

RESEARCH ARTICLE

Pharmacodynamic effects of daclizumab in the intrathecal compartment

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Introduction

Application of the “No evidence of disease activity” (NEDA)¹ outcome to Phase III clinical trials² or longitudinal multiple sclerosis (MS) cohorts³ demonstrated remarkably prevalent (e.g., 78% in 2-year trial of daclizumab or >90% in 7-year natural history cohort) residual MS disease activity on current disease-modifying therapies (DMTs). The mechanism(s) for this residual activity are currently unknown. Based on generally strong effects of DMTs on magnetic resonance imaging (MRI) contrast-enhancing lesions (CELs), which are considered a

Abstract

Objective: It was previously demonstrated that daclizumab therapy normalizes cellular cerebrospinal fluid (CSF) abnormalities typical of multiple sclerosis (MS) in the majority of treated patients. However, CSF cells represent only the mobile portion of intrathecal immune responses. Therefore, we asked whether daclizumab also reverses compartmentalized inflammation and if not, whether residual inflammation correlates with clinical response to the drug. **Methods:** Forty MS patients treated with an intravenous or subcutaneous injection of daclizumab were followed for up to 16 years in two open-label clinical trials. MRI contrast-enhancing lesions (CELs), clinical scales, and CSF biomarkers quantified residual disease. **Results:** Rapid decreases in CELs, sustained throughout the observation period, were observed with daclizumab treatment. Daclizumab therapy induced modest but statistically significant ($P < 0.0001$) decreases in CSF levels of T-cell activation marker CD27 and IgG index. Interleukin 2 (IL-2) CSF levels increased from baseline levels during treatment, consistent with reduced IL-2 consumption by T cells, as a consequence of daclizumab's saturation of high-affinity IL-2 receptors. CSF levels of IL-12p40, chitinase-3-like protein-1 (CHI3L1), chemokine C-X-C motif ligand 13, and neurofilament light chain (NFL) were also significantly reduced by daclizumab. Among them, inhibition of CHI3L1 correlated with inhibition of NFL and with lack of disease progression. **Interpretation:** These observations confirm daclizumab's direct pharmacodynamic effects on immune cells within central nervous system tissues and identify inhibition of CSF biomarkers of myeloid lineage as a stronger determinant of reduction in clinical MS activity than inhibition of biomarkers of adaptive immunity.

biomarker of MS-related inflammation, the prevailing hypothesis implies neurodegeneration, rather than subclinical inflammation as the cause of residual MS activity.

However, we recently demonstrated that intrathecal inflammation, as measured by cerebrospinal fluid (CSF) biomarkers specific for cells of adaptive immunity (i.e., T and B cells and plasma cells) and innate immunity (e.g., microglia/macrophages), is as prevalent in patients who lack CELs (including primary progressive MS [PPMS] patients) as it is in relapsing–remitting MS (RRMS).⁴ This observation prompted a hypothesis that subclinical MS disease activity may be mediated by residual inflammation

compartmentalized to the central nervous system (CNS) and therefore mostly inaccessible to current DMTs. To test this hypothesis, we investigated a group of patients with sustained clinical response to daclizumab (Zinbryta), a humanized monoclonal antibody that selectively binds to the high-affinity interleukin-2 (IL-2) receptor subunit (CD25),^{5–7} and has been recently approved for the treatment of relapsing forms of MS.

Methods

Subjects

All subjects (Table 1) participated in one of the two NIH open-label, baseline versus treatment Phase I/II trials of daclizumab: ZAP-MS trial (intravenous [IV] daclizumab [Zenapax] 1 mg/kg q 4 weeks; ClinicalTrials.gov identifier NCT00071838; $n = 16$) or daclizumab High Yield Process (DAC-HYP) trial (subcutaneous [SC] daclizumab [Zinbryta] 150 mg q 4 weeks; ClinicalTrials.gov identifier NCT01143441; $n = 32$). (Note: ZinbrytaTM has a different form and structure than the earlier form of daclizumab – Zenapax). The DAC-HYP trial consisted of two groups of subjects: Cohort A (subjects switched from Zenapax [either as part of or outside of ZAP-MS trial]) and Cohort B (de novo initiation of Zinbryta). We used all CSF samples we collected in these protocols (Fig. 1).

The study was approved by the NIH Institutional Review Board, and all patients provided written informed consent.

CSF collection and processing

CSF was collected during pretreatment baseline (month 0 [Mo0]), and at different time points after initiation of treatment as outlined in the CONSORT diagram (Fig. 1).

Table 1. Demographic data.

	Zenapax only/and Zinbryta	Zinbryta only
Patients, n (females)	27 (16)	13 (4)
Age at start of treatment (years), median (range)	41.7 (27.9–54.8)	38.9 (26.6–52.5)
Disease duration at start of treatment (years), median (range)	7.5 (0.2–23.4)	8.4 (1.4–32.8)
Total duration of treatment (years), median (range)	82.2 (2.4–178.3)	39.4 (20.0–68.3)
Duration under Zenapax (months), median (range)	22.0 (2.4–134.8)	N.A.
Duration under Zinbryta (months), median (range)	69.6 (27.4–70.6)	39.4 (20.0–68.3)

To assess long-term effects of daclizumab therapy, we collected CSF from all subjects who received daclizumab for more than 12 months (>1 year–11 years; average 5.4 years) and called this time point long-term treatment (LTT). To assess stability of such LTT we also collected CSF sample 1 year later (LTT + 1y).

CSF was processed according to written standard operating procedures (SOP).^{8,9} A portion of CSF was sent to measure IgG index. Research CSF aliquots were assigned alpha-numeric codes, centrifuged at 335g for 10 min at 4°C within 15 min of collection, and stored in polypropylene tubes at –80°C until blinded analyses.

Measurement of daclizumab concentration and other biomarkers

Electrochemiluminescent assays were developed or optimized to quantify the concentrations of serum and CSF biomarkers using the Meso Scale Discovery[®] platform (MSD; Meso Scale Diagnostics, Rockville, MD, <https://www.mesoscale.com/>) as described previously.⁴ The concentration of interleukin 12p40 (IL-12p40) was measured by MSD V-plex using the manufacturer's protocol. The assays for CSF soluble CD21 (sCD21), sCD27, sCD14, sCD163, chitinase-3-like protein 1 (CHI3L1), and chemokine C-X-C motif ligand 13 (CXCL13) and serum/CSF daclizumab were developed through de novo.⁴ Neurofilament light protein (NFL) was measured using a UmanDiagnostics ELISA (UmanDiagnostics AB Sweden). The IL-2 was measured by Singulex[®] IL-2 kit (Singulex, Alameda, CA), following the manufacturer's protocol.

All samples excluding IL-12p40 and NFL were run in duplicate. Each assay contained a minimum of two additional reference samples per plate to evaluate intra- and interassay reliability, which are depicted in Table S1.

Threshold for abnormal values was based on mean + 2 standard deviation (SD) of healthy donors' data⁴ (HD; $n = 43$, yielding 56 different CSF samples as some HD contributed two samples 1 year apart; Table S2). The HD samples were collected by the same group of investigators using identical SOP and prospectively assigned the alphanumeric code that does not allow laboratory personnel to differentiate MS from HD samples, resulting in random distribution of MS and HD samples on assay plates.

After observing no differences between Zenapax- and Zinbryta-treated subjects in a pharmacokinetic marker (CSF daclizumab levels) and pharmacodynamic marker (CSF IL-2), we merged these cohorts for all further analyses, combining data from Mo6.5 in ZAP-MS trial with Mo12 of DAC-HYP trial as short-term treatment (STT).

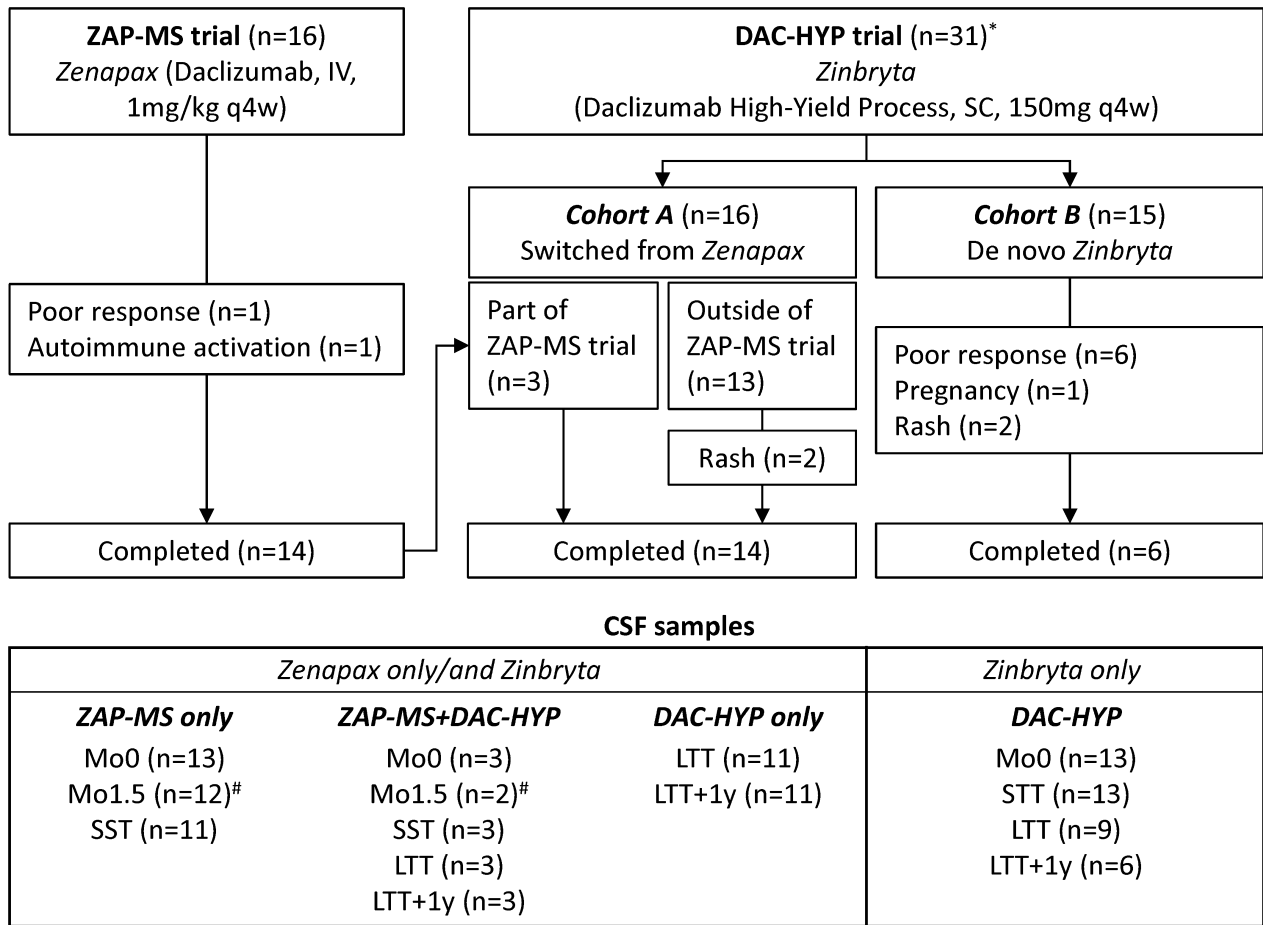


Figure 1. CONSORT diagram. STT, short-term treatment (6.5–12 months); LTT, long-term treatment (>1 to 11 years, on average 5.4 years); LTT + 1y, 1 year after LTT time point (to assess stability of changes induced by long-term daclizumab therapy); IV, intravenous; SC, subcutaneous; Mo, month; *32 subjects were consented, 31 were dosed with Zinbryta; #optional lumbar puncture.

Combinatorial weight-adjusted disability score (CombiWISE)

CombiWISE¹⁰ is a sensitive, continuous composite scale developed and optimized by machine learning that ranges from 0 to 100 and combines EDSS,¹¹ Scripps NRS,¹² 25 foot walk, and nondominant hand data for nine hole peg test. CombiWISE slope was calculated from linear regression equations for patients with more than 3 CombiWISE data points.

MRI

Routine spin-echo and gradient-echo T1-weighted images were collected following intravenous administration of 0.1 mmol/kg gadopentetate dimeglumine as described previously.¹³ CELs were quantified according to the consensus of two neurologists with neuroimmunology/MS

subspecialty training (B. B. and M. K.) based on precontrast T1- and T2-weighted images.

Statistical analyses

Repeated measures analysis of variance (ANOVA; SAS 9.3) was used to evaluate the change in biomarker and clinical score variables from baseline (Mo0) to the follow-up visits. Dunnett’s method was applied to each biomarker and each group to adjust for multiple comparisons with the baseline, but not for multiple biomarkers. The median changes in the results were computed only from patients who have baseline and LTT visit points. We also considered mixed effects model with time as a continuous variable and intercept and slope as random variables. However, because of nonlinear relationships between time and biomarkers, we resolved to treat time as categorical variable (with combining some points) to assess

differences on CSF biomarkers induced by short-term versus long-term daclizumab therapy. Box–Cox transformation was applied to biomarker and clinical score variables.

Results

Pharmacokinetic and pharmacodynamic data

(A) Pharmacokinetic marker: Selected dosing leads to measurable CSF concentrations of daclizumab, representing approximately 0.1% of serum levels

Intravenously administered daclizumab could be detected intrathecally already at Mo1.5 after the first dose (median 15.1 ng/mL in CSF, median 9.97 μ g/mL in serum; Fig. 2A–B, left). Even though this cohort changed the injection method from IV administered version of daclizumab (i.e., Zenapax, 1 mg/kg IV q 4 weeks at Mo1.5, Mo6.5 [STT]) to subcutaneously administered newer version of the drug (i.e., Zinbryta, 150 mg SQ q 4 weeks at LTT and LTT+1y), the median concentrations of serum and CSF daclizumab did not change significantly in this small cohort.

The cohort of subjects treated only with Zinbryta also increased serum and CSF daclizumab concentrations to similar levels at the first follow-up time point (Mo12 [STT]) and kept the level throughout the treatment course (median 15.1 ng/mL in CSF, median 17.8 μ g/mL in serum at STT; Fig. 2A–B, right). CSF and serum daclizumab concentrations correlated strongly (Fig. 2C), supporting the notion that approximately 0.1% of serum daclizumab crosses the blood–CSF barrier.

(B) Pharmacodynamic marker: daclizumab therapy significantly increases serum and CSF levels of IL-2 without any correlation between the two compartments

As daclizumab limits consumption of IL-2 by T cells¹⁴ without limiting the ability of activated T cells or dendritic cells to secrete IL-2,¹⁵ we predicted that daclizumab therapy should lead to increased IL-2 levels in biological fluids. However, this prediction was difficult to prove because the levels of IL-2 were below the detection limits of standard immunoassays. Here, we employed a “digital ELISA” (i.e., Singulex: single molecule counting technology; <https://www.singulex.com/>) that measures low abundance analytes in femtoliter-sized wells with a theoretical detection limit of single molecules.

We observed that serum IL-2 levels significantly increased after the initiation of daclizumab treatment (median 0.71 pg/mL in Zinbryta only cohort at Mo12 [STT], median 0.77 pg/mL in Zenapax only/and Zinbryta

cohort at Mo1.5, Fig. 3A–B) and remained elevated till the end of the study. CSF IL-2 levels also increased at Mo1.5 (median 0.42 pg/mL) in Zenapax only/and Zinbryta cohort and at Mo12 (STT, median 0.34 pg/mL) in Zinbryta only cohort and remained elevated till LTT.

Finally, there was no correlation between CSF and serum IL-2 levels (Fig 3C).

Effects of daclizumab on CSF biomarkers of innate immunity: daclizumab inhibits biomarkers secreted by activated myeloid cells without altering M1 versus M2 phenotypic markers

Because measured pharmacokinetic and pharmacodynamic data were comparable, we combined data from Mo6.5 on Zenapax and data from Mo12 on Zinbryta into a single STT time point. We also calculated the proportion of patients with abnormally high values of biomarkers compared to normal range (mean + 2 standard deviation of HD, Table S2), shown in Figure 4 as pie charts with dark fill representing proportion of patients with abnormally elevated biomarkers.

We investigated the effect of daclizumab on CSF biomarkers previously validated as biomarkers of MS-related inflammation. First, we focused on biomarkers of myeloid lineage (Fig. 4A–D): proposed phenotypical surface markers of M1 (sCD14; expressed on macrophages activated by proinflammatory stimuli) versus M2 macrophages (sCD163; expressed on alternatively activated macrophages), even though we recognize that M1/M2 classification, as well as the ability of these surface markers to reliably reflect M1 versus M2 phenotypes, are oversimplifications. Daclizumab marginally and transiently decreased sCD163 levels, but otherwise did not significantly affect markers of M1 versus M2 lineage (note that the proportion of patients with abnormal values of these biomarkers shifted between STT and LTT, but this apparent shift is due to contribution of patients from different cohorts, see CONSORT diagram).

Next, we measured biomarkers secreted by activated cells of myeloid lineage (macrophages, microglia, and myeloid dendritic cells [DCs]): cytokine IL-12p40 and CHI3L1, a secreted glycoprotein linked to inflammation, tissue remodeling, and fibrosis. Both of these myeloid activation markers decreased significantly (IL-12p40 [median –58.8%, $P < 0.0001$ at LTT] and CHI3L1 [median –16.9%, $P = 0.0007$ at LTT]), during daclizumab therapy and remained in the normal range for the majority of treated patients.

We also measured levels of CHI3L1 in matching Mo0, STT, and LTT serum samples (data not shown), revealing no statistically significant change between baseline and

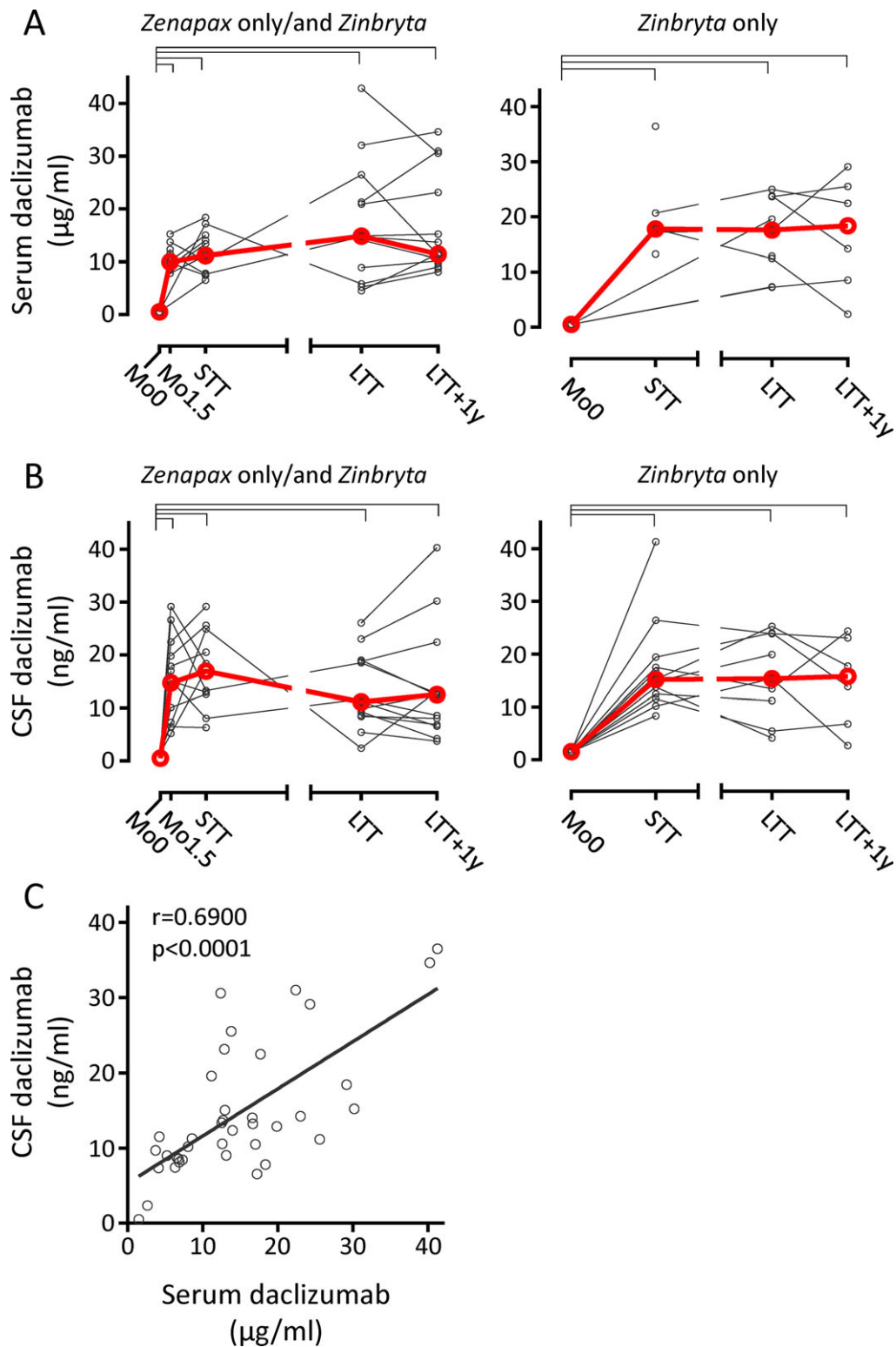


Figure 2. Daclizumab (DAC) concentrations in serum and CSF are comparable between intravenous and subcutaneous regimens. Daclizumab level in serum (A) and CSF (B) by intravenous (Zenapax) and subcutaneous (Zinbryta) injection. Red lines show median values of biomarkers at each follow-up visit. Black brackets represent statistical significance ($P < 0.05$) based on adjusted P -value (Dunnett’s method). Concentration of DAC in serum and CSF was compared to baseline (visit Mo0) and each follow-up visit (Zenapax only/and Zinbryta cohort: Mo1.5, Mo6.5 [STT], long-term treatment [LTT; >1–11 years], and 1 year after [LTT+1y]; Zinbryta only cohort: Mo12 [STT], LTT, and LTT+1y). (C) Pearson’s correlation between CSF and serum DAC concentrations.

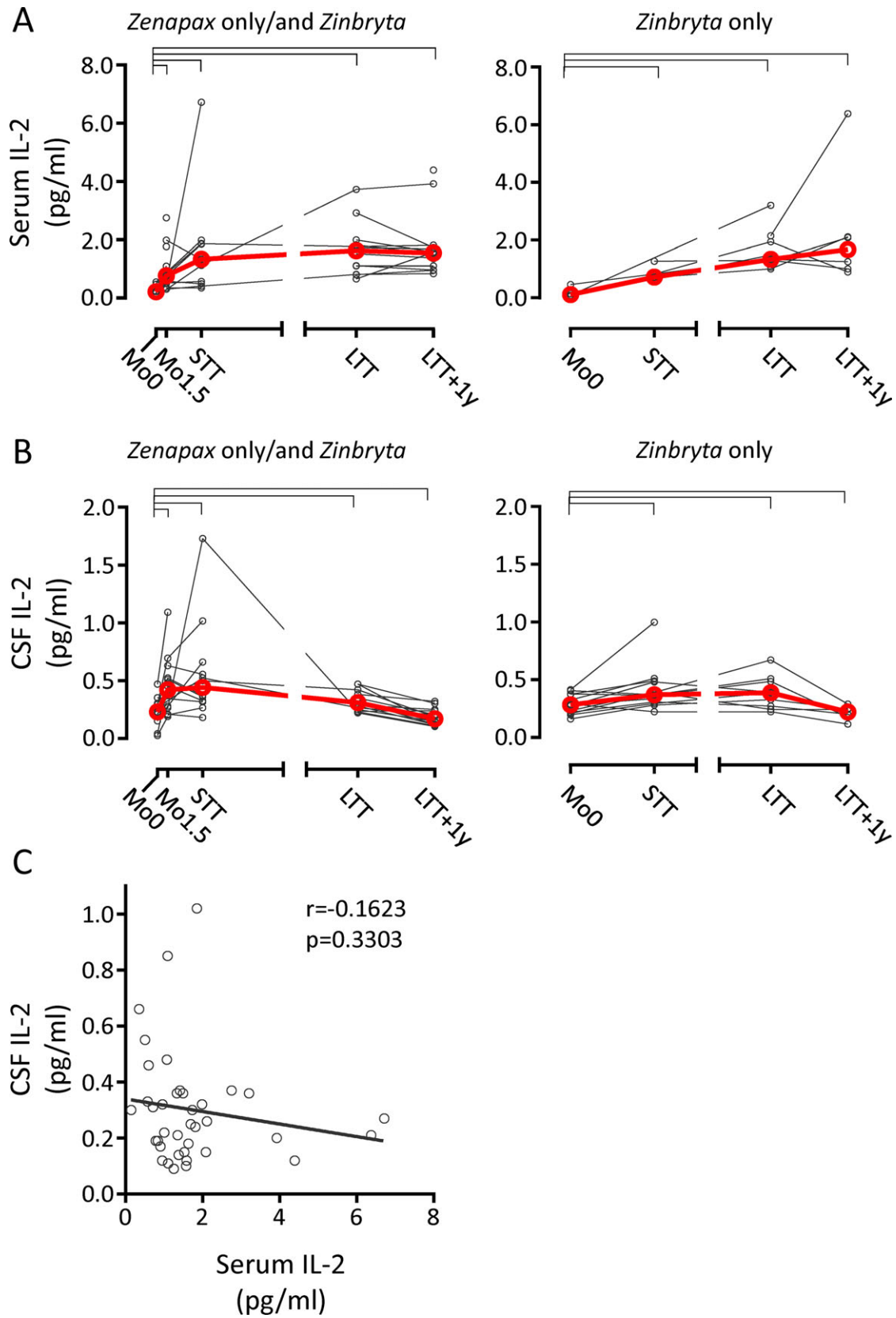


Figure 3. Cerebrospinal fluid (CSF) IL-2 is independently produced intrathecally and its levels are elevated following daclizumab therapy. IL-2 level in serum (A) and CSF (B) by intravenous (Zenapax) and subcutaneous (Zinbryta) injection. Red lines show median values of biomarkers at each follow-up visit. Black brackets represent statistical significance ($P < 0.05$) based on adjusted P -value (Dunnett's method). Concentration of IL-2 in serum and CSF was compared to baseline (visit Mo0) and each follow-up visit (Zenapax only/and Zinbryta cohort: Mo1.5, Mo6.5 [STT], long-term treatment [LTT; >1–11 years], and 1 year after [LTT+1y]; Zinbryta only cohort: Mo12 [STT], LTT, and LTT+1y). (C) Pearson's correlation between CSF and serum IL-2 concentrations. Note that even though the graph does not display the highest measured IL-2 level (1.75 in Fig. 3B), the correlation coefficient and P -value include this outlier.

STT ($P = 0.2922$) or baseline and LTT ($P = 0.2645$). Furthermore, the correlation between serum and CSF levels of CHI3L1 for all measured samples was weak and did not reach statistical significance (Pearson's $r = 0.2066$, $P = 0.0659$).

CSF biomarkers of adaptive immunity are decreased, but remain above healthy donor range in majority of treated patients

To assess effect of daclizumab on cells of adaptive immunity, we measured surface molecules released specifically from B cells (sCD21) and activated T cells (sCD27, shed from activated more than resting T cells and from CD8 T cells more than CD4 T cells).⁴ While sCD21 is a specific B-cell marker, it is highly expressed on naïve cells and its expression diminishes as B cells mature. Thus, class-switched memory B cells express very little, if any, sCD21 and therefore may not secrete this biomarker. Consequently, we also measured IgG index, which quantifies secretion of immunoglobulins by plasma cells and plasmablasts.

While sCD21 was not affected by daclizumab therapy, we observed significant inhibition of sCD27 through the observation period (maximum reduction at LTT median -37.5% , $P < 0.0001$; Fig 4E–F). However, the majority of treated patients had persistent elevations of CSF sCD27 levels above normal range. Finally, the level of IgG index decreased after daclizumab treatment (median -26.3% , $P < 0.0001$ at LTT; data not shown as this finding was partially presented previously¹⁶).

MS-related biomarkers CXCL13 and NFL are normalized in majority, but not all treated patients

CXCL13 is a chemokine typically elevated in MS CSF that is secreted by follicular DCs in lymphatic tissue, including in tertiary lymphoid follicles, but also by activated T and B cells. CSF levels of CXCL13 declined posttreatment (median -70.0% , $P = 0.0527$ at LTT; Fig. 4G), although this change did not reach statistical significance.

The axonal damage marker, NFL, significantly decreased (median -59.3% , $P < 0.0001$ at LTT; Fig. 4H) and

remained in normal range for the majority (but not all) of treated patients through the duration of follow-up.

Linking changes in CSF biomarkers to clinical efficacy of daclizumab therapy

Consistent with strong efficacy on CELs ($P < 0.0001$, median 1.5 at Mo0, median 0.0 at LTT), we observed no significant disability progression, when measured either by EDSS, or more sensitive CombiWISE scale (Fig. 5A–B, Table S3). However, these measurements exclude subjects who withdrew from this long-term study, some for progression of disability (Fig. 1).

When investigating the relationship between CSF biomarkers and disability progression, we observed that only CHI3L1 showed biologically meaningful correlations with the change in clinical disability measures (Fig. 5C–D). CHI3L1 levels correlated with NFL ($r = 0.5052$, $P = 0.0061$) during the observation period (Fig. 5C–D) although change in CHI3L1 was a better predictor of clinical response than change in NFL (Fig. 5C–D). Even higher and highly statistically significant correlation exists between change in NFL and CHI3L1 ($r = 0.6769$, $P < 0.0001$, Fig. 4F). Intriguingly, pretreatment CSF levels of CHI3L1 also correlated with observed clinical response measured as CombiWISE change, although this correlation was not very strong (Fig. 5E).

Discussion

The current study demonstrates that approximately 0.1% of systemically administered daclizumab reaches the intrathecal compartment, consistent with other monoclonal antibodies such as rituximab.^{17,18} The observed rise in serum and CSF IL-2 levels provides evidence for the ability of daclizumab to inhibit consumption of IL-2 by activated T cells¹⁴ in both systemic and intrathecal compartments. Relatively high median CSF concentrations of IL-2 (0.34–0.42 pg/mL) in comparison to measured serum IL-2 concentrations (0.71–0.77 pg/mL) suggest that the source of IL-2 is intrathecal, rather than blood derived. Assuming that IL-2 and IgG use similar passive transport across the blood–brain barrier, based on their molecular

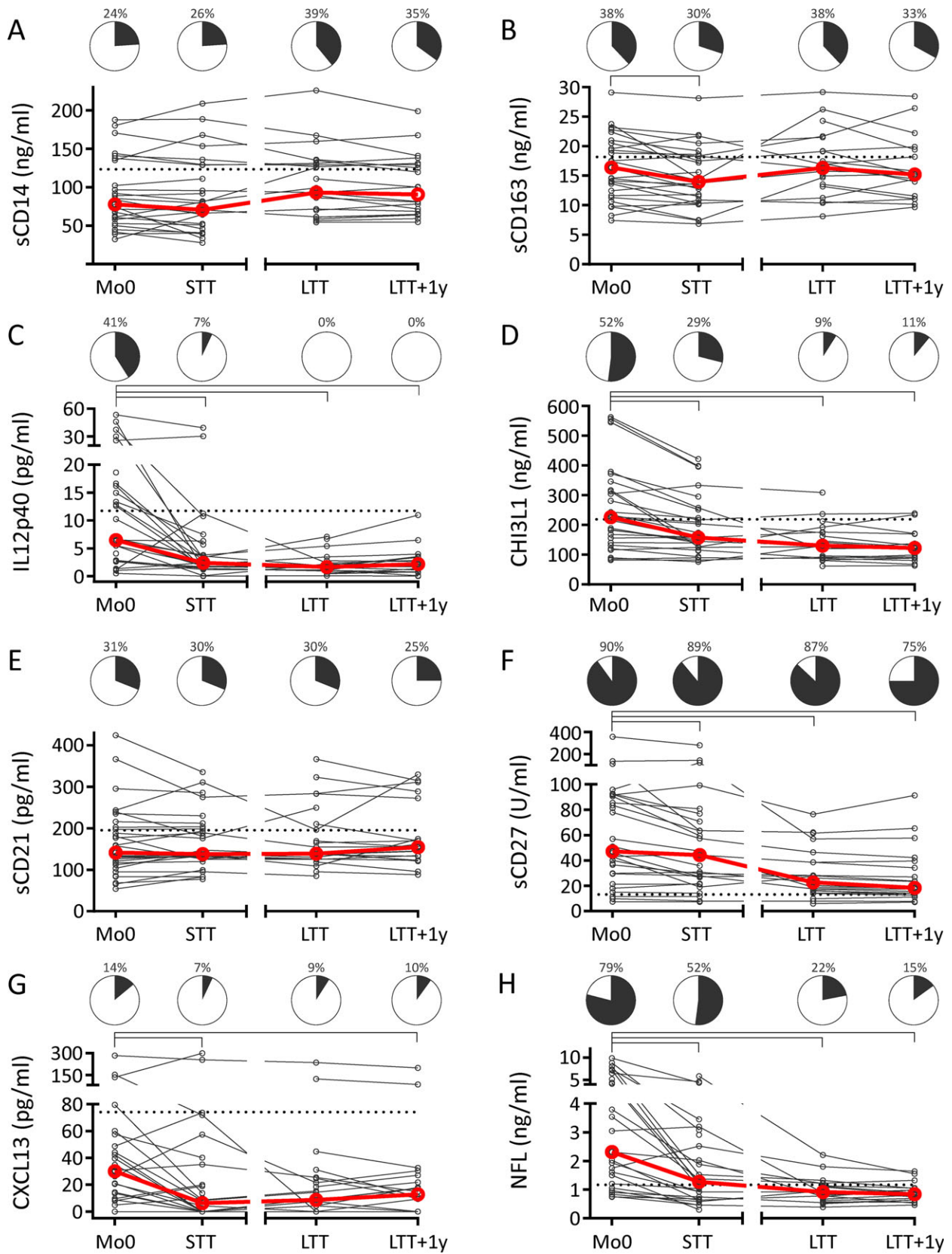


Figure 4. Effects of daclizumab on CSF biomarkers for innate and adaptive immunity. Concentration of cerebrospinal fluid (CSF) innate immune activity related soluble markers, soluble (A) CD14 (sCD14), (B) sCD163, (C) IL-12p40, (D) chitinase 3-like protein 1 (CHI3L1), (E) soluble CD21 (sCD21), (F) sCD27, (G) C-X-C motif chemokine 13 (CXCL13), and (H) neuroaxonal marker neurofilament light chain protein (NFL), were measured in coded samples using electrochemiluminescence assay or ELISA. Red lines show median values of biomarkers at each follow-up visit. Black brackets represent statistical significance ($P < 0.05$) based on adjusted P -value (Dunnett's method). Concentrations of each biomarker was compared between baseline (Mo0) and each follow-up visit (short-term treatment [STT; 6–12 months], long-term treatment [LTT; >1–11 years], and 1 year later of LTT [LTT+1y]). The proportion (%) of patients with values above normal range (dark portion of pie charts) at each follow-up visit is shown above pie charts. Dotted lines represent the upper limit of normal values, calculated as mean + two standard deviations of the healthy donor cohort (sCD14: 123.28 ng/mL, sCD163: 18.1 ng/mL, IL12p40: 11.68 pg/mL, CHI3L1: 224.14 ng/mL, sCD21: 195.33 pg/mL, sCD27: 13.16 U/mL, CXCL13: 74 pg/mL, NFL: 1167.62 pg/mL). Note: the proportion of patients with abnormal values of these biomarkers shifted between STT and LTT, but this apparent shift is due to contribution of patients from different cohorts, see CONSORT diagram.

weights (~15.5 kDa for IL-2 vs. 150 kDa for IgG) we could estimate that up to 1% of serum IL-2 concentrations gain access to the intrathecal compartment, which represents 0.008 pg/mL, or less than 3% of IL-2 levels we measured in the CSF. Our conclusion that measured increase in CSF IL-2 levels is likely derived from the intrathecal compartment is also supported by the observed lack of correlation between blood and CSF IL-2 levels. The source of this intrathecal IL-2 is not known, but may represent activated T cells or dendritic cells. The interesting question is how low measured intrathecal concentration of daclizumab (i.e., 15.1 ng/mL) can inhibit consumption of IL-2 by intrathecal T cells. The answer resides in the previously published observations that in RRMS patients, virtually all CSF T cells had completely blocked CD25 after initiation of dosing by former (i.e., Zenapax)¹⁹ or current (i.e., Zinbryta)⁸ daclizumab formulations. This agrees with another published observation that in contrast to progressive MS, where majority of patients have inflammation compartmentalized to CNS tissue with little turnover between blood and intrathecal compartment, majority of RRMS patients have communicating inflammation, where immune cells are constantly accessing intrathecal compartment from the blood, as already activated T or B cells.²⁰ Consequently, the CD25 on these activated immune cells is blocked in the blood, where the concentration of daclizumab is saturating.

Furthermore, daclizumab potentially inhibits intrathecal immunity but does not normalize biomarkers of adaptive immunity, demonstrating the presence of residual T and B cells/plasma cells in the intrathecal compartment even in clinically stable patients that do not form new MS lesions on MRI imaging for years. Such residual inflammation reflected by T and B cell/plasma cell biomarkers did not correlate with clinical efficacy. The most successful CSF biomarker in this regard was CHI3L1, a marker of activated myeloid cells, previously implicated in tissue remodeling and fibrosis.

The consistency of results between Cohorts A and B (Fig. 1) suggests that the mechanism of action (MOA) is comparable between the two tested daclizumab preparations.

We acknowledge the following limitations: (1) *Potential biases introduced by patient dropouts.* The purpose of the NIH clinical trial “Investigating mechanism of action of DAC-HYP in the treatment of high-inflammatory MS” (NCT01143441) was to define the MOA of the new generation of SC daclizumab (Zinbryta) while allowing patients who experienced strong clinical response to earlier generation of IV daclizumab (Zenapax) before it was taken off the market (Cohort A) to transition to Zinbryta prior to its commercial availability. In recognition that Cohort A was self-selected for high efficacy of daclizumab, we recruited Cohort B of de novo dosed patients to mitigate potential bias. The original protocol to which all patients consented included only CSF examination for Cohort B before and 12 months (STT time point) after initiation of Zinbryta. When it became clear that Zinbryta will not reach regulatory approval before completion of the protocol, in view of patients' requests to continue Zinbryta, we extended the protocol until Zinbryta gained regulatory approval. This extension included two additional LPs (LTT and LTT+1) for patients from both cohorts (A and B). Patients with persistent formation of new lesions during extension were switched to alternative FDA-approved therapies, even though all of them demonstrated therapeutic efficacy of daclizumab when compared to pretreatment (-Mo0) data. Because we do not observe significant differences in any measured biomarkers between STT, LTT, and LTT+1y time points, or between Cohorts A and B, we do not believe that dropouts have a significant bearing on our conclusions. The only results possibly affected by dropouts and small samples size are correlations between CSF biomarkers and therapeutic efficacy, which we therefore consider preliminary and necessitating validation in an independent cohort. (2) *Potential for biases due to investigators' conflicts of interests (COI) in the open-label study.* In response, we implemented safeguards: CSF samples were processed only by investigators who lacked COI using SOP and prospective coding of samples. Clinical and MRI data were generated by different group of investigators who likewise lacked COI. All analyses of CSF biomarkers were blinded; investigators

A

Visit months	EDSS			
	Zenapax only/and Zinbryta (number, median, range)		Zinbryta only (number, median, range)	
Mo -3	n=16	2.0 (0.0 – 3.5)	n=13	2.5 (1.0 – 6.5)
Mo 0	n=16	1.5 (1.0 – 6.5)	n=13	2.5 (1.0 – 6.5)
Mo 1.5	n=16	1.5 (1.0 – 3.5)		
Mo 6/6.5	n=15	1.5 (0.0 – 4.0)	n=13	2.5 (1.0 – 6.5)
Mo 12			n=13	2.5 (1.0 – 6.5)
Mo 18			n=12	2.3 (1.0 – 6.5)
Mo 24			n=8	2.0 (1.0 – 6.5)
LTT	n=14	1.5 (1.0 – 3.0)	n=9	2.5 (1.5 – 6.0)
LTT+0.5y	n=14	1.5 (0.0 – 3.0)	n=9	2.5 (1.0 – 6.0)
LTT+1y	n=14	1.5 (0.0 – 3.0)	n=6	2.5 (1.0 – 6.0)
LTT+1.5y	n=14	1.5 (0.0 – 3.5)	n=6	2.5 (1.0 – 6.0)
LTT+2y	n=14	1.5 (0.0 – 3.5)	n=5	2.5 (2.5 – 6.5)
LTT+2.5y	n=14	1.5 (0.0 – 3.5)	n=6	2.5 (1.0 – 6.5)

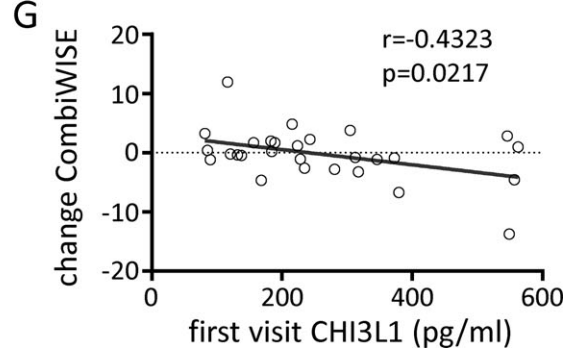
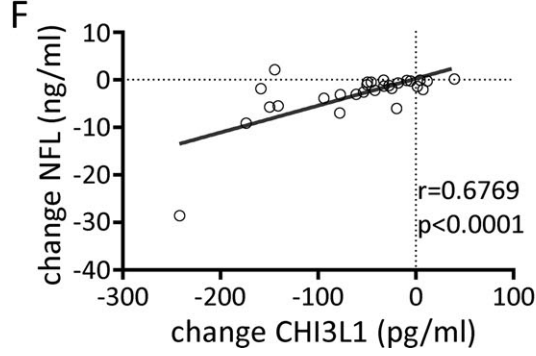
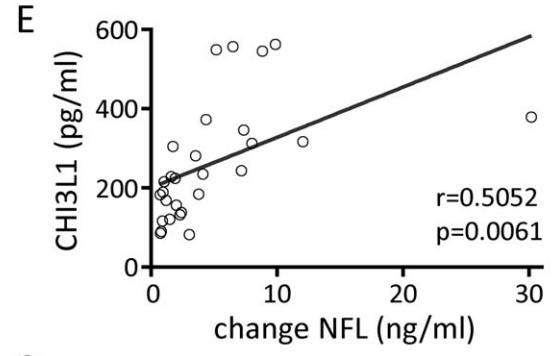
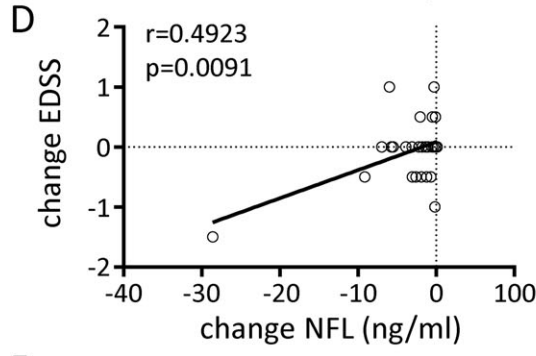
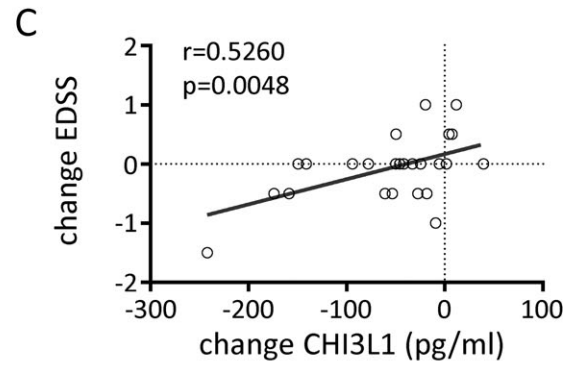
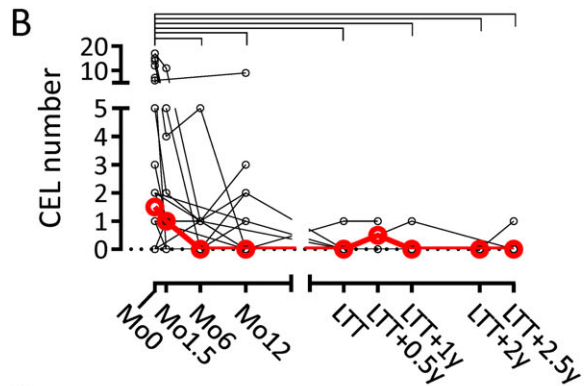


Figure 5. Myeloid marker CHI3L1 is closely associated with clinical efficacy. (A) Change of clinical scores by intravenous (Zenapax) and subcutaneous (Zinbryta) injection. (B) Number of contrast-enhancing lesions (CEL). Red lines show median values of biomarkers at each follow-up visit. Black brackets represent statistical significance ($P < 0.05$) based on adjusted P -value (Dunnett's method). CEL count was compared between baseline (visit Mo0) and each follow-up visit. LTT: long-term treatment (>1 -11 years). (C–G) Pearson's correlations. Change of CHI3L1, NFL, EDSS, and CombiWISE were calculated by subtracting the value of the first visit (Mo0) from the value of the last visit.

who generated CSF data had no access to clinical or imaging data. We included every CSF sample we have ever collected on any daclizumab-treated subject, including those who later dropped out. Finally, data were analyzed by an independent statistician. (3) *Relatively small sample size.* Based on previous CSF studies,^{19,21} we estimated that 15 patients analyzed before and after therapy provide sufficient power to reproducibly identify 20–30% effect sizes. High statistical significance, reproducibility of results between STT, LTT, and LTT+1y time points and between Cohorts A and B support our conclusion that the study is powered to detect biologically meaningful effect sizes. However, small sample sizes could have influenced the power of detecting reproducible relationships between CSF biomarkers and clinical data; thus, we consider these data preliminary and necessitating future validation in an independent cohort.

Notwithstanding these limitations, we conclude that the previously noted normalization of cellular abnormalities in the CSF of MS patients by daclizumab⁸ is not associated with normalization of markers of adaptive immunity in the CNS tissue. This expands the existing knowledge of persistent presence of plasma cells/plasmablasts (reflected by elevated IgG index) in treated MS patients by providing evidence for persistence of T and B cells in the intrathecal compartment of successfully treated MS patients, reflected by elevated CSF levels of sCD27 and sCD21, respectively.⁴ The lack of correlation between these residual markers of adaptive immunity and clinical outcomes (or NFL levels) suggests that mere presence of these cells in the intrathecal compartment does not lead to progressive accumulation of disability associated with axonal damage.

Because sCD27 is released by all T cells (activated $>$ resting and $CD8 > CD4^+$), elevated sCD27 levels may reflect intrathecal accumulation of regulatory T cells (Tregs). However, the most studied population of FoxP3 + Tregs do not secrete IL-2 and therefore cannot contribute to daclizumab-induced rise in CSF IL-2 levels. Furthermore, daclizumab actually inhibits FoxP3 + Treg activation, proliferation, and numbers.¹⁴ Therefore, the daclizumab-induced increase in CSF IL-2 levels indicate that at least part of the residual intrathecal T-cell population includes effector T cells, or regulatory T cells other than FoxP3 + Treg, such as Tr1 cells.²² Remaining CNS T cells may hypothetically retain some advantage over

treatments that abrogate CNS immunosurveillance from the standpoint of patient safety and by permitting “beneficial autoimmunity,” necessary for the maintenance of adult neurogenesis²³ and for neural repair and remyelination.²⁴ This hypothesis requires future comparative analyses of long-term safety and efficacy outcomes.

In contrast to modest inhibitory effects of daclizumab on intrathecal adaptive immunity, the markers of activation of myeloid lineage (e.g., IL-12p40, CHI3L1, and CXCL13) were normalized in the majority of treated subjects. Furthermore, pretreatment levels of CHI3L1 correlated with subsequent clinical efficacy measured by the sensitive disability scale CombiWISE.¹⁰ Finally, treatment-induced reduction of CHI3L1 levels in CSF also correlated with inhibition of axonal damage measured by CSF NFL and with clinical efficacy measured by both EDSS and CombiWISE. CHI3L1 has been reported as a prognostic biomarker in clinically isolated syndromes.²⁵ It is expressed by macrophages/microglia (and also by reactive astrocytes) in actively demyelinating MS lesions and by $CD14^{\text{low}}$ monocytes in the CSF.²⁵ While our dynamic observations suggest pathogenic role of activated members of myeloid lineage (e.g., macrophages, microglia, and myeloid DCs) in axonal damage associated with MS, correlation cannot prove causation. Indeed, activation of myeloid lineage may also be secondary to danger-associated molecular patterns released upon demyelination.²⁶ This interpretation is supported by the fact that we observed only trend, which did not reach statistical significance, for systemic (i.e., serum) inhibition of CHI3L1 levels upon initiation of daclizumab therapy, with no significant correlations between two compartments (data not shown). On the other hand, daclizumab may exert inhibitory effects on activated macrophages, which express CD25 during activation, and it also limits the ability of myeloid DCs to present antigens in immunostimulatory manner.¹⁵ Consequently, whether the activated myeloid lineage drives MS pathology or is an epiphenomenon cannot be determined without therapy that strongly inhibits either myeloid lineage or demyelination and ability to measure its efficacy *in vivo*.

In conclusion, this study provides evidence for the intrathecal pharmacodynamic effect of daclizumab. CSF biomarker studies support broad inhibitory effects of daclizumab on intrathecal immunity, but show residual presence of adaptive immunity in the majority of

treated patients. Nevertheless, the inflammation represented by aberrant activation of myeloid lineage is severely limited by daclizumab, to the extent that this therapy normalizes the biomarker of axonal damage and inhibits progression of disability in this long-term cohort. The emerging hypothesis of pathogenicity of activated myeloid cells in axonal damage represents a rationale for testing treatments that inhibit myeloid lineage in trials targeting residual accumulation of disability on current DMTs.

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Author Contributions

BB designed and supervised the study. JC participated in the evaluation of patients. MK, PK, VZ, JS, AB, and JS acquired data. MK analyzed data and created figures. TW did the statistical analysis. MK and BB did literature search and interpreted data. BB acquired funding. MK and BB wrote the manuscript and all authors reviewed the final version.

Conflict of Interest

Dr. Bielekova is a co-inventor on NIH patent related to daclizumab therapy and as such has received patent royalty payments. Drs. Zhao and Sheridan were employed by AbbVie Biotherapeutics or its predecessors, receiving stock and/or stock options, at the time the study was conducted.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Methodological details of biomarker measurements.

Table S2. Normative data for healthy donors' CSF biomarkers.

Table S3. Clinical scores.