperexpanded T-cell clones at baseline, which persisted and demonstrated further expansion at Month 12 by homeostatic proliferation, identified patients developing secondary autoimmune disorders ($n = 7$ without secondary autoimmunity versus $n = 5$ with secondary autoimmunity). Those processes were followed by an expansion of memory B-cell clones irrespective of persistence, which we detected shortly after the diagnosis of secondary autoimmune disease.

In conclusion, our data demonstrate that (i) peripheral immunological alterations following alemtuzumab are mirrored by longitudinal changes in the CSF; (ii) incomplete T-cell repertoire renewal and reduced thymopoiesis contribute to a proautoimmune state after alemtuzumab; (iii) proteomics and surface immunological phenotyping do not identify patients at risk for secondary autoimmune disorders; (iv) homeostatic proliferation with disparate dynamics of clonal T- and B-cell expansions are associated with secondary autoimmunity; and (v) hyperexpanded T-cell clones at baseline and Month 12 may be used as a biomarker for the risk of alemtuzumab-induced autoimmunity.

Received December 30, 2020. Revised January 04, 2022. Accepted January 27, 2022
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Keywords: alemtuzumab; immune reconstitution therapy; CD52; T-cell repertoire; secondary autoimmunity

Abbreviations: AHSCT = autologous haematopoietic stem cell transplantation; ALEM = alemtuzumab; BCR = B-cell receptor; PBMC = peripheral blood mononuclear cell; RRMS = relapsing–remitting multiple sclerosis; SAID = secondary autoimmune disorder; Simoa = single molecule arrays; TCR = T-cell receptor; T_{reg} = regulatory T cell

Introduction

Alemtuzumab (ALEM) is a humanized monoclonal antibody directed against CD52 that has been approved for the treatment of highly active relapsing–remitting multiple sclerosis (RRMS). ALEM leads to a rapid depletion of CD52-expressing immune cells in peripheral blood with profound quantitative and qualitative effects on immunological networks, principally leading to long-lasting T-cell lymphopenia.[1–4] In contrast to maintenance therapies (e.g. natalizumab and fingolimod), ALEM can induce long-term treatment-free remission and is therefore considered to be an immune reconstitution therapy.[5–8] However, high therapeutic efficacy and treatment freedom is accompanied by potential serious adverse events, of which secondary autoimmune disorders (SAIDs) are the most significant for long-term outcomes.[9–12] SAID was reported in up to 48% of ALEM-treated RRMS patients, with thyroid autoimmunity as the most frequent condition.[11] The onset of SAID is delayed and ranges from 6 to 61 months, peaking in Years 2 and 3 and declining thereafter.[11–13] Frequent ALEM-related SAIDs include glomerulonephritis and immune thrombocytopenia, which in addition to thyroid autoimmunity are all considered to be B-cell-driven and autoantibody-mediated pathologies.[14] As ALEM causes fast, partially overshooting B-cell repopulation with simultaneous T-cell paucity, the most established hypothesis of this lymphopenia-associated autoimmune phenomenon assumes proliferation of autoreactive B cells under insufficient T-cell control.[15,16] However, this hypothesis does not sufficiently explain onset of SAID being delayed by several years and the appearance of clearly T-cell-mediated autoimmunity, for example sarcoidosis and vitiligo.[17–20]

SAID development has also been reported in the context of autologous haematopoietic stem cell transplantation (AHSCT). AHSCT is considered as the prototypic immune reconstitution therapy. Consistent with effects reported for ALEM, naïve B- and T-cell repopulation, a reduction of Th17 cells and a significant surge of regulatory T- and NK-cell subsets have been reported for AHSCT.[5,21–24] Therefore, mechanisms underlying SAID development might show overlaps for both treatment modalities.[5] However, the reported frequencies of SAID after AHSCT in multiple sclerosis patients are much lower than for ALEM and range around 2.8–6.4% of patients.[25–27] Interestingly, ALEM-containing conditioning regimens for AHSCT in patients with multiple sclerosis have been associated with a high incidence (14%) of late immune thrombocytopenia compared to non-ALEM regimens (0–2.8%).[26] Further to this, ALEM used in conditioning regimens treating autoimmune diseases generally has been associated with the occurrence of SAID in a significantly higher number of patients (16%) compared to regimens that did not use lymphocyte-depleting antibodies (0%).[28] For both ALEM and AHSCT, the proliferation of self-antigen-responsive T cells in the context of lymphopenia, so-called homeostatic proliferation, has been implicated as an important factor for SAID development.[27,29,30] For ALEM, early T-cell repopulation is mainly driven by homeostatic proliferation, whereas multiple sclerosis patients with SAID demonstrated reduced thymopoiesis and exhibited clonal restriction of the T-cell repertoire.[29] However, these alterations were demonstrated early following the initiation of ALEM, so how this relates to SAID occurrence peaking in Years 2–3 remains to be elucidated.

In a previous report, IL-21 was shown to promote homeostatic proliferation and IL-21 serum levels correlated with the incidence of SAID in ALEM-treated RRMS patients.[1] Thus, IL-21 measurement was proposed as a biomarker for SAID risk. However, the findings could not be validated in larger prospective cohorts with commercially available IL-21 enzyme-linked immunosorbent assay kits.[31] Baseline thyroid autoantibodies were associated with increased thyroid SAID after ALEM, but lymphocyte repopulation dynamics did not predict SAID occurrence.[32,33] Interestingly, previous treatment and treatment sequence impacts the efficacy and safety profile of ALEM.[34]

Thus, a comprehensive mechanistic hypothesis as well as potential biomarkers/signatures for ALEM-related SAID are currently lacking and are urgently needed in the clinical setting. We here performed in-depth multidimensional immune phenotyping and repertoire analysis predominantly in blood but also in CSF, comparing both compartments [including flow-cytometry, T- and B-cell receptor (TCR/BCR) sequencing, single molecule arrays (Simoa), and proteomics]. This parallel assessment of the TCR and BCR provides a novel hypothesis for the development of SAID, whereas no significant cellular or humoral immune signature was associated with the development of SAID.

Materials and methods

Cohort

This patient collective was established as part of the prospective PROGRAM^{MS} cohort ($n = 106$ patients; for further details see Supplementary material). Ethical approval was granted by the local authority (Institutional Review Board of the Medical Council Westphalia-Lippe, 2014-398-f-S). CSF analysis was conducted in a subgroup of patients who were enrolled in the ALAIN01 study, in which extensive immunoprofiling of ALEM-treated patients was performed. The study protocol has been published previously.[35] The study was approved by local authorities (2014-545-f-A) and has been officially registered (NCT02419378). Besides those 15 patients, CSF analysis was conducted in 3 additional patients after informed consent. For extensive analyses different subcohorts of the PROGRAM^{MS} cohort were analysed, which—aside from the ALAIN01 cohort—were randomly assigned (Table 1 and Fig. 1A).

Biomaterial

For this study, biological parameters from peripheral blood and CSF were investigated. While EDTA–blood and CSF cells were analysed within 1 h of withdrawal, serum, CSF supernatant and peripheral blood mononuclear cells (PBMCs) were cryopreserved following standardized processes as described previously.[36]

Flow cytometry

Flow-cytometric analyses of EDTA blood and CSF cells were performed as a part of routine clinical prectice and for therapy surveillance, as described previously.[36,37] In addition, PBMCs from a subcohort of ALEM patients were analysed according to a flow-cytometric functional immune phenotyping matrix as described before.[37] For this purpose, cryopreserved PBMCs were thawed and stained with distinct sets of fluorochrome-conjugated antibodies (Supplementary material). For intracellular staining, cells were treated with fixation/permeabilization solution (eBiosciences) for 20 min, subsequently washed with permeabilization buffer (eBiosciences) and finally incubated with antibodies directed against intracellular target molecules of interest. To investigate the capacity to produce cytokines, PBMCs were rested overnight in X-VIVO™ 15 Serum-free Hematopoietic Cell Medium (Lonza) and subsequently stimulated with Leucocyte Activation Cocktail, with BD GolgiPlug™ [with phorbol myristate acetate (PMA), Ionomycin and Brefeldin A; BD Pharmigen™] for 4 h before extracellular staining for lineage markers and intracellular staining for cytokines. Flow-cytometric data were analysed using Kaluza Analysis Software Version 2.1 (Beckman Coulter). Immune cell subsets were defined according to a prespecified gating hierarchy (Supplementary material). Gating strategy and representative stainings can be found in Supplementary Figs 1 and 2)

Simoa

CSF supernatant was processed as described above. To ensure high comparability, all samples were analysed on the same day. IL-10, IL-12p70, IFNγ, TNFα, IL-6, IL-17 and GM-CSF were measured in several rounds by Simoa HD-1 (Quanterix) using the Simoa HD-1 Analyzer™ (Quanterix) using human kits for the respective cytokines or the Simoa® NF-light™ Advantage Kit (Quanterix) for serum

Table 1 Baseline characteristics of investigated cohorts

Parameter	Cohort A $n = 106$	Subcohort B $n = 30$	Subcohort C $n = 18$	Subcohort D $n = 12$	*P* subcohort B versus excluded	*P* subcohort C versus excluded	*P* subcohort D versus excluded
Age at baseline, median (IQR)	34 (29–43)	36 (28–46)	34 (28–37)	33 (28–46)	0.277[a]	0.503[a]	0.897[a]
Male sex, *n* (%)	37 (36)	14 (44)	4 (24)	5 (42)	0.200[b]	0.180[b]	0.454[b]
Duration since multiple sclerosis diagnosis, years, median (IQR)	6 (2–10)	5 (2–10)	6 (4–10)	5 (2–10)	0.937[a]	0.656[a]	0.546[a]
Duration since multiple sclerosis onset, years, median (IQR)	7 (3–12)	7 (3–10)	6 (5–11)	7 (3–9)	0.717[a]	0.564[a]	0.731[a]
Number of previous DMT, median (IQR)	2 (1–3)	2 (1–3)	3 (2–4)	2 (1–3)	0.133[a]	0.080[a]	0.514[a]
EDSS at baseline, median (IQR)	2.5 (1.5–4.0)	3 (2.0–3.5)	4 (2.0–4.5)	3 (2.0–4.0)	0.655[a]	0.069[a]	0.103[a]
Number of relapses within last two years before ALEM, median (IQR)	2 (1–3)	2 (1–3)	2 (1–4)	2 (1–3)	0.702[a]	0.431[a]	0.359[a]
Last previous DMT, *n* (%)					0.212[b]	0.304[b]	0.773[b]
None	15 (14)	9 (28)	1 (6)	1 (8)			
Basic	33 (31)	9 (28)	6 (35)	4 (33)			
Escalation	58 (55)	14 (44)	10 (59)	7 (59)			

DMT = disease-modifying therapy; EDSS = Expanded Disability Status Scale.
[a,b]P-values were calculated using the [a]Mann–Whitney rank sum test and [b]Fisher's exact test, respectively.

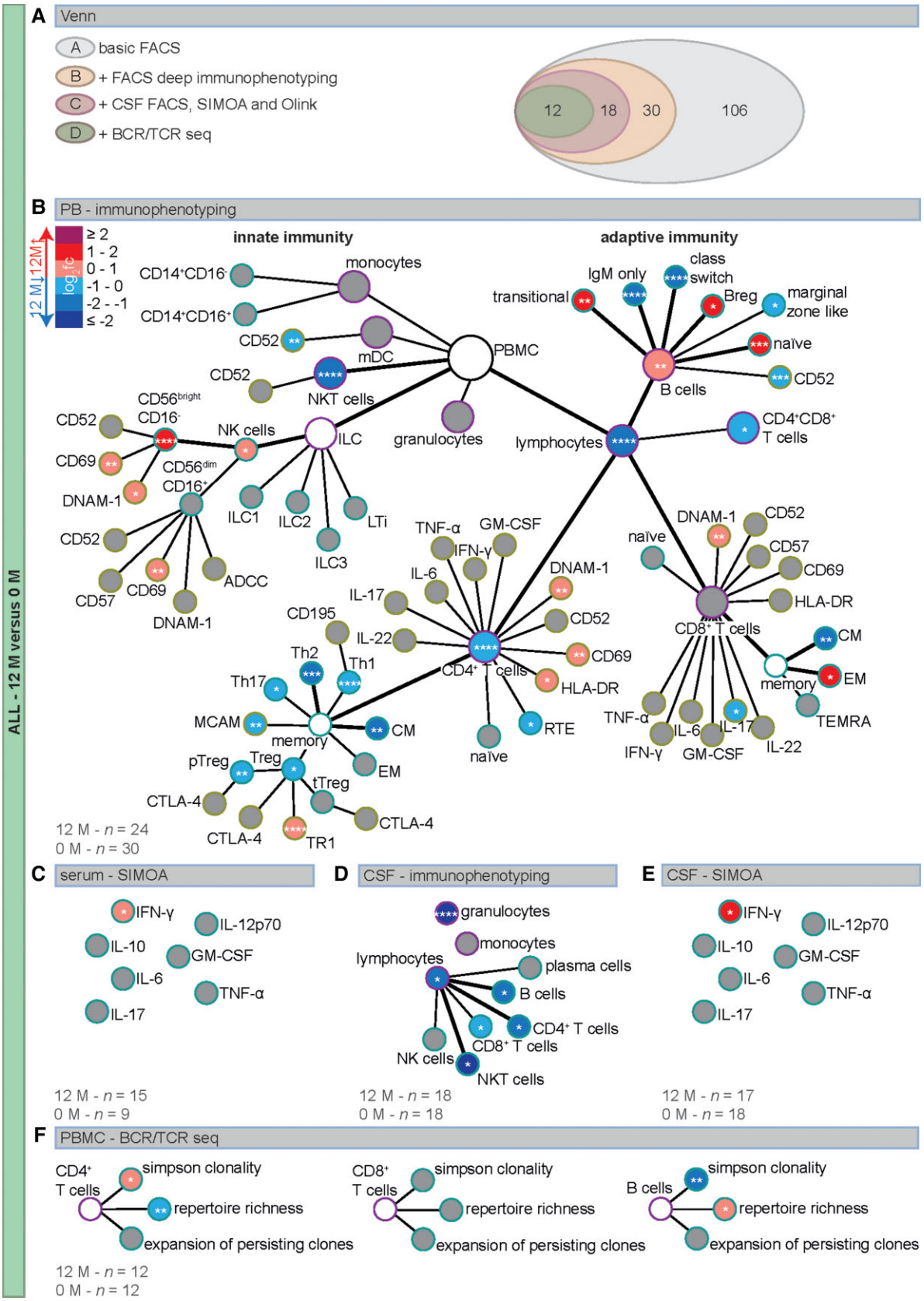

Figure 1 ALEM induces profound changes in the immune repertoire. (**A**) Venn diagram displaying the study cohort and subcohorts with patient numbers and corresponding analyses. For baseline characteristics of the different cohorts see Table 1. (**B**) Hierarchical illustration of the changes to absolute cell numbers of different immune cell subsets in ALEM-treated RRMS patients. All data-points compare values at baseline and after 12 months of treatment; individual values can be found in Supplementary Table 2. Lines display relationships between populations and subpopulations. (Continued)

neurofilament light chain from the same batch according to manufacturer's instructions. Samples were measured in duplicate.

TCR/BCR repertoire sequencing

RNA from Magnetic Activated Cell Sorting-sorted $CD4^+$, $CD8^+$ and $CD19^+$ cells from frozen PBMCs was purified using the RNeasy® Plus Micro Kit (Qiagen) and transcribed into cDNA. $CD4^+$ TCR variable beta chain and $CD19^+$ BCR immunoglobulin heavy chain (IgH) high-throughput sequencing was performed using the immunoSEQ® Assay (Adaptive Biotechnologies), as previously described.[38] The analyses were performed using the R package divo: Tools for Analysis of Diversity and Similarity in Biological Systems to measure the Morisita–Horn index (for quantification of sample overlap).[39] The Efron–Thisted estimator was calculated according to Efron and Thisted[40]; Simpson clonality was calculated as previously described by Pruessmann *et al.*[41] The healthy control and TERI datasets shown in Fig. 3 were processed and analysed from raw data previously published by Klotz *et al.*[38] The AHSCT dataset shown in Fig. 3 was processed and analysed from raw data previously published by Muraro *et al.*[42] All used datasets were sex- and age-matched. For SAID-related TCR beta chain and BCR IgH repertoire sequencing, cells [$CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$) and memory B cells ($CD19^+CD27^+IgD^{+/-}$)] were sorted using a MoFlo XDP High-Speed Cell Sorter (Beckman Coulter). For further sample processing a custom protocol was used, which is comparable to the immunoSEQ® Assay (Adaptive Biotechnologies) and is described in detail in the Supplementary material. Approximately 25 million sequence reads were generated per Illumina Miseq run. Raw sequence reads were processed using the MiXCR software pipeline (https://milaboratories.com/software).[43] MiXCR pre-processed data were post-analysed using VDJtools to evaluate clonal expansions, repertoire diversity, clonotype overlap and clonotype tracking (new and persisting clones; https://vdjtools-doc.readthedocs.io/en/master/install.html, https://immunarch.com/index.html).[44,45]

Proteomics

Cryopreserved serum and CSF samples were sent to Olink for proteomic analysis by the Explore 1536 panel (Olink proteomics) by proximity extension assay as described in a recently published article.[46]

Statistics

Given the heterogeneity of clinical and experimental data, non-parametric tests of association were used throughout this study unless otherwise specified. Comparisons between patient groups were performed using the Mann–Whitney U test (unpaired) or signed-rank test (paired) for continuous variables and Fisher's exact test for categorical variables. Comparisons of multiple groups were made using the Kruskal–Wallis test followed by Dunn's test. Longitudinal analyses of leucocyte compositions in respective patients were analysed using the Friedman test, including Dunn's test. An exploratory analysis of potential markers associated with SAID and treatment response was performed using the volcano plot, which visualizes the *P*-values derived from the testing of a respective parameter and the corresponding $\log_2$ fold change of the medians. Correlation analyses were performed in GraphPad Prism version 9.1 (GraphPad Software, San Diego, California, USA; www.graphpad.com) by linear non-parametric Spearman correlation. For all calculations, a *P*-value below 0.05 was considered statistically significant. Due to the exploratory nature of the dataset, *P*-values were not corrected for type I error. Thus, the findings of this study should be considered as hypothesis-generating and must be independently confirmed.

Data availability

Individual data-points for Figs 1 and 2 can be found in Supplementary Table 2. Further data will be shared upon personal request to the corresponding author.

Results

Alemtuzumab leads to profound immune repertoire changes in the peripheral blood and CSF

Immune depletion and repopulation patterns following ALEM have been reported previously.[2,29,47,48] We first evaluated whether our cohort provides findings compatible with the literature ensuring their transferability. We performed broad immunophenotyping with the complete cohort of 106 ALEM-treated patients (cohort A) and more detailed analyses with subcohorts (cohorts B–D) of those 106 patients (Fig. 1A). The baseline parameters were similar for the different cohorts and consistent with the phase 3 trials (Table 1).[6,7] In cohort A, ALEM treatment led to the predominant depletion of B and T lymphocytes and to a lesser extent innate immune cells (NK, NK-T, monocytes and granulocytes; Supplementary Fig. 3A–J). Absolute and relative lymphocyte counts did not recover to baseline levels during the 24 months of observation (Supplementary Fig. 3A). B cells repopulated quickly, whereas $CD4^+$ and $CD8^+$ T cells remained reduced (Supplementary Fig. 3E–G and J). None of the patients showed the previously described depletion failure with ALEM.[49]

Figure 1 Continued
Bold lines indicate immune compartments with profound changes. Statistically significant changes are coloured and *P*-values are indicated. Colour filling shows the $\log_2$ fold change after 12 months of treatment versus baseline ($n = 30$ for baseline and $n = 24$ for Month 12). (**C**) Serum cytokines measured by Simoa ($n = 9$ for baseline and $n = 15$ for Month 12). (**D**) Change in absolute cell numbers of indicated immune cell subsets in the CSF compartment ($n = 18$ for baseline and Month 12). (**E**) CSF cytokines measured by Simoa ($n = 17$ for baseline and $n = 18$ for Month 12). (**F**) Analysis of BCR and TCR sequencing of $CD4^+$, $CD8^+$ T cells and B cells ($n = 12$ for baseline and Month 12). Simpson clonality measures how evenly receptor sequences are distributed in the repertoire, where 0 represents an even and 1 a monoclonal sample; repertoire richness measures how many clones are present in the repertoire; expansion of persisting clones indicates whether pretreatment clones occupy higher volumes of the repertoire ($n = 12$ for baseline and Month 12). Statistical analysis was performed using the Mann–Whitney test (unpaired comparisons). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns = not significant. ADCC = antibody-dependent cellular cytotoxicity; Breg = regulatory B cell; CM = central memory; CTLA-4 = cytotoxic T-lymphocyte-associated Protein 4; DNAM-1 = DNX-Accessory Molecule-1; EM = effector memory; FACS = flow cytometry; NK = natural killer cell; NKT = natural killer T cell; mDC = myeloid dendritic cells; ILC = innate lymphoid cell; tTreg = thymus-derived Treg; pTreg = peripherally induced Treg; RTE = recent thymic emigrants; seq = sequencing; TEMRA = T effector memory expressing CD45RA.

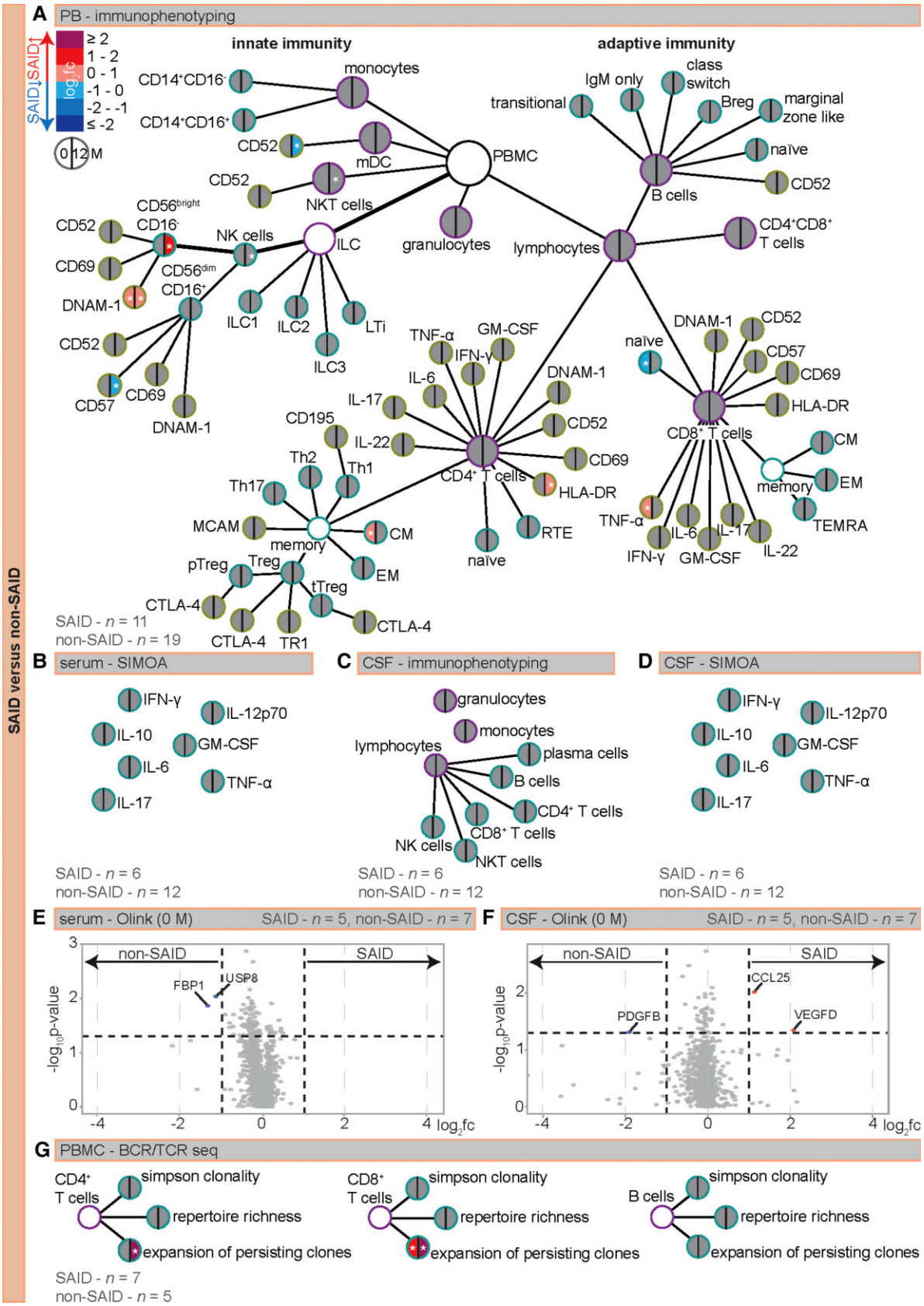

Figure 2 SAID is associated with distinct immune alterations in the TCR repertoire. (A) Hierarchical illustration of the changes to absolute cell numbers of different immune cell subsets in ALEM-treated RRMS patients with or without SAID. Statistically significant differences are coloured and indicated by their *P*-values. All data-points are presented as split circles with the left half indicating the comparison of SAID versus non-SAID patients at baseline and the right half indicating the comparison of SAID versus non-SAID patients after 12 months of treatment; individual values can be found in

(Continued)

More in-depth immunophenotyping in subcohort B demonstrated reduced central memory $CD4^+$ T-cell numbers, inflammatory T helper subsets (T_h1, T_h2, T_h17) and recent thymic emigrants as surrogate markers for thymopoiesis (Fig. 1B) 12 months after ALEM. In the $CD8^+$ T-cell compartment, central memory cells were decreased, whereas effector memory cells were more abundant (Fig. 1B). The production of anti- or pro-inflammatory cytokines was comparable to baseline (cell-type-specific: Fig. 1B, except for less IL-17 in $CD8^+$ T cells; and in serum: Fig. 1C, except for higher IFN-γ levels). In the B-cell compartment, we detected enhanced numbers of immature B-cell subsets (naïve and transitional B cells), whereas memory B-cell subsets (class-switched, IgM only and marginal zone-like memory B cells) were reduced at Month 12 (Fig. 1B). In the regulatory cell subsets, we obsereved a reduction in regulatory T cells ($FoxP3^+$ T_{reg}), which was mainly attributable to peripherally induced T_{reg} (Fig. 1B). In contrast, T regulatory type 1 (T_R1) cell numbers were absolutely and relatively increased. However, relative proportions of both T_{reg} and T_R1 cells were enhanced 12 months after ALEM (Supplementary Fig. 4A). Of note, within the T_{reg} compartment the relative increase was pronounced for $Helios^+$ T_{reg}, which show enhanced immunosuppressive characteristics (Supplementary Fig. 4A).[50,51] T_{reg} (Supplementary Fig. 4) and $CD56^{bright}$ NK cells (Fig. 1B) expressed higher levels of markers that indicate facilitated immunosuppressive function (T_{reg}: Helios, PD-1, GITR[50,52,53]: Supplementary Fig. 4A and B; $CD56^{bright}$ NK cells: DNAM-1: Fig. 1B).[54]

In CSF (subcohort C), ALEM treatment led to significantly reduced absolute cell numbers and a reduced IgG ratio. However, the remaining basic CSF parameters such as total protein and oligoclonal bands demonstrated no relevant changes over the 12 months (cohort A, Supplementary Table 1). Flow-cytometric immunphenotyping showed that ALEM significantly reduced granulocyte, $CD4^+$ T, $CD8^+$ T, B cell and NK-T cell counts (cohort C, Fig. 1D). Cytokine measurements using Simoa showed no significant changes in anti- (IL-10) or pro-inflammatory cytokines (TNF-α, IL-12p70, IL-6, IL-17 and GM-CSF, except for increased IFN-γ levels) in the CSF (subcohort C, Fig. 1E).

Next, we investigated alterations and dynamic changes in the peripheral TCR and BCR repertoires (subcohort D). The $CD4^+$ T-cell compartment showed higher repertoire clonality (Simpson clonality) and reduced richness (Efron–Thisted estimator). Clonality metrics describe the extent to which one or a few clones dominate the sample repertoire, whereas richness measures focus on how many clones are present in the sample repertoire. In marked contrast, B-cell repopulation was characterized by an increased richness and lower clonality after 12 months. The $CD8^+$ TCR repertoire was heterogeneous for the investigated patients (subcohort D, Fig. 1F).

In summary, changes after depletion and during immune repopulation are measurable on numerous immunological levels, both cellular and humoral, which is consistent with the previously reported immunological effects of ALEM.[15,33,47,55] The CSF compartment had not yet been investigated, but broadly mirrored the findings in peripheral blood. Alterations were seen on many adaptive but also innate immune components and support the notion that immune repopulation rebalances towards a more regulatory and anti-inflammatory phenotype. The longitudinal TCR and BCR repertoire changes argue for durable and profound immunological effects. Supplementary Table 2 contains the numeric data that are illustrated in Fig. 1.

Differences in the TCR repertoire characterize patients developing secondary autoimmunity after alemtuzumab

ALEM has proven high efficacy that is sustained for several years without any further treatment.[12] However, uncertainty regarding ALEM therapy arises from the risk of SAID. Hence, biomarkers identifying patients at risk for the development of SAID and deeper insights into the pathophysiology of ALEM-associated SAID are of great clinical interest. The most critical time points in the decision-making process for ALEM therapy are at baseline (whether to start therapy) or at Month 12 (whether to continue therapy), where information on SAID risk would be instrumental. Data, however, suggest that only the initial lymphocyte depletion is decisive for the development of SAID.[56] We therefore investigated the immunophenotypic differences at those time points for ALEM-treated RRMS patients who did or did not develop SAID within the 48 months of follow-up after therapy initiation.

Consistent with previous observations,[33,47] peripheral immune cell subset phenotyping and serological analyses showed only marginal differences between patients with or without SAID. Further, the observed alterations did not affect any clear immunological trait or show any conclusive pattern to generate meaningful hypotheses based on peripheral blood findings (subcohort B, Fig. 2A and B). Additionally, in the CSF, we found no differences in cellular composition (subcohort C, Fig. 2C) or in cytokine signatures (subcohort C, Fig. 2D). To add more depth to the solute-targeted analyses, we performed proteomic analyses by the Olink Explore 1536 panel at baseline in serum and CSF. However, neither serum nor CSF proteins demonstrated relevant differential regulation with only very few proteins marginally passing the selected cut-offs (subcohort C, Fig. 2E and F).

The most prominent alteration between SAID and non-SAID patients among all investigated parameters was the expansion of persisting $CD4^+$ and $CD8^+$ T-cell clones (subcohort D, Fig. 2G). The expansion of persisting clones indicates whether pretreatment

Figure 2 Continued
Supplementary Table 2. Colour filling shows the $\log_2$ fold change of the corresponding comparison ($n = 11$ for SAID and $n = 19$ for non-SAID patients). (**B**) Serum cytokines measured by Simoa ($n = 6$ for SAID and $n = 12$ for non-SAID patients). (**C**) Immunophenotyping of the CSF compartment ($n = 6$ for SAID and $n = 12$ for non-SAID patients). (**D**) CSF cytokines measured by Simoa ($n = 6$ for SAID and $n = 12$ for non-SAID patients). (**E** and **F**) Olink analysis comparing serum and CSF of SAID and non-SAID patients as demonstrated by volcano plot. Volcano plots were constructed by calculating the $\log_2$ fold change of the median and the $-\log_{10}$ *P*-value ($n = 6$ for SAID and $n = 12$ for non-SAID patients). (**G**) Analysis of BCR/TCR sequencing of $CD4^+$, $CD8^+$ and B cells ($n = 7$ for SAID and $n = 5$ for non-SAID patients). Statistical analysis was performed using the Mann–Whitney test (unpaired comparisons). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns = not significant. ADCC = antibody-dependent cellular cytotoxicity; Breg = regulatory B cell; CM = central memory; CTLA-4 = cytotoxic T-lymphocyte-associated Protein 4; DNAM-1 = DNX-Accessory Molecule-1; EM = effector memory; FACS = flow cytometry; NK = natural killer cell; NKT = natural killer T cell; mDC = myeloid dendritic cells; ILC = innate lymphoid cell; tTreg = thymus-derived Treg; pTreg = peripherally induced Treg; RTE = recent thymic emigrants; seq = sequencing; TEMRA = T effector memory expressing CD45RA.

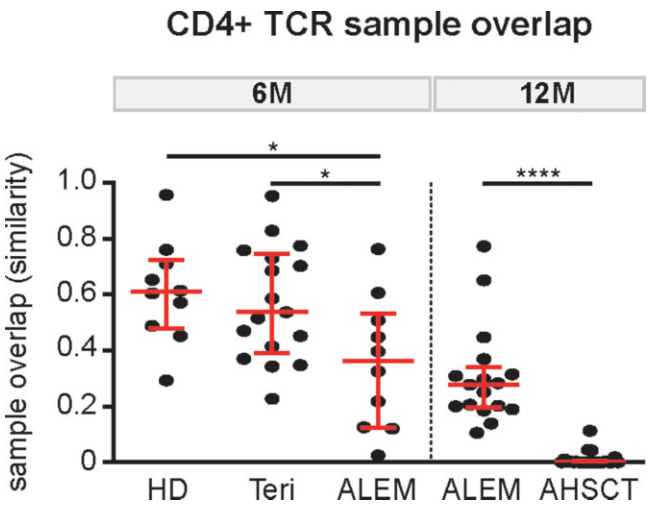

Figure 3 TCR sample overlap in ALEM-treated patients compared to healthy donors, AHSCT and teriflunomide. Dot plot displaying the median sample overlap (similarity of repertoires) of TCR sequencing of $CD4^+$ T cells comparing either baseline and 6-month time points in cohorts of healthy donors, teriflunomide- and ALEM-treated multiple sclerosis patients (*left*) or baseline and 12-month time points in ALEM- and AHSCT-treated multiple sclerosis patients (*right*). Median and IQR are indicated. Statistical analysis was performed by Mann–Whitney test (unpaired comparisons). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns = not significant. HD = healthy donor; Teri = teriflunomide.

clones occupy higher volumes of the repertoire. Supplementary Table 2 contains the numeric data that are illustrated in Fig. 2.

Alemtuzumab leads to incomplete renewal of the $CD4^+$ TCR repertoire

As an expansion of T-cell clones was observed in ALEM-treated SAID patients, ALEM might have unique effects on the T-cell repertoire that contributes to the high risk of SAID development. Immune repertoire exchange due to ALEM was compared to teriflunomide, a platform therapy with no reported cases of SAID,[57] and AHSCT treatment by determining the sample overlap, which describes repertoire similarity according to the Morisita–Horn index.[39] An index of 0 demonstrates that in a given patient the $CD4^+$ TCR repertoires at baseline and the indicated time point do not overlap, whereas an index of 1 represents a perfect overlap of the samples. However, even in healthy donors the sample overlap 6 months from baseline analysis is 0.61 (IQR 0.24) due to ongoing thymopoiesis (Fig. 3, left). In comparison to healthy donors, teriflunomide-treated (TERI) multiple sclerosis patients displayed no relevant differences in the $CD4^+$ TCR repertoire sample overlap (0.54, IQR 0.35) between baseline and 6 months (Fig. 3, left), thus indicating no relevant exchange in the TCR repertoire exceeding physiological parameters. In contrast, ALEM led to a significant but incomplete TCR repertoire exchange (0.36, IQR 0.41) compared to healthy donors and TERI 6 months after first infusion (Fig. 3, left). Twelve months after AHSCT we observed an almost complete TCR repertoire exchange (<0.01, IQR < 0.01) in the $CD4^+$ TCR repertoire, whereas the repertoire remained incompletely exchanged after ALEM (0.28, IQR 0.15; Fig. 3, right). Thus, TERI demonstrated no specific effect on the TCR repertoire, AHSCT led to an almost complete renewal, whereas ALEM induced an incomplete $CD4^+$ TCR repertoire exchange. This unique effects of ALEM on the immune repertoire might be related to the high risk of SAID.

TCR and BCR repertoire changes with specific temporal dynamics are associated with secondary autoimmunity

As we observed specific $CD4^+$ T-cell immune repertoire changes at Month 12 after ALEM treatment, we then investiagted whether the repertoire changes that distinguish SAID patients are restricted to the first year and the $CD4^+$ T-cell compartment. We compared data from five ALEM-treated multiple sclerosis patients with SAID to seven patients without SAID. Clinical details on the investigated cohort are presented in Supplementary Table 3. At 4–6 months, persisting clones occupied significantly larger proportions of both the $CD4^+$ (subcohort D, Fig. 4A, left) and $CD8^+$ TCR (Fig. 4A, right) repertoires in SAID patients. In general, persisting clones (green) made up larger proportions of $CD8^+$ T-cell repertoires than of $CD4^+$ T-cell repertoires (Fig. 4A). In the B-cell compartment, we found that persisting IgG^+ memory B-cell clones occupied only small fractions (1.67% ± 0.42%) of the post-ALEM repertoires in both SAID and non-SAID patients (Supplementary Fig. 5A).

Investigating the dynamics of the cumulative volumes of the 'Top 100' persisting T-cell clones over time, we detected maximum expansion of persisting clones, whereas individual clones were reduced, in both $CD4^+$ (Fig. 4B, middle) and $CD8^+$ TCR (Fig. 4C, middle) repertoires in SAID patients at months 4–6, which then declined over the observation period (Supplementary Fig. 5B and C, left and middle). In non-SAID patients, we found no significant expansion of T-cell clones (Fig. 4B and C, left). Expansion of persistent T-cell clones in SAID patients was significantly different from non-SAID patients at months 4–6 for both $CD4^+$ and $CD8^+$ T cells and at months 18–24 for $CD4^+$ T cells (Fig. 4B and C, right). Regarding B cells, we did not observe significant expansions of persisting memory B-cell clones over the 18–24 months following ALEM treatment for SAID and non-SAID patients (Fig. 4D). However, as the occurrence of SAID peaks around Years 2–3,[11–13] we analysed further time points. To correct for the different timing of SAID occurrence, PBMCs were analysed 1–3 months after SAID diagnosis (Fig. 4E, SAID) and 9–12 months prior to diagnosis (Fig. 4E, before SAID). Comparing repertoire volumes occupied by the 'Top 100' memory B-cell clones, irrespective of their persistence over time, we discovered remarkable expansions shortly after the manifestation of SAID (Fig. 4E). The repertoire dynamics of 'Top 100' memory B-cell clones in the individual SAID patients were visualized at baseline, before and at the first repertoire analysis following manifestation of SAID in Supplementary Fig. 5B and C, right panel. In all five SAID patients we found that hyperexpanded and large B-cell clones occupied significantly increased volumes of the B-cell repertoire after SAID development. (Supplementary Fig. 5D). Hyperexpanded and large clones were defined as clones occupying more than 1% and up to 100% or 0.1% up to <1% of the repertoire, respectively. Thus, SAID patients demonstrate persistence and expansion of T-cell clones as early as after the first ALEM course, peaking around 4–6 months, as well as a consecutive expansion of memory B-cell clones shortly after SAID development.

In SAID patients hyperexpanded T-cell clones are overrepresented even at baseline

As we observed that the 'Top 100' persisting $CD4^+$ and $CD8^+$ T-cell clones of SAID patients showed a strongly pronounced expansion

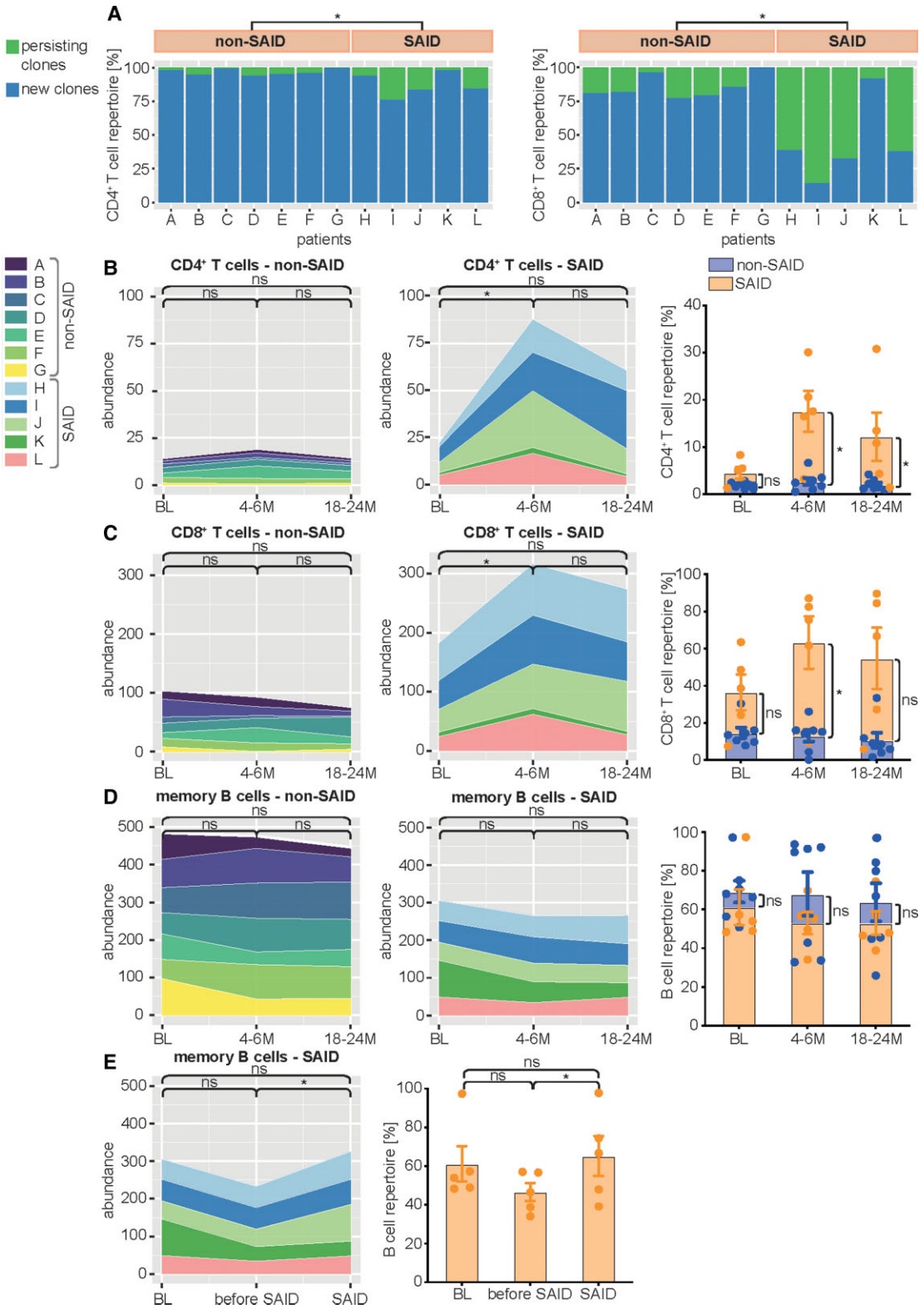

Figure 4 TCR and BCR repertoire changes in multiple sclerosis patients with or without manifestation of SAID after ALEM treatment. (**A**) Proportions of immune repertoire volumes occupied by persisting (green bars) versus new (blue bars) $CD4^+$ (*left*) and $CD8^+$ (*right*) T-cell clones in peripheral blood from non-SAID ($n = 7$, Patients A–G) and SAID patients ($n = 5$, Patients H–L) at 4–6 months after the first ALEM treatment course. Cumulative volumes (abundance) of the 'Top 100' persisting (**B**) $CD4^+$ T cell, (**C**) $CD8^+$ T cell and (**D**) 'Top 100' (irrespective of persistence) $CD19^+$ memory B-cell clones. Longitudinal values (baseline, 4–6 months, 18–24 months) for individual non-SAID (*left*, $n = 7$, Patients A–G), SAID (*middle*, $n = 5$, Patients H–L) and comparison of both (*right*) is depicted. (**E**) Cumulative volumes of 'Top 100' memory B-cell clones (irrespective of persistence) 1–3 months after SAID diagnosis (SAID) and 9–12 months before (pre-SAID). *Left*: Longitudinal values for individual patients; *right*: comparison of pre-SAID and SAID for the different time points ($n = 5$, Patients H–L). Bar graphs depict the median and IQR unless otherwise indicated. Statistical analysis was performed using the Mann–Whitney test (unpaired comparisons) and Wilcoxon signed rank test (paired comparisons). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns = not significant.

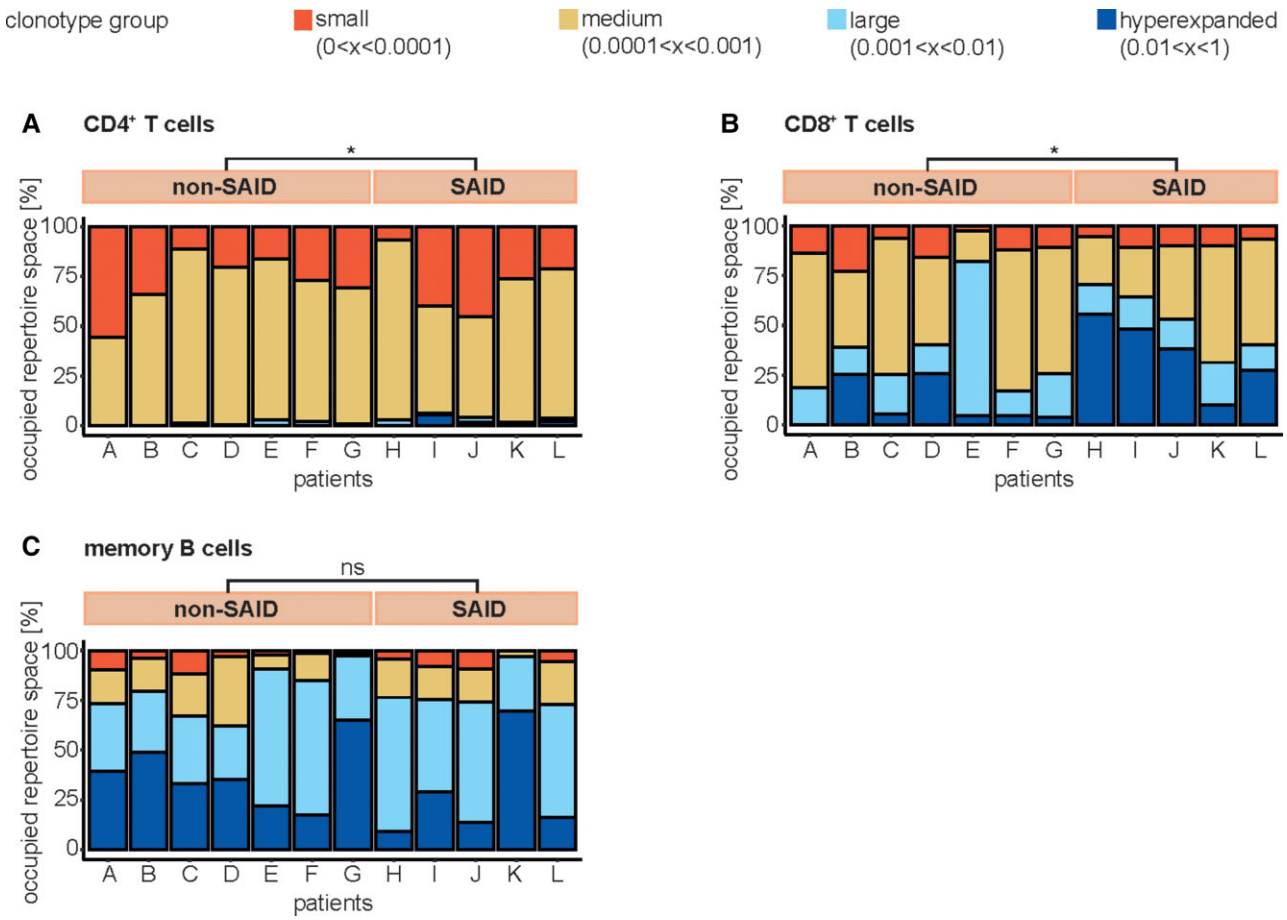

Figure 5 Hyperexpanded T-cell clones can be found in SAID patients already at baseline. Proportions of peripheral (**A**) $CD4^+$ T cell, (**B**) $CD8^+$ T cell and (**C**) $CD19^+$ memory B cell immune repertoire occupied by different clonotype groups in non-SAID ($n = 7$, Patients A–G) and SAID patients ($n = 5$, Patients H–L) at baseline. Bar graphs display proportions of different clonotypes: hyperexpanded T-cell clones (1% to 100% of the repertoire, dark blue) as well as T-cell clones with large (0.1% to <1%, light blue), medium (0.01% to <0.1%, yellow) and small expansion (<0.01%, red). Statistical analysis was performed by Mann–Whitney test (unpaired comparisons). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, ns = not significant.

after the first ALEM treatment course, we investigated whether hyperexpanded clones were already present before ALEM treatment. Remarkably even at baseline, hyperexpanded clones (Fig. 5, dark blue bars) occupied significantly higher proportions of both the $CD4^+$ (Fig. 5A) and $CD8^+$ (Fig. 5B) T-cell repertoire volumes in SAID patients compared to non-SAID patients. At baseline, hyperexpanded clones occupied between 10% and 55% of the $CD8^+$ and between 0% and 5% of the $CD4^+$ T-cell repertoires in SAID patients, whereas their abundance in non-SAID patients was significantly smaller in $CD8^+$ (0%–25%) and not detected within $CD4^+$ TCR repertoires. Differences of hyperexpanded clone abundance at baseline were not significant in the memory B-cell populations (Fig. 5C). Thus, hyperexpanded T-cell clones at baseline might indicate patients at risk for SAID development under ALEM-treatment.

Discussion

ALEM is a highly efficacious therapy for RRMS. However, the high risk of infusion-related adverse events and SAID significantly affects its risk–benefit ratio. We therefore sought a deeper understanding of the pathophysiologic mechanisms underlying SAID and potential biomarkers using a uniqure cohort of well-defined patients with longitudinal follow-up for ≥48 months, adjacent cellular and non-cellular biomaterials as well as longitudinal CSF analysis.

Consistent with previous reports, ALEM treatment led to pronounced adaptive rather than innate immune repertoire changes, which were in favour of regulatory components of the immune system. The CSF compartment mainly mirrored the immunological effects of ALEM in the periphery. Increased numbers of naïve $CD4^+$ T cells and B cells as well as a clonal renewal of $CD4^+$ T- and B-cell repertoires including diversification of the B-cell repertoire resembled features of AHSCT. However, in comparison to AHSCT the clonal renewal by ALEM appeared somewhat incomplete.

Comparing the differentiation of the immune architecture following depletion between SAID and non-SAID, the expansion of persisting T-cell clones at Month 12 was the most prominent alteration, whereas further in-depth cellular and non-cellular phenotyping parameters provided no clear immunological traits or signatures characterizing this difference. In SAID patients, however, hyperexpanded T-cell clones were present even at baseline; their expansion peaked around 4–6 months after ALEM treatment and was followed by a subsequent expansion of memory B-cell clones, which we detected shortly after SAID development.

In 2019, considerable side effects of ALEM including SAID led to a European Medicines Agency review process with subsequent restriction of ALEM use to, among others, RRMS patients without

any pre-existing autoimmune disorder other than multiple sclerosis.[58,59] Some national guidelines on multiple sclerosis therapy are consequently recommending ALEM use only if natalizumab or CD20-depleting agents are contraindicated.[60,61] Given the high efficacy and the advantages of potential treatment freedom, those regulatory processes support the urge for a deeper understanding of ALEM-related SAID, as ALEM is still a valuable treatment option for some patients.

Currently, B- and T-cell-centric hypotheses have been proposed to explain ALEM-related SAID. Considering the B-cell population, a surge of immature B-cell subsets in the absence of T-cell-mediated regulation is believed to give rise to autoreactive B cells and subsequently to antibody-driven SAID.[15,16,62] High IL-21 levels might influence B-cell function and IL-21 has been shown to be a driver for antibody-mediated autoimmunity.[63] Consistent with limited B-cell control by T_{reg}, we found that absolute T_{reg} numbers are significantly reduced at Month 12 after ALEM. The relative increase of T_{reg} as well as enhanced numbers of other regulatory immune cell subsets (T_R1, B cells, NK cells) might still not be sufficient to control emerging autoimmunity. However, we and others did not find differences in T_{reg} numbers and proportions that would discriminate between SAID and non-SAID patients.[33] Further, the B-cell-centric hypothesis does not explain the occurrence of significant—although less so compared to ALEM—frequencies of SAID after AHSCT, where early B-cell hyperpopulation has not been reported.[64] Further, it is not an explanation for the clearly T-cell-mediated SAID after ALEM, for example sarcoidosis or vitiligo.[17–20]

In the context of lymphopenia, some mechanisms that maintain host tolerance are temporarily suspended.[65] T-cell-centric hypotheses suggest that T-cell repopulation during ALEM-induced lymphopenia is dominated by homeostatic proliferation in response to self-antigens, which limits repertoire skewing and predisposes to autoimmune responses. In accordance, previous studies reported IL-21 facilitated homeostatic proliferation of T cells with reduced thymopoiesis and a restricted TCR repertoire as drivers of SAID.[1,29] At Month 12 after ALEM, we consistently observed reduced absolute recent thymic emigrant numbers as a surrogate for diminished thymic function. The work by Jones *et al.* further confirmed this by observing low Sj/β TCR excision circle ratios, which were significantly reduced in patients developing SAID. In our study, RTE numbers were not able to differentiate SAID and non-SAID patients. This might be explained by the fact that TCR excision circle ratios specifically reflect intrathymic T-cell proliferation, whereas recent thymic emigrant numbers are potentially compromised by peripheral cell division.[29] Consistent with our data, the same study reported a restriction of the TCR repertoire in multiple sclerosis patients with ALEM-related SAID in the first 12 months after treatment. In contrast to our study, those observations were restricted to the TCR repertoire and the time before SAID development.

To the best of our knowledge and although provisional in nature due to small sample size, our sequencing data provide for the first time deeper insight into the longitudinal changes of both the TCR and BCR repertoire in the context of SAID and support the synthesis of both hypotheses. Consistent with enhanced homeostatic proliferation as a driver for SAID, we observed a significantly increased persistence and expansion of $CD8^+$ more than of $CD4^+$ T-cell clones, which peaked around 4–6 months after ALEM and slowly declined over the observation period of 18–24 months. This implies that thymopoiesis does contribute to T-cell repopulation, at least in the long term.[66] Interestingly, core study data indicated that the SAID risk is mostly defined by the first ALEM treatment course, thus supporting the relevance of those early immunological processes.[56] The B-cell compartment, however, demonstrated no relevant expansion of persisting clones. Irrespective of clonal persistence, we found a clonal expansion of memory B-cell clones shortly after SAID diagnosis compared to pre-SAID time points. Whether those dynamics are just coincidence or are causally linked processes leading to autoimmunity remains to be elucidated. However, it is tempting to speculate that expanded T-cell clones might provide T-cell help for autoreactive B cells that bind the same auto-antigens resulting in B-cell proliferation and maturation, auto-antibody production and consequently SAID.[67] As the regeneration of $CD4^+$ T cells requires an average of 35 months,[9,68] the number of T-cell clones might not be sufficient to induce clonal B-cell expansion before years 2 or 3, thus potentially explaining the late occurrence of B-cell-mediated autoimmunity. This hypothesis is supported by our observation of delayed clonal memory B-cell expansion shortly after SAID diagnosis. As B-cell repopulation kinetics were not different in patients with or without SAID,[33,47] the observed B-cell hyperpopulation may be indicative of an autoimmunity permissive environment rather than representing the underlying pathophysiology.

For T-cell-mediated SAID, the pathophysiologic pathway might be different with expansion of autoreactive $CD4^+$ or $CD8^+$ T cells that directly induce autoimmune responses. Vitiligo is induced by antigen-specific and clonally expanded $CD8^+$ T cells.[69] In ALEM-related vitiligo, we previously found a predominance of single CD8 T-cell clones supporting a directly T-cell-induced pathogenesis after ALEM treatment.[20] In this case, the proposed strategy of CD20 depletion after ALEM to mitigate SAID should be less efficient.[70]

As the ALEM treatment procedure is standardized, patient-specific prerequisites are the most likely factor to be decisive for SAID risk and whether SAID pathogenesis is B- or T-cell-mediated. Consistent with this, we found hyperexpanded T-cell clones even at baseline in patients that went on to develop SAID. As previously suggested, impaired or dysfunctional thymopoiesis may contribute to this proautoimmune state and in combination with exaggerated homeostatic proliferation may facilitate SAID development under ALEM.[29] The individual genetic risk profile might be the basis for these factors and thus for the increased susceptibility to SAID in parallel to that of multiple sclerosis. In support of this, linked genetic risk factors and genetic associations have been described for multiple sclerosis and other autoimmune disorders such as Graves' disease, Goodpasture syndrome, immune thrombocytopenia and vitiligo.[10,71–74] As SAID is not a feature among patients receiving ALEM for cancer,[75] the high SAID rates in multiple sclerosis patients might reflect this proautoimmune background. Further, anti-thyroid autoantibodies detected before treatment predisposed patients to ALEM-related thyroid autoimmunity.[32]

The SAID frequencies after ALEM are about 10 times higher than after AHSCT. We here observed that in comparison to AHSCT the clonal renewal through ALEM was rather incomplete. In the context of extensive lymphopenia, this incomplete renewal might lead to increased probability of persisting autoreactive T-cell clones and their expansion by homeostatic proliferation. Further, we and others observed reduced thymopoiesis after ALEM treatment,[29] whereas AHSCT has been reported to enhance thymic function.[66] Thus, the ALEM-characteristic changes to the TCR repertoire might be associated with higher SAID risk. Consistent with this, cyclophosphamide leads to contrary effects on the TCR repertoire with reduction of high-frequency T-cell clones, high TCR diversity and sample overlap[76] and is associated

with lower SAID risk when included in AHSCT conditioning regimens than ALEM.[26]

In addition, previous treatments might increase the probability of autoreactive T cells that escape ALEM-mediated depletion. Consistent with this, fingolimod pretreatment was associated with a higher risk of SAID.[34] Fingolimod blocks lymphocyte egress from lymphoid tissues, which are supposedly less susceptible to ALEM,[77] and might thus prevent the depletion by ALEM.[78]

We are aware that the exploratory nature of our analyses, the small sample size, particularly also the subcohorts for extensive analyses and a potential sample bias in our tertiary centre represent our study's limitations. Sample size might have particularly affected multiparameter analyses such as Olink, although this supports the relevance of cellular analyses. The immunological changes in our ALEM cohort were largely comparable to previous reports,[2,29,47,48] thus we expect that our findings are transferable to ALEM treatment in general. Particularly for the TCR/BCR sequencing data, a validation in larger cohorts is required based on this hypothesis-generating project. Differences in single parameters might be related to variations in patient cohorts, marker selection and detection methods.

Our findings imply that hyperexpanded T-cell clones, already present at baseline, may be predictive for the development of SAID with ALEM treatment. Following exploration of these findings in a larger cohort, the analysis of hyperexpanded T-cell clones may be used as a biomarker to exclude patients prone to SAID from ALEM therapy at baseline. However, sequencing technologies are expensive and cut-offs for normal values do not exist. Thus, a large-scale application of our findings in clinical practice might rather be reserved for the future.

In conclusion, our findings support ALEM-specific immune repertoire changes (restriction of TCR repertoire, reduced thymopoiesis, homeostatic proliferation, disparate dynamics of clonal T- and B-cell expansion) that provide a conceptual basis for ALEM-related SAID development in predisposed patients, combining current B- and T-cell-centric hypotheses. The deeper understanding of the immunological changes by ALEM may be instrumental in guiding its optimal use as a durable therapeutic strategy.

Acknowledgements

We thank all patients who participated in this study. The authors thank Gabriele Berens, Kirsten Weiß, Arne Seeger, Tobias Schilling, Tanja Wandelt, Christiane Schulze Weppel, Aline Kiese, Alina Teich and Lena Schünemann (University of Münster), as well as Katharina Raba and Nora Hinssen (Heinrich Heine University Düsseldorf) for excellent technical assistance. Also, the advice of Dr Vera Balz (Heinrich Heine University Düsseldorf) regarding NGS library preparation is highly appreciated. Further we thank Dipl.-Stat. Maria Eveslage (Institute of Biometry and clinical research, University of Münster) for statistical advice.

Funding

This work was supported by the Else-Kröner Fresenius Foundation (2018_A03 to T.R.), the German Research Foundation (RU 2169/2-1 to T.R.; SFB TR128 projects A9, A10, B1, B6, Z2), the Bundesministerium für Bildung und Forschung (01GI1603D to T.R., H.W. and S.G.M.; FKZ01FI1603A to H.W., L.K. and C.C.G.; 01EC1901A to T.R.), in part by DFG grant number ME4050/7-1, under the frame of E-Rare-3, the ERA-Net for Research on Rare Diseases and Genzyme Therapeutics Ltd, UK (Alemtuzumab: Reprogramming the Immune Repertoire in MS, GZ-2016-11570, to N.G.; Alemtuzumab in autoimmune inflammatory neurodegeneration: mechanisms of action and neuroprotective potential, 2014-000709-10, ALAIN to T.R., H.W. and S.G.M.).

The funding by Genzyme had no impact on conducted experiments or drafting the content of this manuscript.

Competing interests

T.R. has received honoraria and consultation fee support from Celgene/BMS, Biogen, Roche, Sanofi Aventis, Alexion, Novartis, and Teva and has also received personal support from Merck Serono. S.P. received travel reimbursements and lecturing honoraria from Sanofi Genzyme and Biogen, Merck and Mylan and received research support from Diamed. A.S.M. and S.Ba. received travel expenses and research support from Novartis. C.N., C.B.S., A.W. and M.H. report no conflict of interest. T.M. reports no competing interests. N.M. received honoraria for lecturing and travel expenses for attending meetings from Biogen Idec, GlaxoSmith Kline, Teva, Novartis Pharma, Bayer Healthcare, Genzyme, Alexion Pharmaceuticals, Fresenius Medical Care, Diamed and BIAL, and has received financial research support from Euroimmun, Fresenius Medical Care, Diamed, Alexion Pharmaceuticals and Novartis Pharma. M.L., M.R., F.S. report no conflict of interest. J.K. received honoraria for lecturing from Biogen, Novartis, Mylan and Teva and financial research support from Sanofi Genzyme. C.C.G. received speaker honoraria from Mylan, Bayer Healthcare and Sanofi-Genzyme and travel/accommodation/meeting expenses from Bayer Healthcare, Biogen, EUROIMMUN, Novartis, Sanofi-Genzyme and Roche. She also received research support from Biogen and Novartis. L.K. received compensation for serving on Scientific Advisory Boards for Genzyme. She received speaker honoraria and travel support from Novartis, Merck Serono and CSL Behring. She receives research support from Novartis. P.M. received travel support and speaker honoraria from unrestricted educational activities organized by Novartis, Bayer HealthCare, Bayer Pharma, Biogen Idec, Merck-Serono and Sanofi Aventis; he also discloses consulting to Magenta Therapeutics and Jasper Therapeutics. T.S.H. received research and travel support from Biogen and Novartis Pharma. N.S. received research and travel support from Biogen and Novartis Pharma. G.M.z.H. reports no conflict of interest. S.B. has received funding for travel expenses for attending meetings from Merck Serono and honoraria from Biogen Idec, Bristol Meyer Squibbs, Merck Serono, Novartis, Roche, Sanofi Genzyme and TEVA. His research is funded by Deutsche Forschungsgemeinschaft (DFG) and Hertie foundation. N.G. received honoraria for speaking at scientific meetings, serving at scientific advisory boards, travel and/or research support from Biogen, BMBF ('Connect Generate'), HHU-Forschungskommission, International Progressive MS Alliance (BRAVEinMS), Novartis, Roche, Sanofi-Genzyme and the Wellcome Trust. S.G.M. has received honoraria for lecturing, travel expenses for attending meetings and financial research support from Almirall, Bayer Health Care, Biogen, Diamed, Fresenius Medical Care, Genzyme, Merck Serono, Novartis, Novo Nordisk, ONO Pharma, Roche, Sanofi-Aventis and Teva. H.W. receives honoraria for acting as a member of the Scientific Advisory Boards for Biogen, Evgen, Genzyme, MedDay Pharmaceuticals, Merck Serono, Novartis, Roche Pharma AG and Sanofi-Aventis as well as speaker honoraria

and travel support from Alexion, Biogen, Cognomed, F. Hoffmann-La Roche Ltd, Gemeinnützige Hertie-Stiftung, Merck Serono, Novartis, Roche Pharma AG, Genzyme, TEVA and WebMD Global. H.W. is acting as a paid consultant for Actelion, Biogen, IGES, Johnson & Johnson, Novartis, Roche, Sanofi-Aventis and the Swiss Multiple Sclerosis Society. His research is funded by the German Ministry for Education and Research (BMBF), Deutsche Forschungsgemeinschaft (DFG), Else Kröner Fresenius Foundation, Fresenius Foundation, the European Union, Hertie Foundation, NRW Ministry of Education and Research, Interdisciplinary Center for Clinical Studies (IZKF) Muenster, Biogen, GlaxoSmithKline GmbH, Roche Pharma AG and Sanofi-Genzyme.

Supplementary material

Supplementary material is available at *Brain* online.

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