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Treg Susceptibility to Cladribine-Induced Depletion Correlates With Therapy Response in Patients With Multiple Sclerosis

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Abstract

Background and Objectives

Immune reconstitution therapies for multiple sclerosis (MS) are based on selective lymphocyte reduction, followed by repopulation and rescue of immune tolerance. Among these therapies, cladribine is an adenosine analog that interferes with cell division and depletes several lymphocyte subtypes. Regulatory T cells (Tregs), physiologically devoted to immune suppression, are dysfunctional in the context of MS. In this study, we explored the effects of cladribine on Treg dynamics and phenotype.

Methods

In vivo, deep immunophenotyping was conducted on peripheral blood of patients with MS (n = 11), longitudinally collected before and after 6 and 12 months of cladribine therapy. In vitro, expanded Tregs were treated with cladribine and analyzed for their phenotypic, molecular, and metabolic profiles.

Results

In vivo, Tregs were overall less sensitive than conventional T cells (Tconvs) to the depleting effects of cladribine. This phenomenon was particularly evident in the subset of the resting (rest) Tregs. At baseline, while activated (act) Tregs presented markers of proliferation, senescence, and survival, restTregs highly expressed the antiapoptotic protein Bcl2 and the quiescence marker Bach2. In vitro, cladribine strongly reduced Treg viability while inducing a program of senescence and dysfunction and compromising their metabolic fitness. When Treg dynamics were analyzed ex vivo in relation to neuroinflammation and response to therapy, restTregs exhibited resistance to depletion in nonresponders, in association with increasing expression of Bcl2.

Discussion

These results indicate that the efficacy of cladribine therapy may require reduction and repopulation of the Treg compartment, an event that may be hindered by restTreg resistance, which is supported by antiapoptotic signals.

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Supplementary Material

Introduction

Regulatory T cells (Tregs) are a class of CD4 T lymphocytes characterized by the ability to preserve immune and tissue homeostasis, whose development is determined by the transcription factor FOXP3. Tregs are a highly proliferative population in both mice and humans.

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Glossary

dCK = deoxycytidine kinase; **ECAR** = extracellular acidification rate; **GSEA** = gene set enrichment analysis; **HD** = healthy donor; **MS** = multiple sclerosis; **NFL** = neurofilament; **NfL** = neurofilament light chain; **OCR** = oxygen consumption rate; **PBMC** = peripheral blood mononuclear cell; **Tregs** = regulatory T cells.

Human Tregs can be distinguished into the 3 fractions of resting, activated, and nonsuppressive Tregs: while resting Tregs are those with the highest proliferative potential, activated Tregs contain the highest proportions of cycling cells in vivo.[1] In autoimmune diseases, including multiple sclerosis (MS), perturbations in Treg frequencies, functions, and/or stability have been variably reported.[2,3] Of note, Tregs from patients with MS are characterized by poor proliferative potential due to impaired glycolytic metabolism.[4,5]

Cladribine administration represents an immune reconstitution therapy for MS.[6,7] Cladribine is an adenosine analog prodrug that exerts immunomodulatory effects through the selective targeting of lymphocyte subsets, based on its differential intracellular metabolism. On internalization, cladribine is phosphorylated by the deoxycytidine kinase (dCK) to generate its active moiety, a modified nucleotide that is incorporated into DNA, thus blocking its duplication.[6] Lymphocytes are characterized by a high ratio of dCK expression compared with the dephosphorylating enzyme NT5C2 and are thus susceptible to cladribine activity.[8] Both proliferating and quiescent cells may respond to cladribine,[9] although activated cells are much more sensitive than resting cells, because of increased dCK expression.[8] However, some activities of cladribine may be independent of dCK: indeed, cladribine was still able to inhibit cytokine secretion by T cells even in the presence of deoxycytidine, a competitive inhibitor of dCK.[10] Being an adenosine analog, cladribine may exert some of its effects through the binding of adenosine receptors on the lymphocyte surface,[11] known to have immunomodulatory properties.

Whether Tregs represent a preferential or a neglected target for cladribine is not clear. Tregs do not show a higher dCK relative content compared with other T-cell subsets.[8] However, Tregs are highly proliferative and may be particularly susceptible to the effects of the drug. It was reported that total Treg counts declined from week 5, reaching the nadir at week 24 and remaining low up to week 48 from the start of therapy. $CD45RA^-$ memory Tregs (including both nonsuppressive Tregs and activated Tregs) seemed less sensitive to depletion than naïve Tregs at advanced time points.[12] A recent analysis has confirmed that Tregs decline together with other T-cell subsets up to 24 months after therapy initiation.[13]

In this study, we dissected the effects exerted by cladribine on the distinct Tregs subsets, by analyzing the number and phenotype of circulating Treg fractions. Our results point to a relative resistance of Tregs to depletion, except for the activated Treg compartment, which is characterized by a higher proliferative rate. In vitro, cladribine profoundly reduced the viability of activated Tregs in a dCK-dependent manner, while concomitantly compromising their metabolic fitness. Conversely, the resistance of resting Tregs was associated with the high expression of the antiapoptotic gene Bcl2 by this subset.

Methods

Patients and Samples

In this study, we enrolled 11 patients with MS, eligible for cladribine therapy, at IRCCS Fondazione Santa Lucia of Rome. From each patient, a peripheral blood sample was obtained before starting the therapy (T0) and after 6 (T1) and 12 (T2) months of treatment. Clinical data were collected, including dosage of neurofilament (NFL) in serum. Samples from healthy donors (HDs) were also obtained from the Blood Transfusion Center of Azienda Ospedaliera San Camillo Forlanini, to perform in vitro experiments. All patients and donors provided written informed consent before sampling, in accordance with the Declaration of Helsinki.

Peripheral blood mononuclear cells (PBMCs) were obtained from fresh heparinized blood by density gradient centrifugation using Lympholyte (Cedarlane). Cells were collected in complete RPMI-1640 medium with 10% FBS (Life Technologies), 2 mmol/L L-glutamine (Sigma-Aldrich, St. Louis, MO), penicillin/streptomycin, nonessential amino acids and sodium pyruvate (all from EuroClone), and 50 μmol/L 2-ME (Sigma-Aldrich). PBMCs, collected on different days, were frozen and then thawed collectively for simultaneous analysis.

Treg Isolation, Expansion, and Culture

Highly purified Tregs were isolated from PBMCs of HDs by immunomagnetic separation with the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II (Miltenyi Biotec) and expanded in vitro for 14 days using the Treg Expansion Kit (Miltenyi Biotec). In brief, Tregs were cultured at a bead-to-cell ratio of 4:1 in complete RPMI with 5% AB human serum plus 500 U/mL interleukin-2 (IL-2). Cells were split every 2–3 days and, at day 14, were cultured for further 24 or 48 hours in the presence of the following compounds, either single or in combination: cladribine (Clad) 0.1, 1, 10 μM; deoxycytidine (dC) 250 μM; A2AR antagonist (SCH-58261, antago) 0.1, 1, 10 μM; A2AR agonist (CGS-21680, ago) 0.01, 0.1, 1 μM (all from Sigma-Aldrich). Then, cells were analyzed by flow cytometry or used for other applications.

Flow Cytometry

Thawed PBMCs from patients with MS and in vitro–expanded Tregs were analyzed by flow cytometry. First, cells were stained with fixable viability dye eFluor780 (Thermo Fisher Scientific, Waltham, MA) in PBS for 30 minutes at room temperature. After washing, surface antibodies were added and cells were incubated in PBS 2% FBS for 20 minutes at 4°C. Then, cells were fixed and permeabilized with the FOXP3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) for 30 minutes at 4°C. Finally, cells were washed, and intracellular antibodies were added in permeabilization buffer for 30 minutes at room temperature. The list of antibodies is given in eTable 1.

RNA Extraction and RNA-seq

In vitro–expanded Tregs were treated or not for a further 24 hours with cladribine (1 μM). Then, beads and dead cells were removed with the MACSiMAG Separator and Dead Cell Removal Kit (both from Miltenyi Biotec). RNA was extracted using a Single Cell RNA Purification Kit (Norgen Biotek). Samples were sequenced on an Illumina NextSeq500 platform. After sequencing, quality control checks were performed to assess the data quality and remove low-quality reads or artifacts. The quality-filtered reads were aligned to the human genome (GRCm38) using the STAR aligner with default parameters (version 2.6.1d). The aligned reads were then used to obtain gene-based read counts using the featureCounts module (version 1.6.4) and the Ensembl GRCm38 annotation. To compare the expression levels across samples, raw read counts were normalized using the TMM (trimmed mean of log-ratio values) method. Genes with counts per million mapped reads greater than 1 in at least 2 libraries were considered for further analysis. The edgeR package (version 3.26.5) in R statistical software was used to perform differential gene expression analysis. *p* Values were adjusted using the Benjamini-Hochberg method. Gene set enrichment analysis (GSEA) was performed using GSEA software (version 3.0) from the Broad Institute of MIT. The gene list was ranked based on log2 fold changes. GSEA was conducted in the preranked mode, with the scoring scheme set to "classic" and using 1,000 permutations. The gene signature used for analysis was retrieved from the Molecular Signatures Database (MSigDB v6.2).

Seahorse Analysis

The metabolic profile has been evaluated on expanded Treg cells treated with or without cladribine (1 μM). Real-time measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were performed using an XF^e-96 Extracellular Flux Analyzer (Seahorse Bioscience). OCR was assessed in the XF medium (nonbuffered DMEM with 10 mM glucose and 1 mM sodium pyruvate) basally and in response to 2 μM oligomycin, 1.5 μM FCCP (carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone), and 1 μM antimycin and rotenone (Sigma-Aldrich). ECAR was measured in XF medium in basal conditions and after exposure to 10 mM glucose, 2 μM oligomycin, and 100 mM 2-DG (all from Sigma-Aldrich).

Neurofilament Analysis

Quantification of neurofilament light chain (NfL) levels in serum samples was performed using an ultrasensitive immunoassay based on the Single Molecule Array (SiMoA) technology (Quanterix, Billerica, MA), as previously described.[14] In brief, serum samples stored at −80°C were thawed at room temperature and centrifuged at 10,000×*g* for 5 minutes to remove debris. NfL levels were measured using the NF-Light Advantage Kit (Quanterix, item 103400) on the fully automated SiMoA SR-X Analyzer (Quanterix Corporation, Massachusetts), following the manufacturer's 2-step digital protocol. All calibrators and serum samples were assayed in duplicate, with appropriate standards and internal quality controls included in each run, according to the manufacturer's instructions. To reduce interassay variability, all longitudinal samples from each patient were analyzed within the same run. The lower limit of quantification and the limit of detection were established based on the kit specifications. NfL concentrations in patients with MS were compared with those measured in a control group of age-matched and sex-matched healthy individuals.

Statistical Analysis

Analysis was performed using Prism 10 (GraphPad Software) for statistical tests and graphical presentations. The statistical methods used for each analysis are described in the figure legends. Statistical significance between 2 or more groups of experimental replicates from in vitro experiments was calculated using the Student *t* test and 1-way or 2-way ANOVA. In cases of multiple comparisons, *p* values were corrected using the Tukey method. Data are presented as means ± SD. *p* Values <0.05 were considered statistically significant.

Ethics Approval Statement

Human studies were performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and approved by the ethical committee of IRCCS Fondazione Santa Lucia (CE/PROG.886).

Data Availability

All data generated or analyzed during this study are included in this published article. The RNA-seq accompanying this article is deposited on Zenodo: 10.5281/zenodo.17778156.

Results

Tregs, Except ActTregs, Are Relatively Resistant to Cladribine Depletion In Vivo

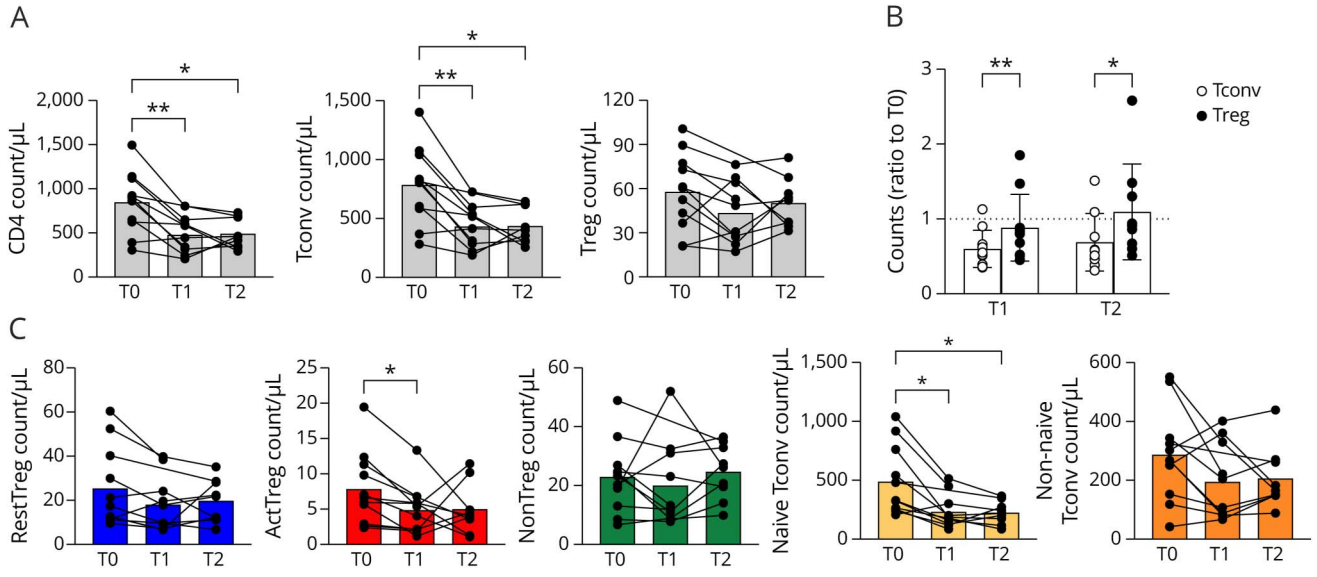
Along an immune reconstitution therapy, 3 phases can be recognized based on immune cell dynamics: reduction, repopulation, and reconstitution (the 3 Rs).[7] Others have already shown that Treg decline is an early event in cladribine therapy, starting in the reduction phase at 5 weeks.[12] With the aim to determine Treg dynamics during repopulation and reconstitution phases, we analyzed Treg frequency and counts at baseline (T0), at 6 months (T1), and at 12 months (T2), in a cohort of patients with MS (n = 11) undergoing cladribine

therapy. First, we quantified, in the $CD4^+$ T-cell gate, the percentage of $CD25^+CD127^{low}$ ($FOXP3^+$) Tregs. The full gating strategy is shown in eFigure 1A. We found that Treg percentage significantly increased from T0 to T1 and remained high at T2 (eFigure 1, B and C). Then, we investigated whether such relative increase was due to a higher resistance of Tregs to cladribine depletion, compared with conventional T cells (Tconvs). In line with the findings by others,[12,13] $CD4^+$ T-cell count declined significantly on therapy, but when Tconvs and Tregs were separately analyzed, only the Tconv reduction reached statistical significance (Figure 1A). Furthermore, the magnitude of such reduction (measured as the T1/T0 and T2/T0 ratios of cell counts) was significantly greater in Tconvs than in Tregs at both time points (Figure 1B).

Next, we enumerated the 3 fractions of resting Tregs (restTregs, $CD45RA^+$ $FOXP3^{low}$), activated Tregs (actTregs, $CD45RA^-$ $FOXP3^{hi}$), and nonsuppressive Tregs (nonTregs, $CD45RA^-$ $FOXP3^{lo}$), according to a previously established classification[1] (eFigure 1D). As controls, Tconvs were subdivided into naïve ($CD127^+CD45RA^+$) and non-naïve (excluded from the naïve gate) cells. In the Tconv compartment, only naïve Tconvs showed a significant decline from T0 to T1 and T2, in line with previous data highlighting higher susceptibility of naïve vs memory T cells to cladribine in vivo.[12] Within the Treg compartment, the overall composition in restTregs, actTregs, and nonTregs did not change (eFigure 1E). However, when we analyzed the cell count of each subset, as a measure of cladribine-induced depletion in vivo, we found that, while actTreg counts significantly dropped from T0 to T1, restTregs and nonTregs did not (Figure 1C). At both T1 and T2, nonTregs and restTregs, but not actTregs, declined significantly less compared with naïve Tconvs as a reference (eFigure 1F).

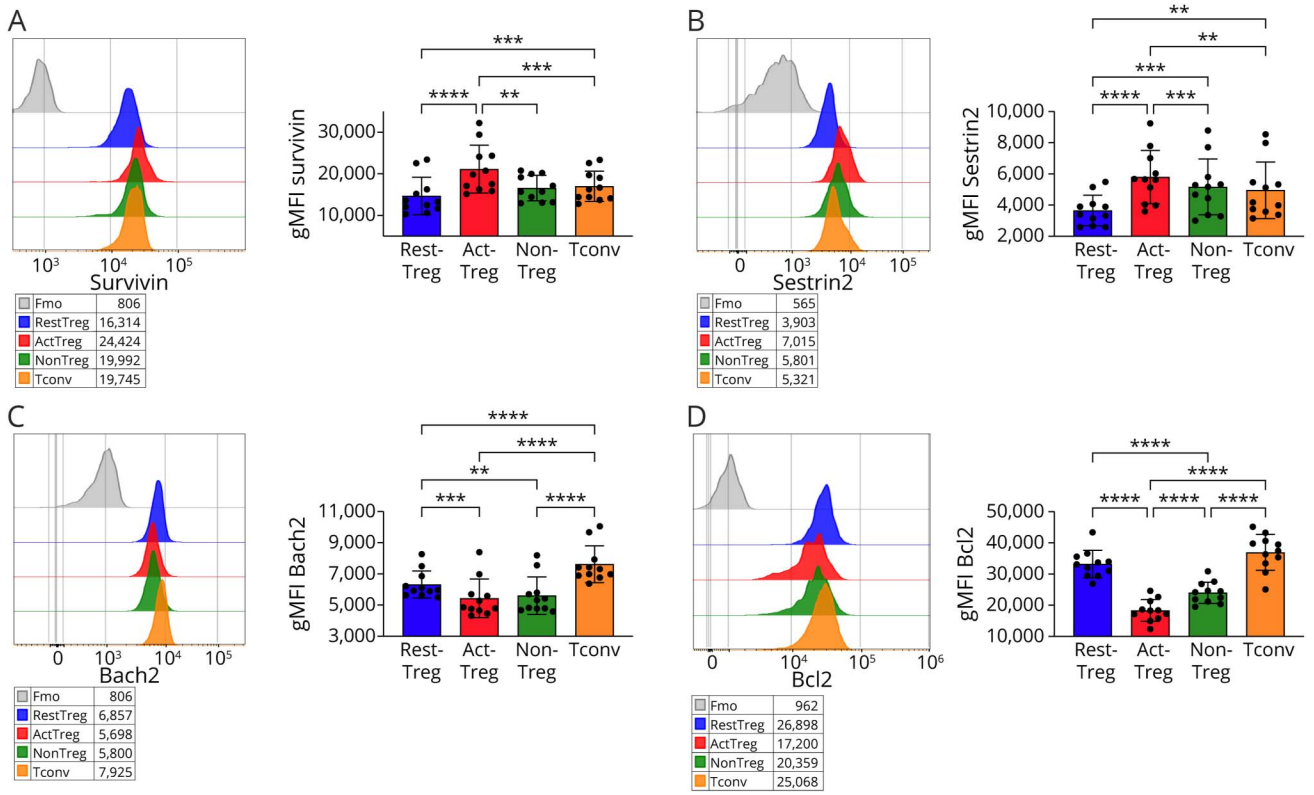
These results suggest that, within the Treg population, distinct subsets have variable susceptibility to cladribine depletion. To investigate the mechanisms behind this different responsiveness, we analyzed the expression of several markers associated, respectively, with cell proliferation (Ki67), survival (survivin), senescence (Sestrin2), quiescence (Bach2), and resistance to apoptosis (Bcl2) at baseline, in Treg subsets and Tconvs as control. This analysis revealed that, in line with previous studies,[1] actTregs were by far the most proliferative subset (eFigure 2A). However, they also expressed both survivin (Figure 2A) and Sestrin2 (Figure 2B) to the highest extent. Survivin is induced after T-cell activation, especially downstream of the signaling of TNF receptor superfamily members, and antagonizes apoptosis.[15] Sestrins are induced in activated T cells in response to metabolic stress.[16] Conversely, restTregs expressed higher levels of Bach2 (Figure 2C) and Bcl2 (Figure 2D). Bach2 is expressed by restTregs to support their quiescence and long-term maintenance.[17] Bcl2 is an antiapoptotic protein that is highly expressed in Tregs and supports their stability against Th17-like polarization.[18,19] Overall, this analysis suggests that actTregs and restTregs rely on different programs for their maintenance, respectively, Sestrin2-related senescence and Bach2-related quiescence, which may be differentially targeted by cladribine.

Figure 1 Tregs, Except for ActTregs, Are Relatively Resistant to Cladribine-Induced Reduction In Vivo in Patients With MS



Peripheral blood mononuclear cells (PBMCs) from 11 patients with MS were collected and frozen before (T0) and after 6 (T1) or 12 (T2) months of oral cladribine treatment. Cells were thawed and analyzed by flow cytometry. (A) Absolute numbers of CD4 T cells, Tconvs, and Tregs in PB of patients with MS at T0, T1, and T2. (B) Ratios of Tconv and Treg absolute counts were calculated at T1 and T2 with respect to T0. The dotted line at 1 represents the null variation. (C) Absolute numbers of restTregs, actTregs, and nonTregs (gated as shown above), and naïve ($CD127^+CD45RA^+$) and non-naïve Tconvs in the peripheral blood of patients with MS at T0, T1, and T2. In all plots, each dot represents a single patient. Bars indicate the mean and SD. *$p < 0.05$ and **$p < 0.01$, by RM 1-way ANOVA or mixed-effects analysis, with the Geisser-Greenhouse correction and Tukey multiple comparisons test or uncorrected Fisher LSD.

Figure 2 ActTregs Are Terminally Differentiated Cells at Baseline



Histogram overlays (left) and bar graphs (right) showing the geometric mean fluorescence intensity (gMFI) of survivin (A), Sestrin2 (B), Bach2 (C), and Bcl2 (D), as evaluated by intracellular staining in the subsets of restTregs, actTregs, nonTregs, or Tconvs, in the PB of patients with MS (n = 11) at T0, before cladribine therapy. Fmo represents the fluorescence-minus-one control. In all bar graphs, each dot represents a single patient. Bars indicate the mean and SD. $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$, by RM 1-way ANOVA, with the Geisser-Greenhouse correction and Tukey multiple comparisons test.

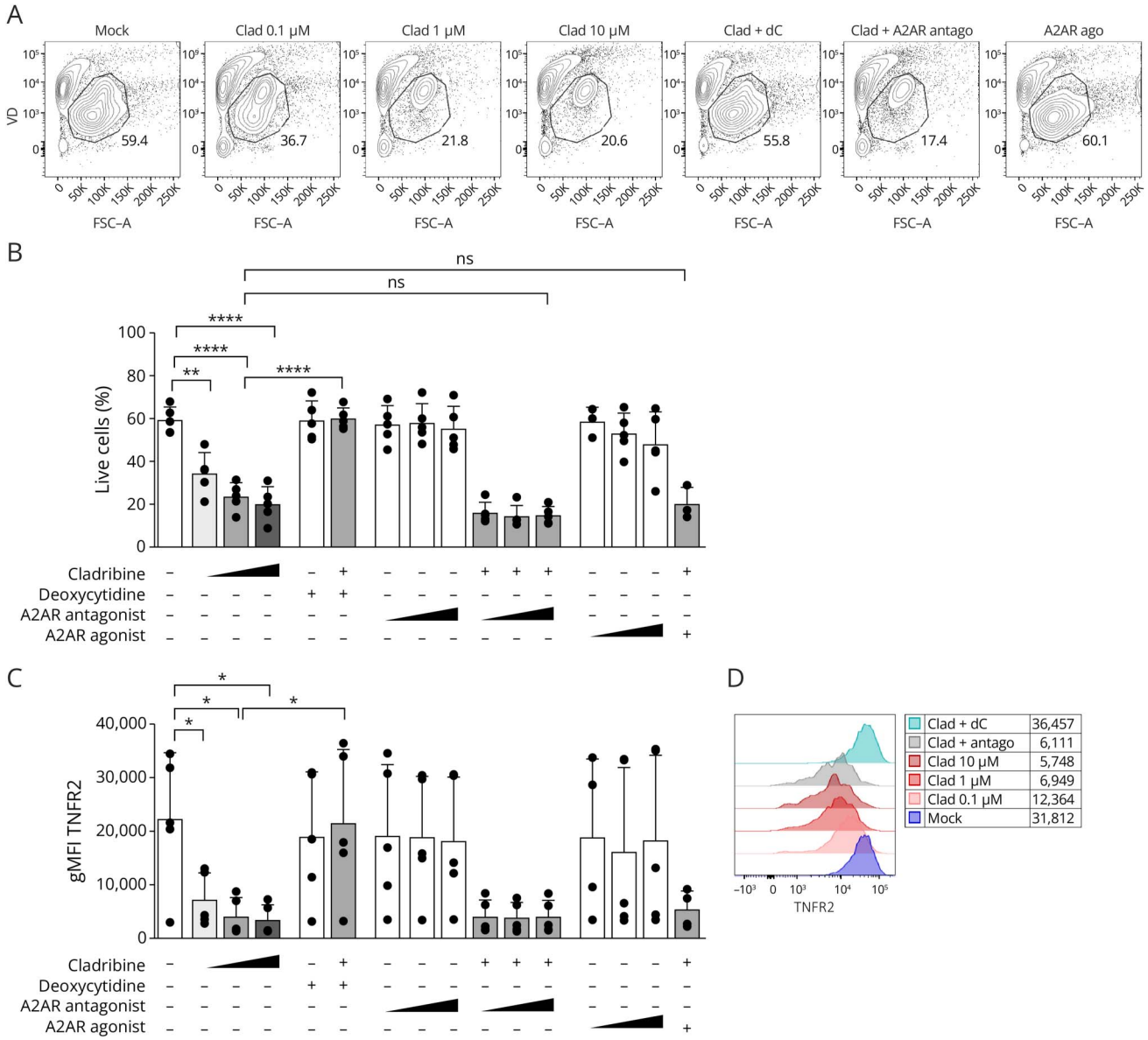
The TNF receptor superfamily has key roles in Treg expansion and activation.[20] When we analyzed the expression of 2 members, TNFR2 and OX40, on the surface of Treg subsets, we confirmed that both receptors were more represented on actTregs (eFigure 2, B and C). Of interest, TNFR2 expression positively correlated ($p = 0.0519$) with the expression of Sestrin2, and not with survivin or Ki67, in Tregs (eFigure 2D), suggesting that in patients with MS, actTregs may exhibit a dysfunctional activation profile wherein TNFR2 expression is associated with senescence.

Cladribine Directly Restrains ActTregs In Vitro

To study the direct and acute effects of cladribine selectively on actTregs, we set up an in vitro system of human primary Treg expansion and cladribine treatment. To this aim, $CD25^{+}CD127^{lo}$ Tregs were immunomagnetically purified from the blood of healthy donors (n = 5) and expanded in vitro for 14 days, reaching a purity of >96% with high expression of FOXP3 and CD25 (eFigure 3A). In this setting, we confirmed that both TNFR2 and OX40, markers of actTregs, were upregulated as early as day 1 and remained at high levels until the end of culture (eFigure 3B). Because Tregs can sense adenosine through the A2AR,[21] we took advantage of this experimental setting to verify the involvement of A2AR in the effects of cladribine, which may interact with this receptor as an adenosine analog.[11] In primary in vitro–expanded Tregs, A2AR was undetectable by surface staining (not shown) and thus was analyzed in fixed and permeabilized Tregs, where it was found to be modestly induced, peaking at day 6 (eFigure 3C). When scaled concentrations of cladribine were added, according to previous protocols,[10] for a further 48 hours, the cell viability strongly declined in a dose-dependent fashion. Of note, the dCK inhibitor deoxycytidine (dC) completely reversed this effect. Conversely, an A2AR antagonist did not affect cell viability while an A2AR agonist failed to mimic or to inhibit the cladribine toxicity (Figure 3, A and B). When looking at the Treg phenotype, we could not find any significant change in the expression of CD25, FOXP3, OX40, or A2AR (eFigure 4). Conversely, the expression of TNFR2 (despite heterogeneity among single donors) was significantly reduced by cladribine, and this effect again was abolished by dCK but not A2AR inhibition (Figure 3, C and D). Overall, these findings demonstrate that cladribine directly reduced the viability and TNFR2 expression of activated Tregs in a dCK-dependent and A2AR-independent mechanism.

To define the molecular and metabolic profiles associated with cladribine exposure, in vitro–expanded Tregs (from 3

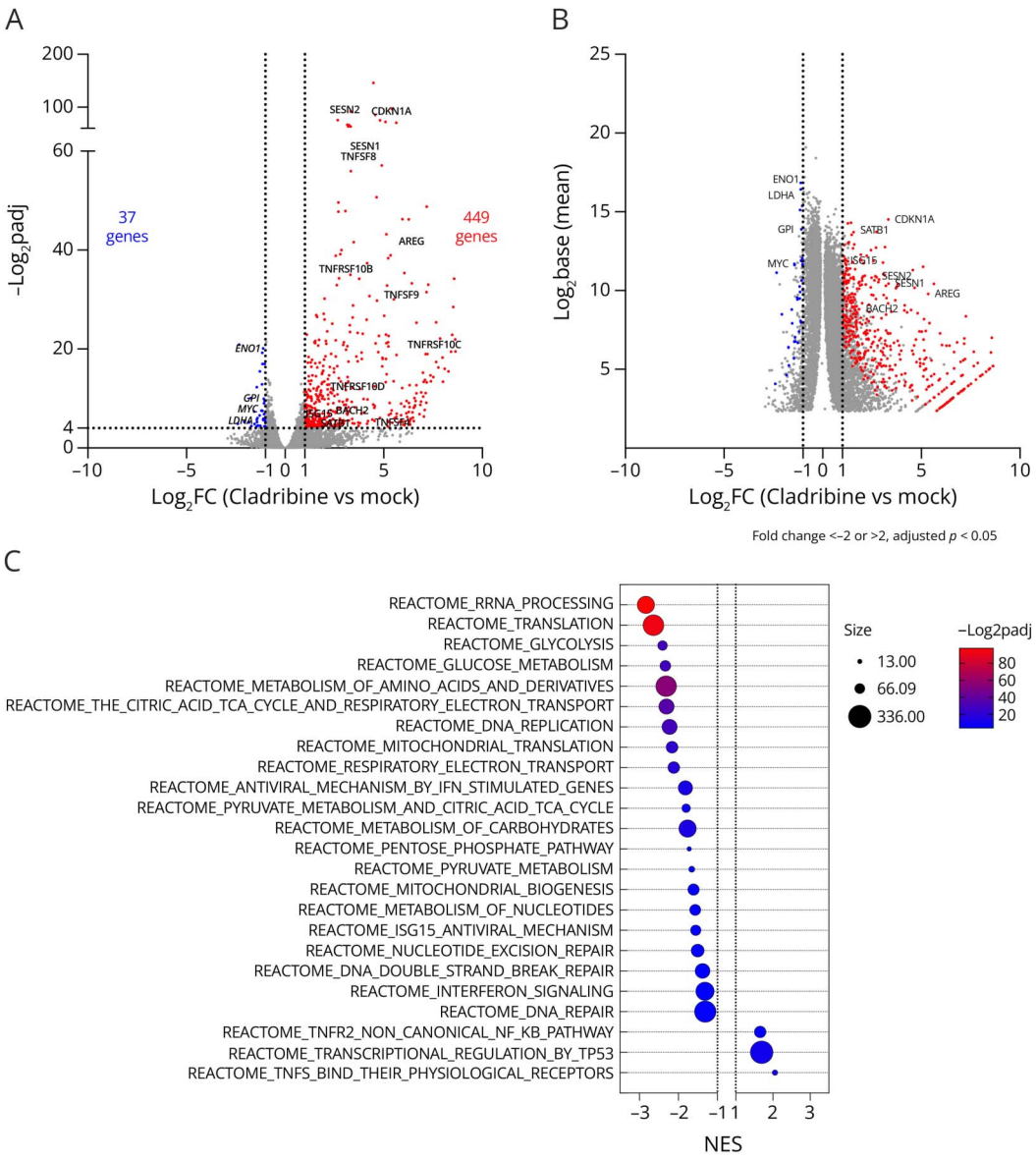
Figure 3 Cladribine Reduces Viability and TNFR2 Expression of In Vitro–Expanded Tregs in a A2AR-Independent and DCK-Dependent Fashion



In vitro–expanded Tregs were cultured for further 48 hours in the presence of the following compounds, either single or in combination: cladribine (Clad) 0.1, 1, 10 µM; deoxycytidine (dC) 250 µM; A2AR antagonist (antago) 0.1, 1, 10 µM; A2AR agonist (ago) 0.01, 0.1, 1 µM. Then, cells were analyzed by flow cytometry. (A) Representative contour plots showing the percentages of live cells under different culture conditions. Dead cells were discriminated with a viability dye (VD). (B) Cumulative analysis of the frequency of live cells under the indicated culture conditions. (C and D) Cumulative analysis (C) and representative histogram overlays (D) of the geometric mean fluorescence intensity (gMFI) of TNFR2 in the indicated conditions. Bars represent means and SD. Data are pooled from 3 independent experiments with Tregs expanded from 5 HDs. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$ by the paired t test.

donors) were treated for a further 24 hours with cladribine and then analyzed by bulk RNA-seq analysis or bioenergetic profiling assay. At this earlier time point, we confirmed a significant decline in cell viability and TNFR2 expression on treatment (not shown); nevertheless, an adequate yield of surviving cells was secured for downstream applications. Significantly upregulated genes (449) greatly outnumbered downregulated genes (37) in cladribine-treated Tregs (Figure 4A), and both sets included highly expressed genes (Figure 4B). Upregulated genes included several genes of the TNFR superfamily, such as *TNFSF4* (OX40L), *TNFSF8* (CD153), *TNFRSF10B/C/D* (all TRAIL receptors), and *TNFSF9* (41BBL), but not *TNFRSF1B* (TNFR2) as expected. Cladribine-treated Tregs also expressed higher levels of genes related to senescence (*SESN1* and *2*), quiescence (*BACH2*), regenerative function (*AREG*), cell cycle regulation (*CDKN1A*, p21), interferon response (*ISG15*), and repression of suppressive function (*SATB1*[22]). Conversely, genes related to glycolysis (*MYC*, *GPI*, *LDHA*, *ENO1*) were found downregulated in cladribine-treated Tregs. Pathway

Figure 4 Cladribine-Selected Tregs Upregulate TNFR Signaling Programs

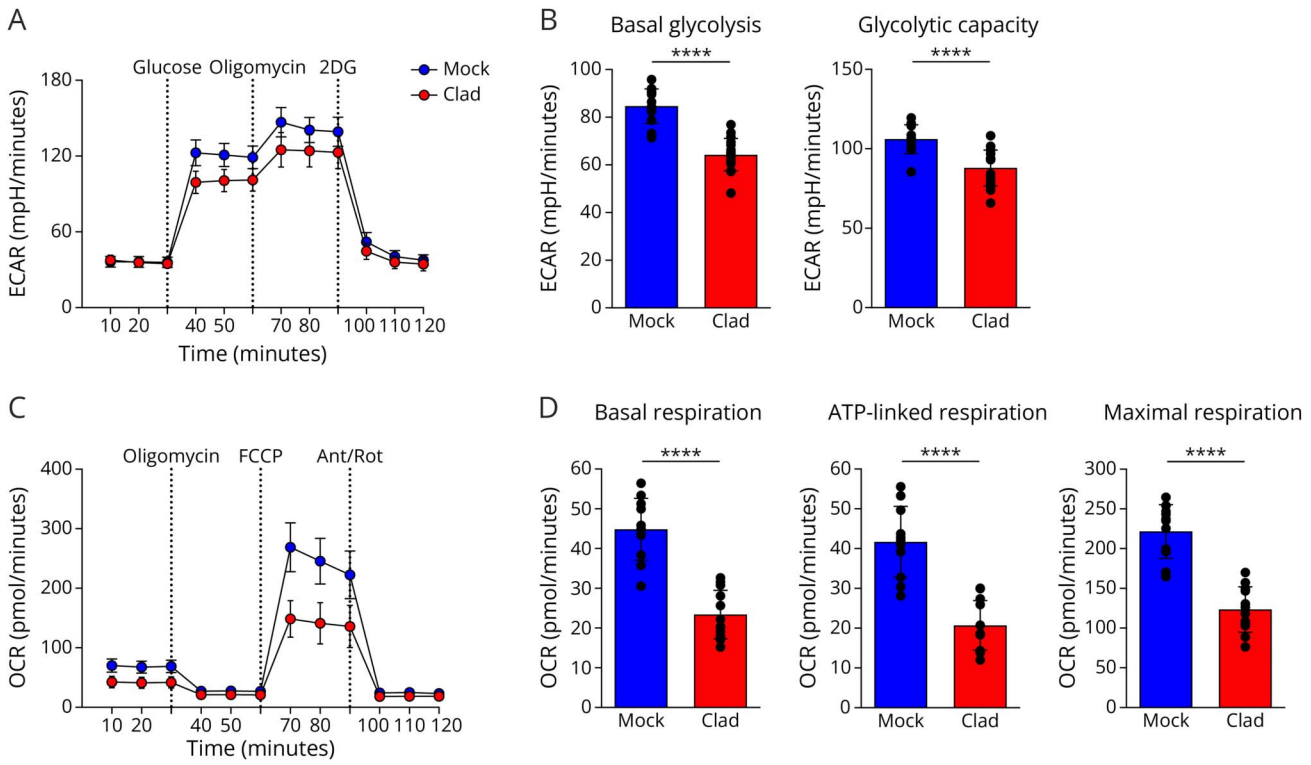


Volcano plot (A) and MA plot (B) showing the fold change in gene expression by RNA-seq of Tregs obtained from the PB of 3 independent donors, expanded in vitro and then treated or not for a further 24 hours with cladribine. Genes with adjusted *p* value < 0.05 and with fold change >2 (red) or <−2 (blue) are highlighted. (C) Reactome pathway enrichment analysis was performed with the differentially expressed genes between cladribine-treated and mock-treated Tregs. Pathways with normalized enrichment scores (NES) > 1 or <−1 and adjusted *p* value < 0.05 are shown.

analysis revealed that several pathways related to protein synthesis and all the major metabolic routes (including both glycolytic and oxidative metabolism) were severely affected in cladribine-treated Tregs while TNFRSF-dependent NF-κB activation and p53-dependent transcriptional regulation were increased (Figure 4C). It is important to note that this profile represents the molecular program of those Tregs that survived cladribine exposure, thus being selected by the treatment. The analysis of the bioenergetic profile confirmed the profound impairment of both glycolysis and mitochondrial respiration in cells surviving cladribine treatment, as evidenced by the consistent cladribine-induced inhibition of metabolic parameters closely associated with these 2 intracellular energy pathways (Figure 5). Of interest, the expression of *DCK* declined in Tregs on cladribine exposure, leading to a reduced DCK/NT5C2 ratio, which speaks in favor of reduced susceptibility to the drug in surviving cells (eFigure 5).

Altogether, these data show that cladribine treatment induces the death of activated Tregs in vitro and establishes in surviving cells a profile characterized by senescence, TRAIL-related signaling pathway, cell cycle arrest, and metabolic quiescence.

Figure 5 Cladribine Compromises Metabolic Fitness of In Vitro–Expanded Tregs



The graphs represent the metabolic profile of in vitro–expanded Tregs treated for 24 hours with cladribine (at least n = 17 from 3 independent experiments for ECAR and at least n = 12 from 2 independent experiments for OCR) or mock-treated (at least n = 13 from 3 independent experiments for ECAR and at least n = 11 from 2 independent experiments for OCR). Extracellular acidification rate (ECAR) kinetic profile (A) and indices of basal glycolysis and glycolytic capacity (B), as well as oxygen consumption rate (OCR) kinetic profile (C) and indices of basal, ATP-related, and maximal respiration (D), were measured. Data are expressed as mean ± SEM, unpaired *t*-test; ****$p < 0.0001$.

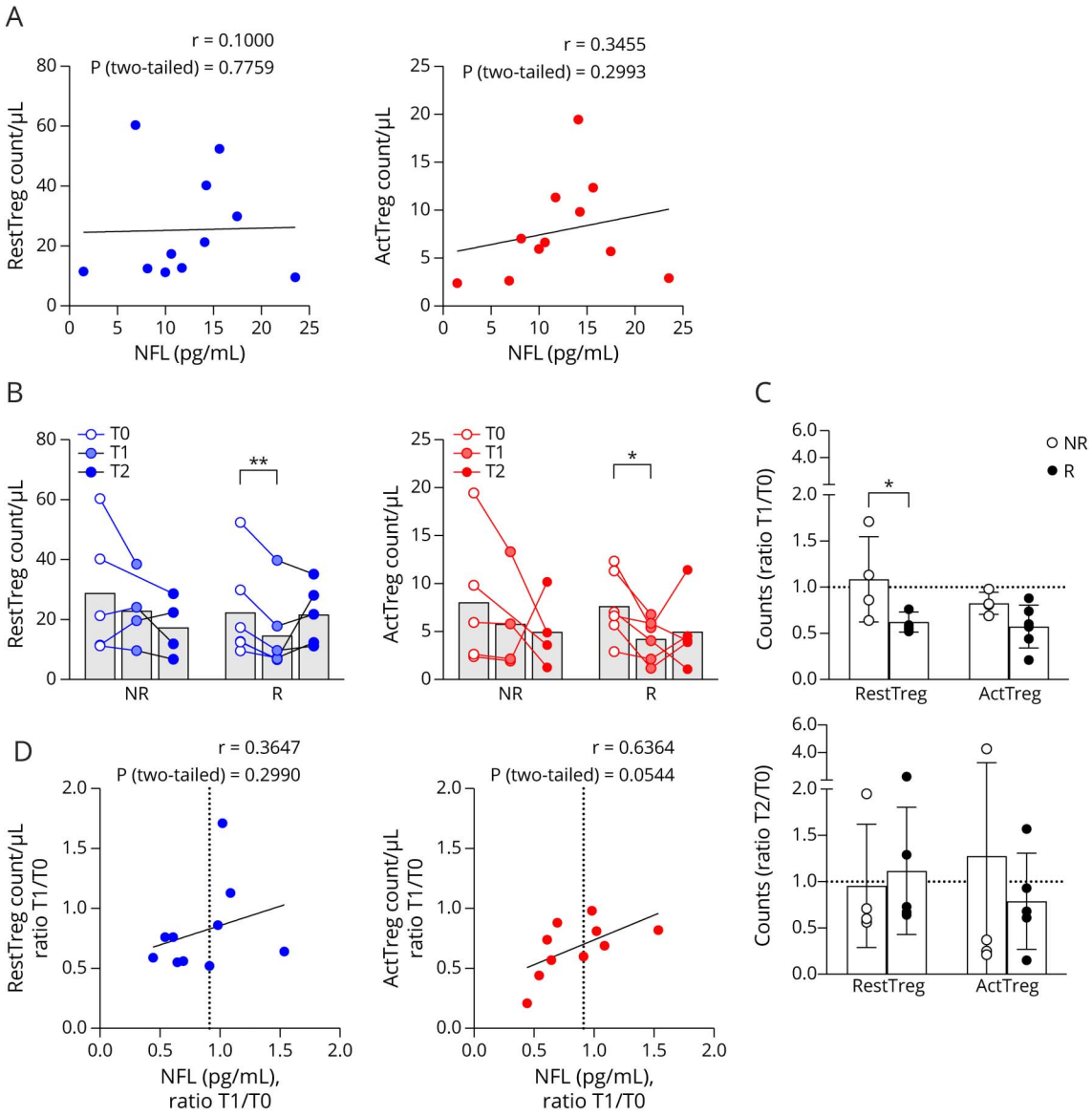
Treg Reduction Correlates With Clinical Response to Cladribine Therapy In Vivo in Patients With MS

Several data indicate that, in MS, Treg defects may arise from aberrant functions, stability, and metabolic fitness, rather than reduced numbers.[3] Moreover, conflicting results are available regarding the correlation between Treg frequency/count and disease severity, as evaluated with the EDSS score.[23-25] In our cohort of cladribine-treated patients, we searched for correlations between Treg count/phenotype and disease severity, using a recently implemented biomarker, the concentration of NfL measured in the serum[26] (eTable 2). At baseline, we could not find any correlation between NFL and the counts of Tregs or Tconvs (eFigure 6A). On cladribine treatment, according to previous studies,[13] we found that NFL overall decreased at both T1 and T2 compared with T0; however, this event was not consistent in the whole cohort. Therefore, we stratified the patients into 2 subgroups, based on the median of T1/T0 ratios of NFL concentrations, resulting in the identification of 2 subgroups: responders (R, ratio ≤0.912) and nonresponders (NR, ratio >0.912) (eTable 2). Of interest, we observed that both Treg and Tconv reductions were statistically significant at T1 only in R patients (eFigure 6B), and the extent of Treg decline (regarding the T1/T0 ratio of cell count) was significantly more pronounced in R patients compared with NR patients (eFigure 6C), despite the absence of a direct correlation with NFL reduction (eFigure 6D).

To ascertain which Treg subpopulation mostly contributed to this association, we separately analyzed restTreg and actTreg counts and ratios. Again, no correlation emerged between the count of any Treg subset and NFL concentration at baseline (Figure 6A), and both restTregs and actTregs showed a decrease from T0 to T1 only in R patients (Figure 6B). However, only restTregs showed a significantly statistical difference in their T1/T0 ratio between R and NR patients (Figure 6C). Of interest, the extent of actTreg decline positively correlated ($p = 0.0544$) with the magnitude of NFL reduction (Figure 6D), suggesting that actTreg contraction induced by cladribine may represent a favorable event in the clinical response to therapy.

Given that naïve Tconvs are strongly reduced by cladribine in vivo (Figure 1C), we investigated whether these cells, similarly to restTregs, were differentially affected in R vs NR patients. We found that the naïve Tconv counts declined at T1 exclusively in R patients (eFigure 7A). However, contrary to restTregs, the T1/T0 ratio was not significantly different between R and NR patients (eFigure 7B), denoting that

Figure 6 ActTreg Decline Correlates With Response to Cladribine Therapy in Patients With MS



(A) Spearman correlations were calculated between the serum concentration of neurofilament (NFL) and the count of resting or activated Tregs, in the PB of patients with MS at T0. Lines indicate linear regressions. (B) Cumulative analysis of restTreg or actTreg counts at the 3 tested time points of cladribine therapy. Patients with MS were stratified into nonresponders (NR) or responders (R) if the T1/T0 ratio of NFL concentrations were, respectively, > or ≤0.912 (the median value). Each dot represents a single patient. Bars indicate the means. *$p < 0.05$ and **$p < 0.01$, by mixed-effects analysis, with the Geisser-Greenhouse correction and uncorrected Fisher LSD. (C) Ratios of restTreg and actTreg absolute counts were calculated at T1 and T2 with respect to T0 in patients with MS stratified into NR and R as detailed above. The dotted line at 1 represents the null variation. Bars indicate the means and SD. *$p < 0.05$ by the mixed-effects model and uncorrected Fisher LSD. (D) Spearman correlations between the T1/T0 ratios of NFL concentrations and the T1/T0 ratios of restTreg or actTreg counts, in the PB of patients with MS (N = 11). Lines indicate linear regressions. Dotted lines show the median cutoff of NFL ratios (0.912).

quantitative differences may exist in the relative resistance of restTregs and naïve Tconvs in relation to clinical response.

We next explored whether cladribine treatment affected specific phenotypes of actTregs or restTregs, namely proliferation rate (as percent of cycling Ki67$^+$ cells), effector cell survival (survivin expression), senescence (expression of Sestrin2), quiescence (Bach2), and resistance to apoptosis (Bcl2), along with TNFR2 expression. Among all these examined parameters, only Bcl2 expression (which was higher in restTregs than in actTregs, as previously depicted in Figure 2D) further increased significantly, only in restTregs, from T0 to T1 and T2 (eFigure 8, A and B). Of note, this was not a unique feature of restTregs because Bcl2 expression also increased in naïve Tconvs at T1, especially in the NR group (eFigure 9, A and B).

The relative increase in Bcl2 expression from T0 to T2 tended to positively correlate ($p = 0.0666$) with restTreg maintenance (Figure 7A), suggesting that Bcl2 expression may indeed contribute to the resistance of this subpopulation to cladribine therapy, at least in the long term (at T2).

Finally, we searched for association between Bcl2 expression/variations in restTregs and the clinical response to therapy. First, we noticed that the rise in Bcl2 expression in restTregs reached statistical significance only in NR patients (Figure 7B). Second, at T0 ($p = 0.0876$) and T1 ($p = 0.0368$), Bcl2 expression in restTregs positively correlated with disease severity, as assessed by NFL measurement (Figure 7C). Bcl2 expression in naïve Tconvs also positively correlated with disease severity at T1 (eFigure 9C); however, it was not associated with naïve Tconv maintenance at any time point (eFigure 9D), indicating that Bcl2 may have a stronger impact on restTregs.

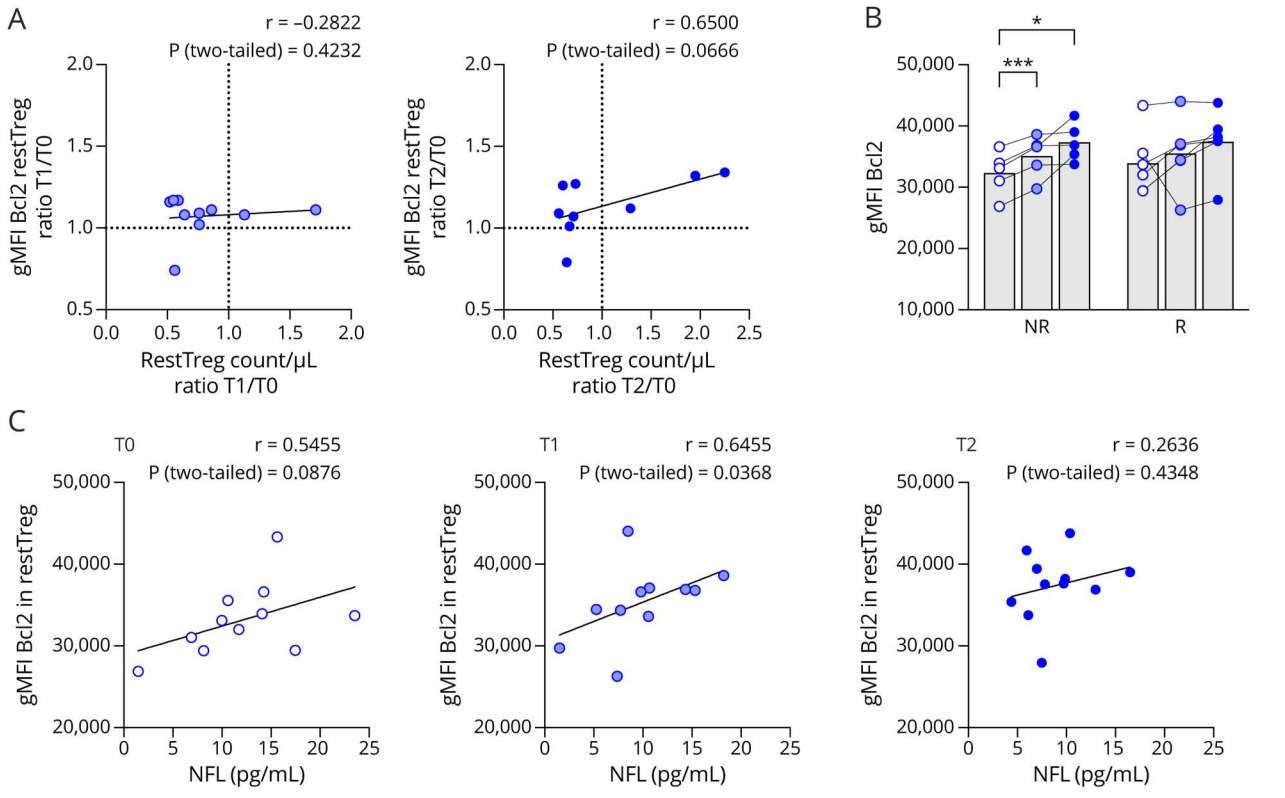
Overall, these results indicate that Bcl2 expression in restTregs represents an unfavorable factor in MS, likely in relation to the resistance of these cells to depletion.

Discussion

Accumulating data from experimental models of neuroinflammatory diseases clearly demonstrate that Tregs exert protective effects in such contexts: indeed, Treg depletion or functional inhibition has been shown to exacerbate disease symptoms or interfere with the recovery phase.[27] However, in an inflammatory environment, Treg suppression may not work correctly, because the target cells of Tregs are insensitive to suppression,[28] and/or Treg themselves are dysfunctional and acquire the ability to produce proinflammatory functions.[29] In this study, we show that, in patients with MS undergoing cladribine therapy, Tregs (and especially the resting Tregs) are less susceptible to reduction, but such resistance is associated with a poorer response to therapy. In resting Tregs, a high expression of the antiapoptotic molecule Bcl2 correlates not only with the persistence of their number but also with a more severe disease.

In patients with MS, the Treg defects appear to be more related to dysfunctional activity and/or stability, rather than reduced frequencies/numbers.[3] A meta-analysis of several studies has shown no association between MS diagnosis and alterations in the total Treg frequency.[30] A number of studies have analyzed the percentages and features of the Treg fractions, and one study has even reported that patients with MS have an increased frequency of actTregs, but fewer restTregs, compared with controls.[31] However, the cycling Ki67$^+$ Tregs, which are mostly included in the actTreg subpopulation, are

Figure 7 Bcl2 Expression in Resting Tregs Correlates With Disease Severity



(A) Spearman correlations between the T1/T0 or T2/T0 ratios of Bcl2 expression in restTregs (in terms of gMFI) and ratios of restTreg counts, in the PB of patients with MS (N = 11). Lines indicate the null variation (ratio = 1). (B) Cumulative analysis of Bcl2 gMFI in gated restTregs at the 3 tested time points of cladribine therapy in patients with MS stratified into nonresponders (NR) or responders (R). Each dot represents a single patient. Bars indicate the means. *$p <$ 0.05 and ***$p < 0.001$, by RM 2-way ANOVA, with the Geisser-Greenhouse correction and uncorrected Fisher LSD. (C) Spearman correlations between the serum concentration of neurofilament (NFL) and the gMFI of Bcl2 in restTregs at the 3 tested time points. Lines indicate linear regressions. gMFI = geometric mean fluorescence intensity.

significantly reduced in MS.[4] It has been established that $CD25^{hi}$ $CD45RO^{+}$ Tregs from patients with MS contain a population of IFNγ-producing cells, under the control of beta-catenin signaling, which promotes Treg dysfunctions and autoimmunity.[29,32] The FOXP3 splicing variant containing exon 2, which is expressed in highly suppressive and stable Tregs, is indeed less represented in Tregs from patients with MS.[5,33] These Tregs also expressed higher levels of PD1[33], which may indicate a dysfunctional and exhausted status. In this study, we could not directly assess Treg functions in patients with MS compared with healthy controls. However, in our cohort, the actTreg subset expressed high levels of the senescence marker Sestrin2 at baseline, which is indicative of terminally differentiated cells with impaired activation.[16]

Conflicting data are available regarding the association between Treg enumeration and disease severity. A high frequency of circulating Tregs,[23,25] a strong expansion rate of Tregs ex vivo,[4] or increased counts of resting Tregs[34] were found to inversely correlate with different measures of MS severity. However, other studies have reported a positive correlation between Treg frequency and EDSS scores in patients with MS.[24] In our study in which NFL was adopted as a marker of neuroinflammation, we could not find any correlation between NFL and counts of Tregs or Treg subsets. However, the longitudinal variation of NFL from T0 to T1 in individual patients allowed estimation of the clinical response to cladribine and stratification of the patients into R and NR groups. This approach revealed that (1) the decline in actTreg counts was a favorable event in the clinical response to therapy, (2) a higher resistance of restTregs to cladribine depletion (measured as the T1/T0 ratio of cell counts) appeared in NR patients compared with R patients, and (3) Bcl2 expression in restTregs was associated with the maintenance of the cell counts in this subset, a more severe disease, and a worse response to therapy.

In this study, we focused on the Treg compartment, based on the observation of its relative resistance to cladribine in vivo. However, variations in Tconvs, especially in the naïve subset, may also contribute to treatment response. Indeed, similar to restTregs, naïve Tconv counts are reduced by cladribine in R patients while upregulating Bcl2 in NR, and their Bcl2 expression correlates with disease severity. However, in contrast to restTregs, Bcl2 expression in naïve Tconvs does not correlate with the maintenance of their numbers. Hence, naïve Tconvs and restTregs seem to share some features of their response to cladribine; however, these have a stronger impact on the restTreg compartment.

From our data, it is not possible to establish any causal relationships between Treg variations and disease dynamics. Indeed, on the one side, an enhanced amelioration of neuroinflammation in responders may result in the attenuation of signals that would instigate immune regulation, thus leading to Treg contraction. On the other side, it is possible that Treg contraction may actively participate in disease amelioration. This possibility may appear counterintuitive, if we consider the well-established protective roles exerted by Tregs in autoimmune and inflammatory diseases. However, it should be considered that Tregs are dysfunctional in MS, and that in an immune reconstitution therapy (such as cladribine treatment), the substitution of dysfunctional with functional cells may be key to success.[7] Another possible explanation is that the contraction of circulating Tregs reflects their enhanced infiltration into the CNS, where they may exert their protective effects locally.

When acutely exposed to cladribine, in vitro–expanded Tregs underwent cell death in a dCK-dependent fashion. This observation confirms previous predictions of Treg susceptibility to cladribine, which were based on their DCK/NT5C2 ratio.[8] Among the markers that we analyzed, only the TNFR2 declined, again in a dCK-dependent manner, in cladribine-treated Tregs. However, no changes were observed at the mRNA level, suggesting that cladribine effect was post-transcriptional, potentially through the selection of $TNFR2^{low}$ cells. Such surviving cells also displayed molecular and bioenergetic features suggestive of a state of dysfunction. The TNF/TNFR superfamily has been shown to sustain Treg proliferation, effector function, and stability.[35] Notably, TNF signaling through TNFR2 exerts multiple and significant effects in promoting Treg expansion and suppressive function.[20] TNFR2 stimulation seems particularly relevant for Treg survival and acquisition of an effector program.[36] In a mouse model of CNS autoimmunity, TNFR2 expression selectively by Tregs was necessary to suppress effector T cells within the tissue, thus controlling disease severity.[37] The finding that TNFR2 is pivotal for immune regulation may offer a potential explanation for the observation that, in contrast to other autoimmune diseases, therapies that target TNF/TNFR blockade can have deleterious effects on patients with MS.[38] The question of whether the positive effects of TNFR2 signaling are preserved in Tregs in the context of MS remains to be determined.

In summary, in this study, we uncovered a novel mechanism involved in cladribine-based therapy for MS. According to our data, the circulating compartment of restTregs is restrained along therapy and this associates with clinical response.

Our study has several limitations. First, the cohort size of only 11 patients with MS is small, and our findings require confirmation in larger, independent studies. Second, we could not determine the fate of Tregs, particularly the restTregs, after the decline of their counts in blood. Cell-tracing studies are needed to ascertain whether the restTregs, highly expressing Bcl2, are functionally impaired in patients with MS and whether they are eliminated by cladribine and effectively replaced by newly derived, competent Tregs.

Author Contributions

I. Cammarata: major role in the acquisition of data. G. Sartori: major role in the acquisition of data. T. Giacomelli: major role

in the acquisition of data. V. Pinna: major role in the acquisition of data. A. Pinzon Grimaldos: major role in the acquisition of data. G. De Rosa: major role in the acquisition of data. G Matarese: analysis or interpretation of data. C. Procaccini: major role in the acquisition of data; analysis or interpretation of data. D. Mishra: major role in the acquisition of data. D. Di Mitri: analysis or interpretation of data. C. Gasperini: major role in the acquisition of data; study concept or design. G. Guerrera: major role in the acquisition of data. M. Sambucci: major role in the acquisition of data. L. Battistini: drafting/revision of the manuscript for content, including medical writing for content; study concept or design. S. Piconese: drafting/revision of the manuscript for content, including medical writing for content; study concept or design; analysis or interpretation of data.

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