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**Permalink**

https://escholarship.org/uc/item/4z9020bg

**Journal**

The FASEB Journal, 37(8)

**ISSN**

0892-6638

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**Publication Date**

2023-08-01

**DOI**

10.1096/fj.202300573r

Peer reviewed

# HHS Public Access

# Therapy of autoimmune inflammation in sporadic amyotrophic lateral sclerosis: Dimethyl fumarate and H-151 down-regulate inflammatory cytokines in the cGAS-STING pathway

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## Abstract

In sporadic amyotrophic lateral sclerosis (sALS), IL-17A- and granzyme-positive cytotoxic T lymphocytes (CTL), IL-17A-positive mast cells, and inflammatory macrophages invade the brain and spinal cord. In some patients, the disease starts following a trauma or a severe infection. We examined cytokines and cytokine regulators over the disease course and found that, since

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the early stages, peripheral blood mononuclear cells (PBMC) exhibit increased expression of inflammatory cytokines *IL-12A*, *IFN-γ*, and *TNF-α*, as well as granzymes and the transcription factors *STAT3* and *STAT4*. In later stages, PBMCs upregulated the autoimmunity-associated cytokines *IL-23A* and *IL-17B*, and the chemokines *CXCL9* and *CXCL10*, which attract CTL and monocytes into the central nervous system. The inflammation is fueled by the downregulation of *IL-10*, *TGFβ*, and the inhibitory T-cell co-receptors *CTLA4, LAG3,* and *PD-1,* and, in vitro, by stimulation with the ligand PD-L1. We investigated in two sALS patients the regulation of the macrophage transcriptome by dimethyl fumarate (DMF), a drug approved against multiple sclerosis and psoriasis, and the cyclic GMP-AMP synthase/stimulator of interferon genes (cGAS/STING) pathway inhibitor H-151. Both DMF and H-151 downregulated the expression of granzymes and the pro-inflammatory cytokines *IL-1β, IL-6, IL-15, IL-23A, and IFN-γ*, and induced a pro-resolution macrophage phenotype. The eicosanoid epoxyeicosatrienoic acids (EET) from arachidonic acid was anti-inflammatory in synergy with DMF. H-151 and DMF are thus candidate drugs targeting the inflammation and autoimmunity in sALS via modulation of the NFκB and cGAS/STING pathways.

## Plain Language Summary:

Amyotrophic lateral sclerosis (ALS) is a paralyzing disease affecting both young and old subjects. About 5–10% of ALS cases are genetically inherited (familial ALS), while 90–95% of cases develop "sporadic" ALS (sALS) without a positive family history. Neuropathological studies demonstrate that sALS is an inflammatory disease involving an attack by cytotoxic T cells, mast cells, and inflammatory macrophages on the neurons in the brain and spinal cord. Peripheral blood mononuclear cells (PBMC) of sALS patients show increasing "autoimmune" (i.e. self-directed) messages against autologous DNA and microbial molecules. We treated sALS patients' immune cells with the drug dimethyl fumarate (DMF), which is an approved drug against the autoimmune disease multiple sclerosis, and the molecule H-151, which blocks autoimmunity in model systems. Both DMF and H-151 decreased the expression of cytokines and granzymes. The effect of DMF was increased by epoxyeicosatrienoic acids. DMF and H-151 together with certain fatty acids in the diet are thus candidates for a clinical trial targeting the autoimmune inflammation in ALS that resists current therapies.

cGAS is a sensor of mislocated autologous DNA and microbial nucleic acids. cGAS synthesizes the cyclic nucleotide cGAMP, which activates STING in endoplasmic reticulum (ER). STING translocates to Golgi, where it is palmitoylated and phosphorylated by TBK1 and recruits IRF3. TBK1 phosphorylates IRF3 and NFκB transcription factors that drive inflammatory cytokine transcription. Inhibiting STING with H-151 may thus be a therapeutic strategy for treating sALS.

### Keywords

## 1.0   Introduction

Amyotrophic lateral sclerosis (ALS) is a chronic, progressive neurodegenerative disorder of upper and lower motor neurons associated with an inflammatory attack in the brain and spinal cord (Fig.1). ALS has familial (fALS) and sporadic (sALS) forms, and lacks effective therapy. The cause of fALS is attributed to the mutations of the genes for protein homeostasis, and RNA metabolism, involving the genes *C9orf72,* superoxide dismutase *(SOD1),* TAR DNA-binding protein 43 (*TDP-43),* and *FUS-TLS*, and altered cytoskeletal dynamics (1).

The cause of sALS is unknown, but epidemiological data show an association of sALS with prior trauma (2) and microbial infections (3). Damage-associated molecular pattern (DAMP) molecules, including autologous nucleic acids released by trauma, and microbial agents with pathogen-associated molecular patterns (PAMPs) are potential (auto)inflammatory stimuli. The role of microbial infection was noted in the study of a genetically identical twin pair with both twins possessing common ALS mutations, but only the twin suffering a cat bite and a severe systemic Pasteurella infection later developed ALS, whereas the other twin remains healthy 11 years later (4). The ALS twin displayed spontaneous production of the cytokines IL-6 and TNFα, and epigenetic modification through differential methylation of immune-related regions near *EGFR* and *TNFRSF11A* genes.

TDP-43, a nuclear DNA/RNA-binding protein, accumulates and aggregates in neurons of sALS patients, triggers the release of mitochondrial DNA, and activates cyclic GMP-AMP synthase (*cGAS*), which synthesizes 2', 3'-cGMP-AMP (cGAMP), and activates the stimulator of interferon genes (cGAS-STING) pathway (5). In this pathway, the endoplasmic reticulum (ER)-membrane adaptor stimulator of interferon genes (*STING*) is released from ER and traffics to Golgi, is palmitoylated and phosphorylated by TANK-binding kinase 1 (*TBK1*), and recruits the transcription factor interferon regulatory factor 3 (*IRF3*), which is phosphorylated. Phospho-IRF3 dimerizes, translocates to the nucleus, and induces interferons and interferon-stimulated genes *TNF, IL-1β, and IL-6*. STING also inactivates *IkB* and activates *NFkB* (6) (7).

There are multiple lines of evidence supporting the roles of autoimmunity and inflammation in sALS. The central nervous system (CNS) gray matter is infiltrated by *IL-17* - positive cytotoxic T lymphocytes (CTLs) (8) (9) (10) (11), which are also found in the blood, and *IL-17A* mRNA and protein are increased in the blood and cerebrospinal fluid. Moreover, IL-17A-positive mast cells and inflammatory monocyte/macrophages (MM) infiltrate the spinal cord gray matter. MM are cyclooxygenase-2 (*COX-2*)-, interleukin-1β (*IL-1β*)-, tumor necrosis factor-α (*TNF-α*)-, and nitric oxide synthase (*NOS)*-positive and appear to phagocytize neurons (12) (Fig.1)

The rationale for an anti-inflammatory therapy of sALS is supported by the increasing inflammatory and autoimmune transcripts in peripheral blood mononuclear cells (PBMC) over the disease course, and the sALS neuropathology. We initially speculated that the programmed cell death ligand 1 (PD-L1) would engage the inhibitory receptor PD1 and down-regulate inflammation but, in ALS patients, PD-L1 engagement was aberrantly pro-

inflammatory. Motivated by the increased evidence of autoimmunity and inflammation, we tested in ALS macrophages a) dimethyl fumarate (DMF), the drug approved for the therapy of two autoimmune diseases, multiple sclerosis and psoriasis, b) the inhibitor of the cGAS-STING pathway H-151 (5) (6), and c) the eicosanoid epoxyeicosatrienoic acids (EET). We demonstrate that DMF with epoxyeicosatrienoic acids (EET) and H-151 have anti-inflammatory and anti-autoimmune effects in immune cells, which suggest their therapeutic usefulness in ALS.

## 2.  Materials and Methods

### 2.1.  sALS and control patients

We investigated the peripheral blood mononuclear cell (PBMC) transcriptome of eight sporadic ALS (sALS) patients over the disease course: **#1** (M, 38 y/o, "progressing relatively fast" with the loss of 0.56 FRS points per month); **#2** (M, 41 years, "progressing relatively slow" with the loss of 0.22 FRS points per month); **#3** (M, 70-year-old, loss 0.80 points per month), **#4** (M, 60 y/o, loss 1.03 points per month), **#5** (M, 65 y/o, loss 1.00 points per month), **#6** (M, 59 y/o, loss 0.57 points per month), **#7** (M, 65 y/o, loss 0.41 points per month), **#8** (M, 68 y/o, loss 0.85 points per month). We also tested the transcriptome of four healthy controls (**Ctr #1** M, 81y/o; **Ctr #2** F 51 y/o; **Ctr #3** F 67 y/o; **Ctr #4** F, 56 y/o) (Fig. 2). The diagnosis of sALS was established by primary neurologists according to upper and lower motor neuron signs, electromyography, and exclusion of other disorders and familial ALS. We evaluated the clinical stage according to the ALS functional rating scale (ALSFRS), rated on a scale from 0 to 48 with a score of 48 corresponding to control patients (Table 1).

We investigated *in vitro* immune regulation of differentiated macrophages from sALS patient **#9** (M, 40 y/o, ALS-FRS 16, loss of 1.33 points per month, duration 24 months) with a typical ALS presentation, and sALS patient **#10** (F, 65 y/o, ALS-FRS score 39, loss of 0.37 points per month, duration 24 months) with dysarthria and weakness of facial muscles followed by sensory changes, characteristic of the ALS-related disorder facial onset sensory and motor neuronopathy (FOSMN). The latter patient had a history of severe impetigo infection (a source of microbial nucleic acids) in childhood. We investigated protein abundances in natural killer (NK) cells of an identical but ALS-discordant twin pair, the **sALS twin #1** (M, 77y/o, loss 0.57 points per month), and the identical **healthy twin #2** (Fig. 2).

### 2.2.  Treatment of sALS macrophages by dimethyl fumarate (DMF) with epoxyeicosatrienoic acids, and the cGAS-STING inhibitor H-151

We investigated immune regulation in macrophages differentiated *in vitro* from PBMCs of the rapidly progressing younger sALS patient **#9** and the slowly-progressing older sALS patient **#10.** We tested possible therapeutic molecules dissolved in dimethyl sulfoxide (DMSO) and IMDM medium (Fisher): the soluble epoxide hydrolase inhibitor (sEHI) TPPU (1 uM) (13), dimethyl fumarate (DMF) (Sigma-Aldrich) (0.1 uM), epoxyeicosatrienoic acids (EET) (1 uM), and H-151 (InvivoGen, San Diego, CA 92121) (10 ug/ml and 1 ug/ml). Epoxyeicosatrienoic acids (EET) methyl esters were synthesized in the laboratory of B.

Hammock, UC Davis from arachidonic acid methyl ester according to the method of Falck et al (Methods in Enzymology, vol 187, pages 357– 365, 1990).

### 2.3. Immunohistochemistry of spinal cord tissues

The immunochemical staining of sALS paraffin-embedded spinal cord tissues, after antigen retrieval using pH 6-buffer steam and blockade with goat serum, was performed using DAKO Envision Doublestain system with primary antibodies to the macrophage marker CD68, (DAKO), granzyme B, and IL-17A (Fig. 1).

### 2.4. RNA-Seq analysis

We isolated RNA using the Quick-RNA miniprep kit (Zymo Research) and prepared RNA libraries using the Illumina TruSeq Stranded mRNA kit. We quantified final libraries using the Qubit BR dsDNA Assay (Life Technologies) and performed QC with the D1000 Assay on a 2200 Tape Station (Agilent Technologies). We sequenced PBMC libraries using an Illumina HiSeq 4000 to obtain 50 bp reads and evaluated the quality of the RNA-seq data using FastQC. We aligned the reads to the human genome (hg19) using STAR v2.5.0a (14). We quantified raw read counts using HTSeq v0.6.1 (15) and transformed them to Reads Per Million (RPM) for each sample. We analyzed the time course of the expression of cytokines, chemokines, granzymes, and transcription factors after disease onset.

For analyses of patient macrophages treated with candidate drugs, we aligned reads to hg38 using the RNAseq processing workflow TOIL (16). Briefly, TOIL employs STAR for alignment and quantifies reads using both RSEM (RNAseq by Expectation Maximization) and Kallisto. We used RSEM upper quartile normalized counts for subsequent analyses. For each set of samples, we calculated expression log fold changes by comparing each patient's treated cells to the DMSO-treated control. Heatmaps were generated with the package *pheatmap* in R, and principal component analysis (PCA) was performed using the R function *prcomp*.

### 2.5. Global proteomic analysis of NK cells

NK cells were isolated using a CD56+ selection kit (Stem Cells Technologies, Vancouver, BC, Canada) from the blood of a twin-pair discordant in the ALS diagnosis. The protein abundance ratio of NK cells was measured using a native hybrid quadrupole-Orbitrap mass spectrometer and high pH reverse-phase chromatography fractionation mass spectroscopy. We analyzed the protein abundance ratio of the NK immune- and cell adhesion- proteins in the affected over the healthy twin.

## 3.0 Results

### 3.1. Inflammatory cytokines are upregulated in early disease and autoimmune cytokines and granzymes in late disease.

The key sALS immunopathology of the ALS spinal cord gray matter involves the invasion by IL-17A-positive and granzyme B-positive cytotoxic CD8 cells, which engulf the neurons (Fiala, 2010) (Fig.1). Since this inflammatory neuropathology is derived from the circulating PBMCs, we analyzed by RNAseq the transcriptome of prospectively-collected PBMCs over

the ALS disease course since the disease onset. Two sALS patients provided prospective samples and 8 patients and 4 control subjects (shown at baseline) provided one sample (Fig. 2).

The cytokines *tumor necrosis factor-α (TNFα)* and *interferon-γ (IFNγ)* were up-regulated since disease onset in relation to controls shown at baseline and were down-regulated 25- and 15-months post-diagnosis, respectively. Conversely, the anti-inflammatory cytokines *IL-10* and *transforming growth factor beta 1* (*TGFβ1*) transcripts decreased over the disease course. The autoimmunity cytokine *IL-17 B* increased in late disease, together with the expression of the pro-inflammatory chemokines *CXCL9* and *CXCL10,* which attract immune cells into the CNS (Fig. 3A).

Increased expression of inflammatory cytokines, especially from the IL-23 and IL12 cytokine families, signal to the downstream transcription factors STAT1, STAT3, and STAT4. (17). Target genes of *STAT 3* and *STAT4* include the cytokine IFNG and granzymes. Granzymes are serine proteases toxic to neurons, which are released from natural killer (NK) cells and cytotoxic T-cells (18). Granzymes were induced following the induction of *STAT4,* which serves as the transcription factor for the granzyme B promoter (19). Correspondingly, the expression of granzymes decreased late in the disease course after 30 months, following a decrease in STAT4 expression at 25 months (Fig. 3B).

### 3.2.  Systemic inflammation over the ALS disease course is fueled by the down-regulation of T-cell inhibitory co-receptors and up-regulation of stimulatory T-cell co-receptors

T-cell inhibitory co-receptors such as cytotoxic T-lymphocyte associated protein 4 (CTLA4) and Programmed cell death protein-1 (PD-1) are negative regulators of T-cell immune responses, while the stimulatory co-receptors show the opposite effect (20). In ALS patients' PBMCs, *CTLA4* showed gradual downregulation over the disease course. Another inhibitory co-receptor *LAG3* showed initial upregulation with a sharp decline at 40 months post-onset. In contrast, the stimulatory co-receptors *OX40* and *GITR* showed dramatic upregulation at 40 months post-onset. As expected (21), in the whole cohort, late-surviving patients had a relative increase in the transcription factor *FOXP3*, a master regulator of the regulatory pathway in the development and function of immunoregulatory T cells (Tregs) (Fig. 3C).

### 3.3.  Proteomic analysis indicates overexpression of granzymes, kinases, cell adhesion, and apoptotic proteins in the natural killer cells of the ALS twin in comparison to the healthy twin

To identify the changes in natural killer (NK) T cell populations of an ALS discordant and genetically identical twin-ship, we performed proteomics and evaluated proteins with relevant functions according to the ratio of the ALS twin/the healthy twin (Fig. 4). As expected, granzyme B, a protease toxic to neurons, was increased in the ALS twin. In addition, the surface proteins CD44 (cell-surface glycoprotein involved in cell-cell interactions), CD8 cytotoxic T-cell antigen, and the histocompatibility antigen HLA-DRA were also increased, suggesting increased T-cell activity in the ALS twin.

We performed proteomic analysis of immune regulation and cell death. Signaling by GTPases RAC1, RAC2, and RHOA stimulates cytoskeletal reorganization and inflammatory pathways (22). Signaling by these GTPases is important for regulating cell proliferation, differentiation, and survival. These GTPases, as well as MAPK proteins and SRC kinase, were more abundant in NK cells of the ALS twin than in the healthy twin. Notably, SRC kinase inhibitors have been shown in a large phenotypic screen to protect from motor neuron degeneration, and MAPKp38 inhibition has also been proposed as a therapeutic strategy for ALS (23) (24).

The cell junction and cell adhesion proteins actin (ACTB), talin1 (TLN1), integrin β3 and VIM were also more abundant in the ALS twin than in the healthy twin. Certain cell surface proteins are thought to serve as potential biomarkers for ALS and may be the target for autoantibodies. In addition, anti-ACTB antibodies may be a biomarker for ALS disease severity.

Finally, the immunophilin proteins, which bind to the immunosuppressive molecules FK506 and rapamycin, were higher in the ALS twin, as were caspase 3, death agonist protein BID, and superoxide dismutase-1 (SOD-1), which promote apoptosis (Fig. 4). Taken together, these results suggested multiple dysregulated pathway functions in the NK cells of an ALS patient compared to his healthy identical twin.

### 3.4. Aberrant immune regulation by PDL-1, PD-1 antibody in ALS macrophages

Given the pro-inflammatory signatures seen in the ALS time-course and the concomitant downregulation of T-cell inhibitory co-receptors, we hypothesized that manipulating immune checkpoint receptor-ligand interactions may reduce inflammatory cytokine production in the ALS macrophage transcriptome. PDL-1 (CD274) is a ligand on cancer cells and antigen-presenting cells that stimulates the inhibitory co-receptor programmed cell death protein 1 (PD-1, aka CD279) to block inflammatory activation of CTLs. We investigated the effects of PD-L1 (8 ug/ml) (Biolegend, San Diego, CA) and PD-1 antibody (2.3 ug/ml) (Abclonal Technology, Woburn, Mass) (in comparison to 1% dimethyl sulfoxide (DMSO)) on induction of the macrophage inflammatory cytokines, transcription factors, and regulatory co-receptors (*CTLA4, LAG3, PDCD1*). Contrary to the established function of PD-L1 to activate PD-1 and inhibit immune responses, treatment with PDL-1 generated a pro-inflammatory effect in sALS patients' macrophages by upregulating the expression of the inflammatory cytokines. Treatment with the PD-1 antibody, which blocks PD-1/PD-L1 interactions, was expected to be pro-inflammatory, but instead tended to be anti-inflammatory, decreasing the expression of IL1B and IL6 (Fig.5). In contrast, treatment of the same patient's cells with DMF resulted in strong downregulation of all examined cytokines, granzymes, and NFκB target genes.

### 3.5. Inhibition of inflammatory cytokines, granzymes, and NFκB in ALS macrophages by therapeutic molecules DMF and H-151

Given the autoimmune features of sALS, we next tested dimethyl fumarate (DMF) (Fig.6), a drug approved for the therapy of two autoimmune diseases, multiple sclerosis and psoriasis, without or with epoxyeicosatrienoic acids (EET) and the cGAS-STING inhibitor H-151

(Fig. 7). We investigated these effects in the macrophage cultures of two ALS patients: the younger patient **#9** (M, 40 y/o, ALS-FRS 16, severe loss of 1.33 ALS FRS points per month, duration 24 months, with typical ALS features) and the older patient **#10** (F, 65 y/o, ALS-FRS score 39, less severe loss of 0.37 points per month, duration 24 months, with FOSMN). After 24-hr treatment with either drug or DMSO, we collected total RNA and performed RNA sequencing. We calculated the log fold change of gene expression in DMF or H151-treated cells compared to DMSO. A negative log2 fold change indicates decreased expression of the gene upon drug treatment.

**a)  Inhibition by dimethyl fumarate (DMF) with or without epoxyeicosatrienoic acids (EET) and TPPU**—We investigated the effect of DMF and H-151 on the expression of several categories of genes: inflammatory cytokines, granzymes, NFκB, and IFN target genes (Fig. 6). In both patients, we observed that DMF downregulated the inflammatory cytokines *IL1β, IL15, and IFNγ*. The downregulation of the expression of pro-inflammatory cytokines as well as granzymes and NFκB target genes such, as *IL6* and *TNFAIP3*, by DMF appeared to be potentiated by the addition of EET, an epoxy fatty acid derived from the metabolism of arachidonic acid, known to have anti-inflammatory effects, and, in some transcripts, by the soluble epoxide hydrolase inhibitor (sEHI) TPPU.

**b)  Inhibition by cGAS/STING inhibitor H 151**—To investigate the role of the cGAS/STING pathway, we next examined the effect of the STING inhibitor H-151 on cytokines, NFκB target genes, and interferon signaling (e.g. IRF3 and IRF7) target genes in macrophages with or without PBMC (Fig. 7). H-151 in a dose-responsive fashion (1 and 10 ug/ml) downregulated a subset of pro-inflammatory cytokines, including IL1B and TNF, and the effects were potentiated by the presence of PBMC in macrophage cultures. The direct NFκB target gene NFKBIA was also downregulated, suggesting decreased NFκB signaling activity. Importantly, downstream targets of cGAS/STING signaling such as ISG15, MX1, and IFIT1 were downregulated, supporting effective STING inhibition by H-151.

### 3.6:  Comparison of the effects of DMF vs. H-151 on ALS patient macrophages.

We first evaluated the reproducibility of biological replicates in the same cultures (R = 0.94) and in different cultures of the same patient (R= 0.98), in comparison to the cultures of different patients (R= 0.87) and different treatments (R =0.83). Thus, we observed high concordance (R > 0.9) of biological replicates of the same patient (Fig.8).

To examine the differences between DMF and H-151 treatment, we performed principal component analysis (PCA) on more recently collected samples from patient **#10**, which appear to cluster together. PCA on this subset showed that DMF vs. H-151 treated samples separated along Principal Component 2 (Fig. 9A). This analysis identified a subset of inflammatory genes that most strongly drove this distinction: IFNG, CCL15, CXCL9, GZMB, and IGFBP15 were all down regulated in H151-treated samples more than in DMF-treated samples (Fig. 9B). In contrast, H-151 samples exhibited an increased expression of genes important in oncogenesis, such WNT10A, WNT7A, PLEKHB1, and SLC22A17 (Solute Carrier Transporter), which have been implicated in or suggested as therapeutic targets for ALS (Fig. 9B) (25) (26). Serendipitously, DMF +/− EET+/− TPPU inhibited

these WNT and PLEKHB targets. Future investigation and eventually clinical trials need to determine whether the combination of H-151 with DMF would be synergistic against autoimmune inflammation in sALS patients.

## 4.0  Discussion

### 4.1  Clues about the origin of sporadic ALS.

In a previous study of a genetically identical, ALS discordant twin pair, both twins had common ALS mutations, but only the ALS twin suffered a cat bite leading to a severe systemic Pasteurella infection and later developed ALS, whereas the other twin remains healthy 11 years later (4). The ALS twin displayed spontaneous production of the cytokines IL-6 and TNFα, and epigenetic modification through differential methylation of immune-related regions near *EGFR* and *TNFRSF11A* genes.

### 4.2  Progressive inflammation over the course of sporadic ALS: An initial inflammation with interferon-γ and TNF-α and a later autoimmune inflammation with IL-17B

We examined in a cohort of 8 sALS patients over the disease course the transcripts of inflammatory and autoimmune cytokines (Fig. 3). The prospective results show an early up-regulation of the inflammatory cytokines *IFN-γ and TNF-α*, which are signaling through *STAT3* and *STAT4*, followed by a later increase of CD4 T cells and up-regulation of *IL-17B* on Th17 cytotoxic T cells and the chemokines associated with *STAT1* signaling. The inflammatory cytokines stimulating IL-17 expression in Th17 cells, i.e. *IL-1, IL-6,* and *IL-23A*, are upregulated in response to aggregated superoxide dismutase-1(SOD-1) *in vitro* (11). Over the sALS disease course, the stimulatory co-receptor *OX-40 and GITR* increased, whereas *IL-10*, *TGFβ1,* the inhibitory co-receptors *LAG3* and *CTLA4,* and regulatory T cells (Tregs) decreased. We also analyzed the relation of inflammation to autoimmunity in PBMC and NK cells from a pair of identical, ALS-discordant twins. The increase of inflammation was also observed in this twin-ship proteomic study with an overall increase in the sALS twin's NK cells of inflammatory proteins, including granzymes, GTPases, and cell adhesion proteins (Fig 4).

### 4.3  Anti-inflammatory therapies of ALS

The heterogeneity of inflammatory mechanisms in sALS have suggested that diverse immunotherapeutic strategies may be necessary. The IL-6 receptor antibody tocilizumab (12) showed temporary effects in a subset of sALS patients with an inflammatory Th1/Th17 signature but no effects in a second subset with a B cell signature (4). An i*n vitro* study showed that induced pluripotent stem cell (iPSC)-derived M2 macrophages from sALS patients suppress activation of M1 macrophages and boost ALS Tregs (27). In a mouse model, grafting protective macrophages at disease onset extended survival and decreased inflammation (28). In patients with chronic autoimmune inflammation, macrophages have both beneficial immunosuppressive and pathogenic tissue-destructive roles (29).

We initially speculated that activation of the inhibitory co-receptor PD-1 by recombinant PD-L1 would be anti-inflammatory, i.e. therapeutic. However, both recombinant PD-L1 ligand and recombinant PD-1 receptor were strongly pro-inflammatory, as observed in

systemic sclerosis (30) and ALS (31), whereas PD-1 antibody was anti-inflammatory, demonstrating aberrant regulation of the ALS patients' immune system (Fig 5).

### 4.4 DMF and H-151 are anti-inflammatory drugs in sporadic ALS patients

We abandoned the PD-L1 strategy and chose a strategy with dimethyl fumarate (DMF), an approved drug against two autoimmune diseases, multiple sclerosis and psoriasis, and the inhibitor of the cGAS-STING pathway H-151 implicated in autoimmunity. Both drugs, DMF (in combination with EET) (Fig. 6) and H151 (Fig. 7) inhibited the inflammatory cytokines (*IFN-γ, IL-1β, IL-15, IL-23A, and TNF*), the granzymes (B, M, and H), and the transcription factors *NFkB1* and *NFkB2* in macrophages of ALS patients. According to the literature, only DMF has been used in a clinical setting in a combination with riluzole in a randomized multi-site Australian clinical trial in 107 sporadic ALS patients (32). In that study, the DMF therapy (240 mg twice daily P.O.) was safe but, in comparison to placebo, not significantly active against ALS progression according to ALS FRS. Our *in vitro* results using the EET/DMF combination in ALS macrophages suggest that DMF may be effective when combined with a diet rich in certain polyunsaturated fatty acids (PUFAs).

## Conclusions:

Both DMF (in synergy with EET and TPPU) and H-151 inhibit in immune cells of sALS patients inflammatory and autoimmune signaling, which support their investigation in a clinical trial.

## Acknowledgments

## Data Availability Statement

Data are available from the corresponding authors.

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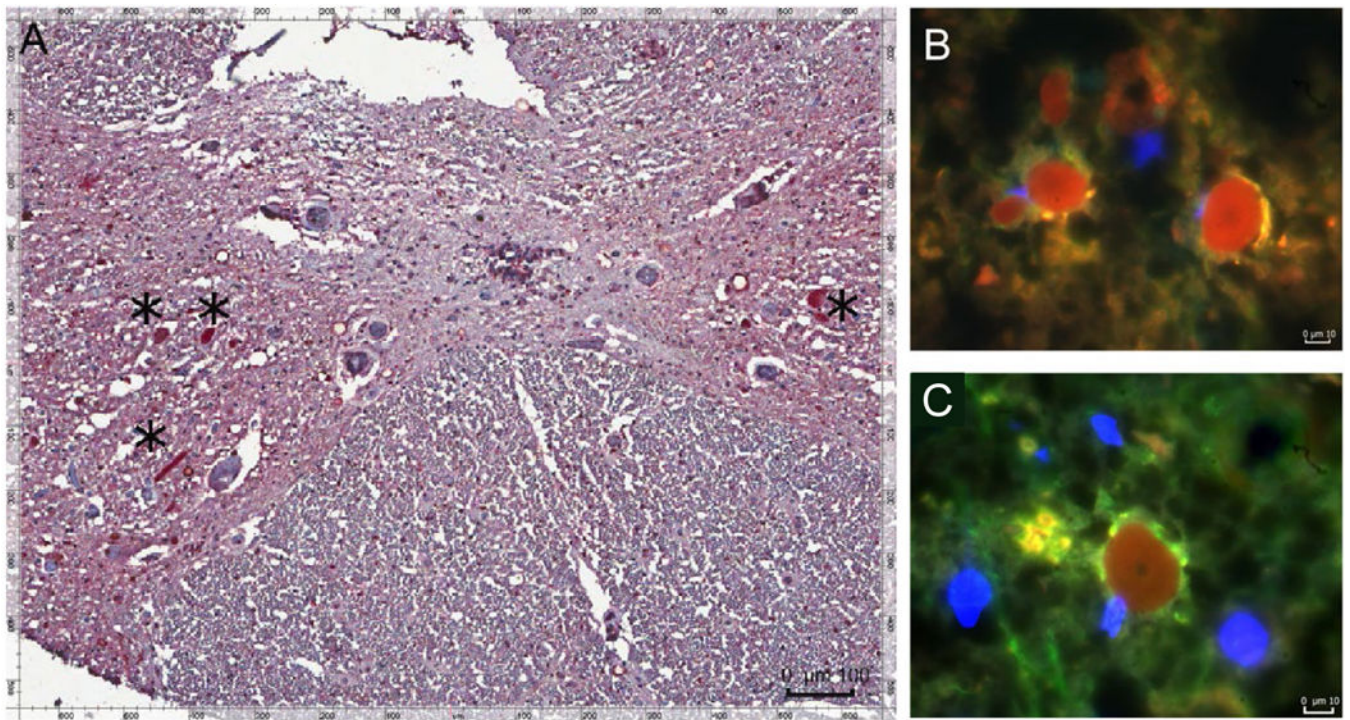
**Fig. 1. IL-17A- and granzyme B-positive CD3 cells invade the ALS spinal cord gray matter.**
Immunohistochemistry **(A)** and immunofluorescence (**B, C**) demonstrate (**A**) IL-17A-
positive cells (indicated by **\***) in the gray matter; (**B**) Granzyme B-positive CD3 cells (red/
green); (**C**) IL-17A-positive CD3 cells (red/green) (sporadic ALS patient #8; reprinted from
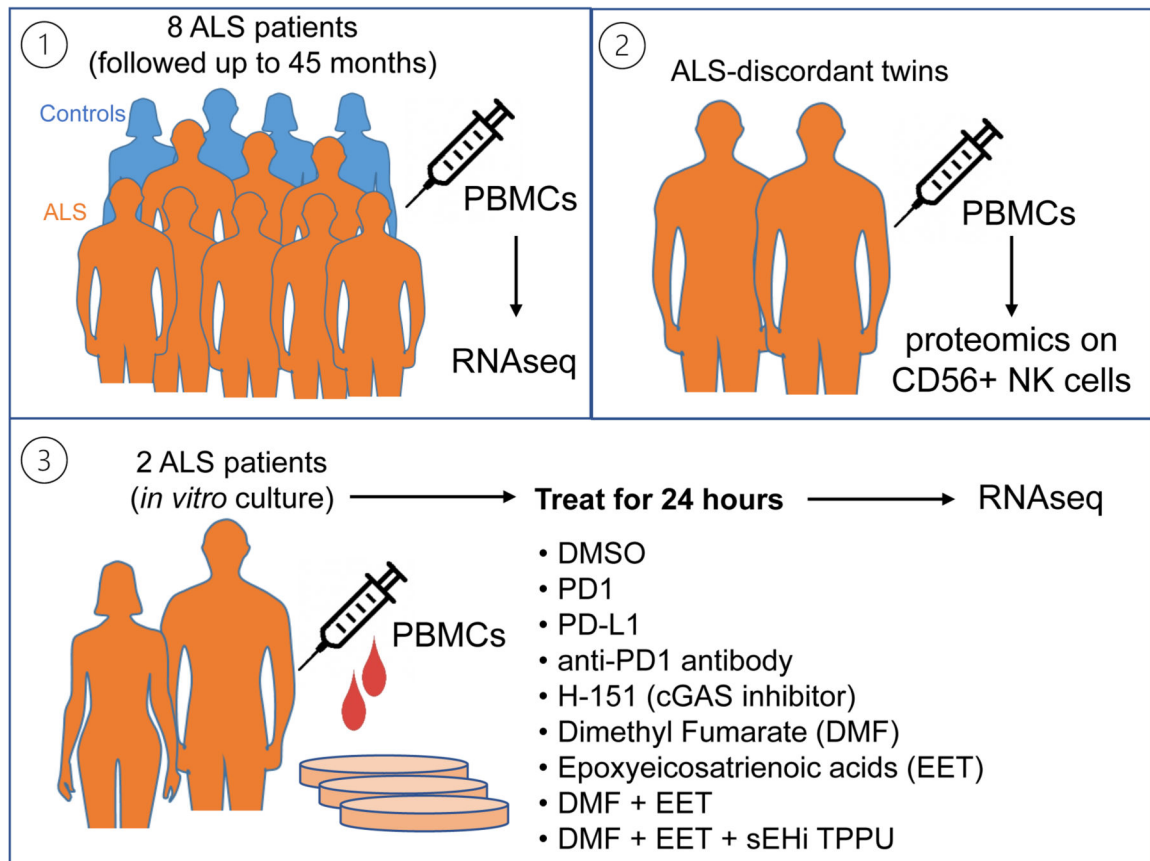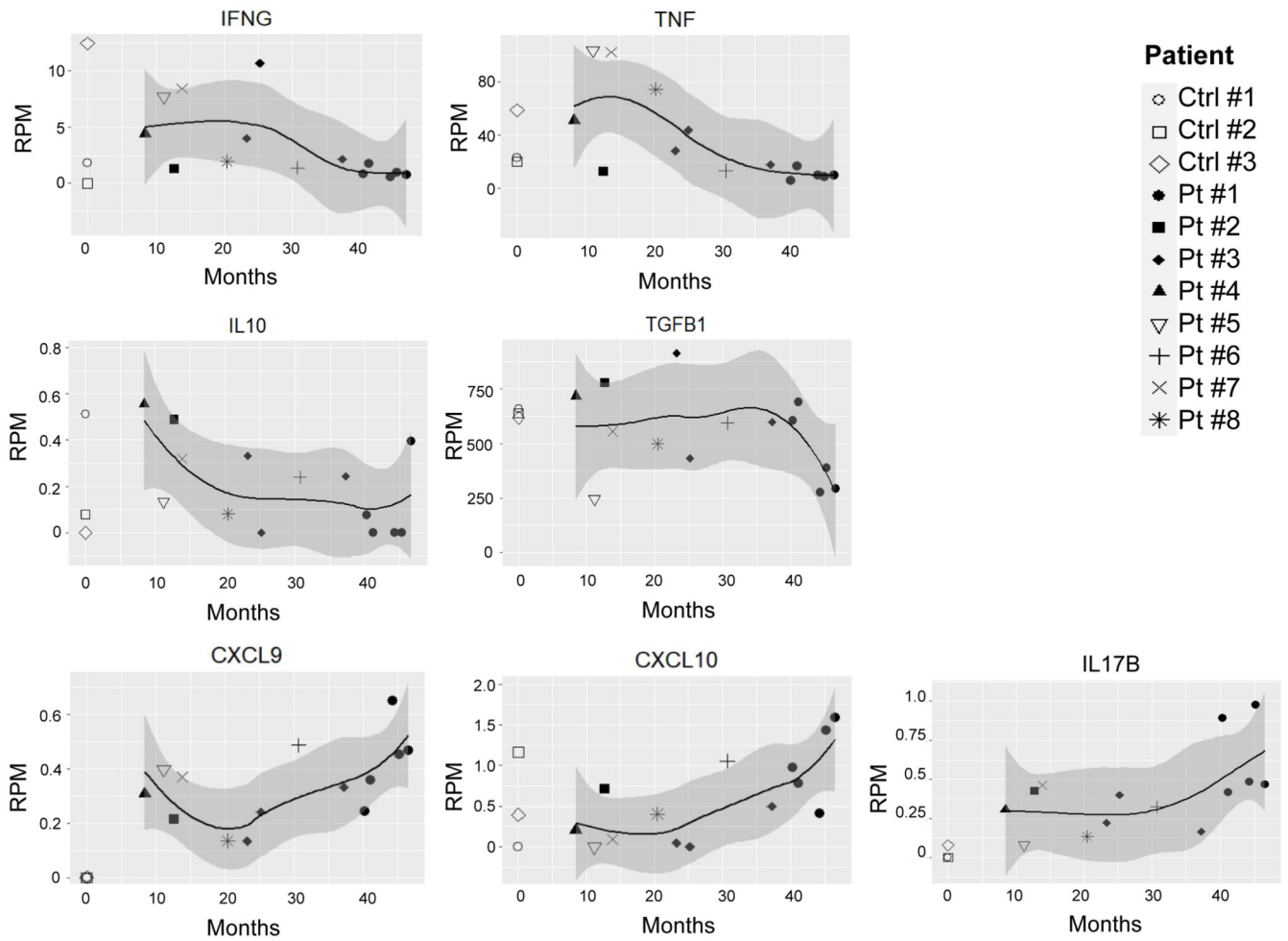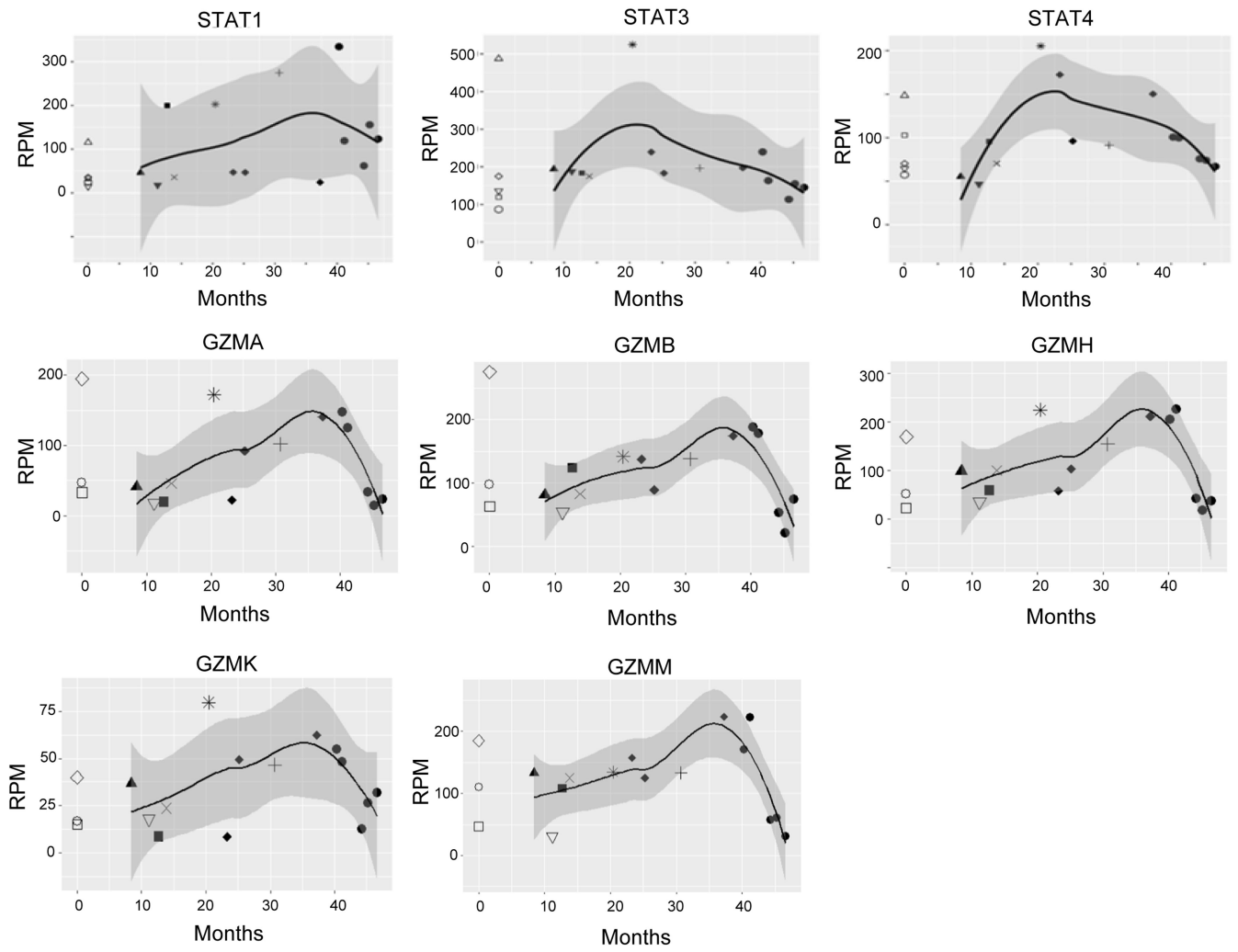Fiala, M. et al JNI 2010).

**Fig. 2. Study populations.**

Transcriptomic analysis of PBMCs from **1)** 4 controls and 8 ALS patients over the disease course, **2)** one ALS-discordant pair, or **3)** two ALS patients' PBMCs and macrophages treated *in vitro* with candidate drug combinations.

## A Cytokines and cytokine regulators

## B. Transcription factors and granzymes

## C Co-receptors

### 1. Stimulatory co-receptors
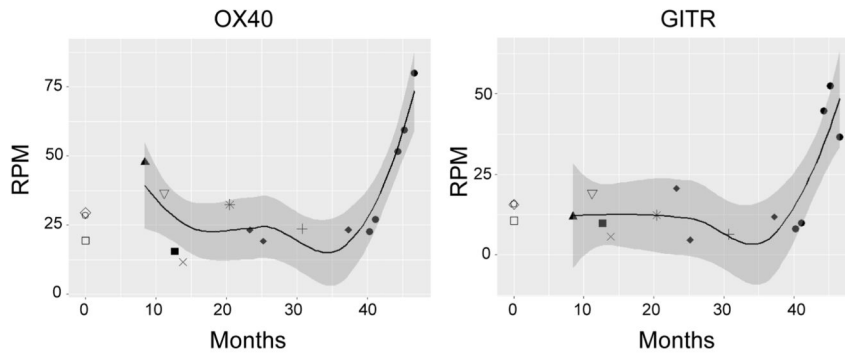


### 2. Inhibitory co-receptors
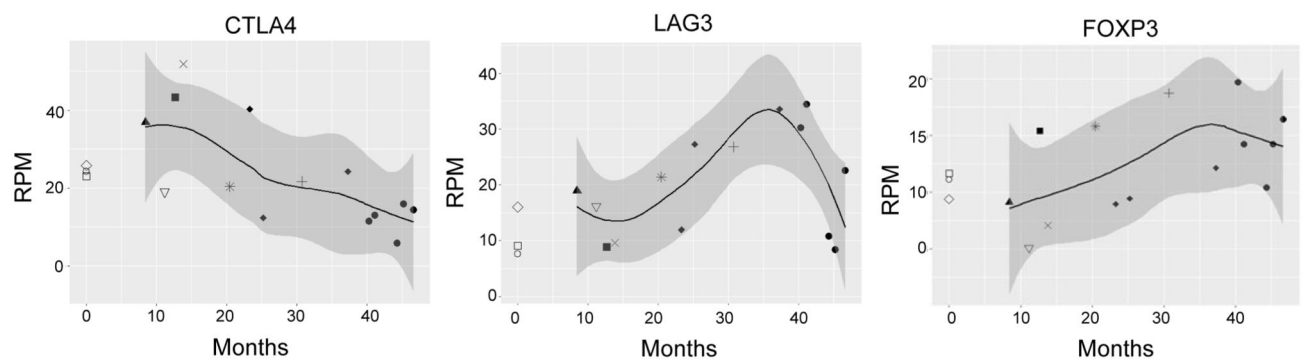


**Fig. 3. ALS PBMC transcriptome over the disease course:**
A Cytokines and cytokine regulators. The transcriptome of ALS patients is shown over the disease course; the transcriptome of controls is shown at baseline (data on the ALS patients #1 to #8 according to the time after onset).

B Transcription factors and granzymes.

C Co-receptors
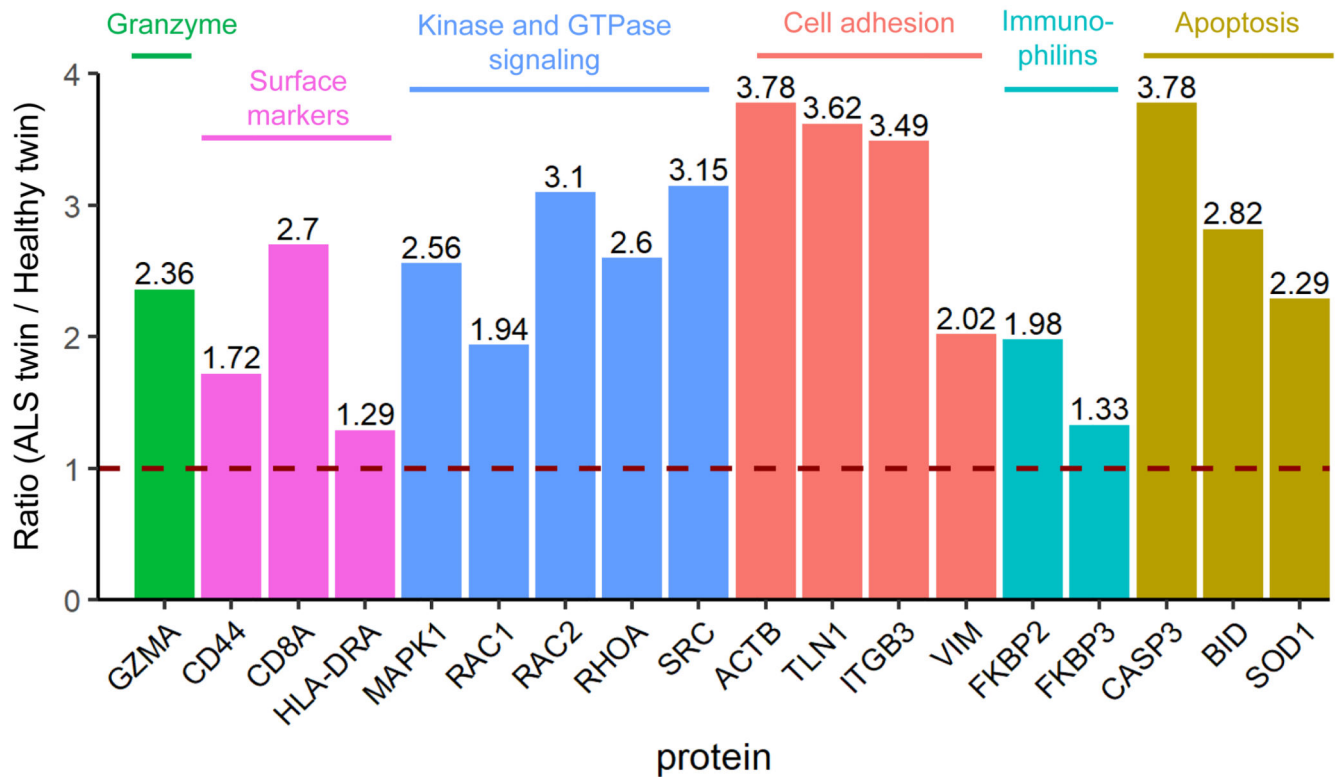
**Fig. 4. Natural killer (NK) cell proteome in the twin-ship:**
Ratio of the inflammatory proteins in the ALS twin vs. the healthy twin. Note the overexpression of granzymes, RAC, MAPK signaling, cell adhesion, immunophilins, and apoptotic proteins in the ALS twin.
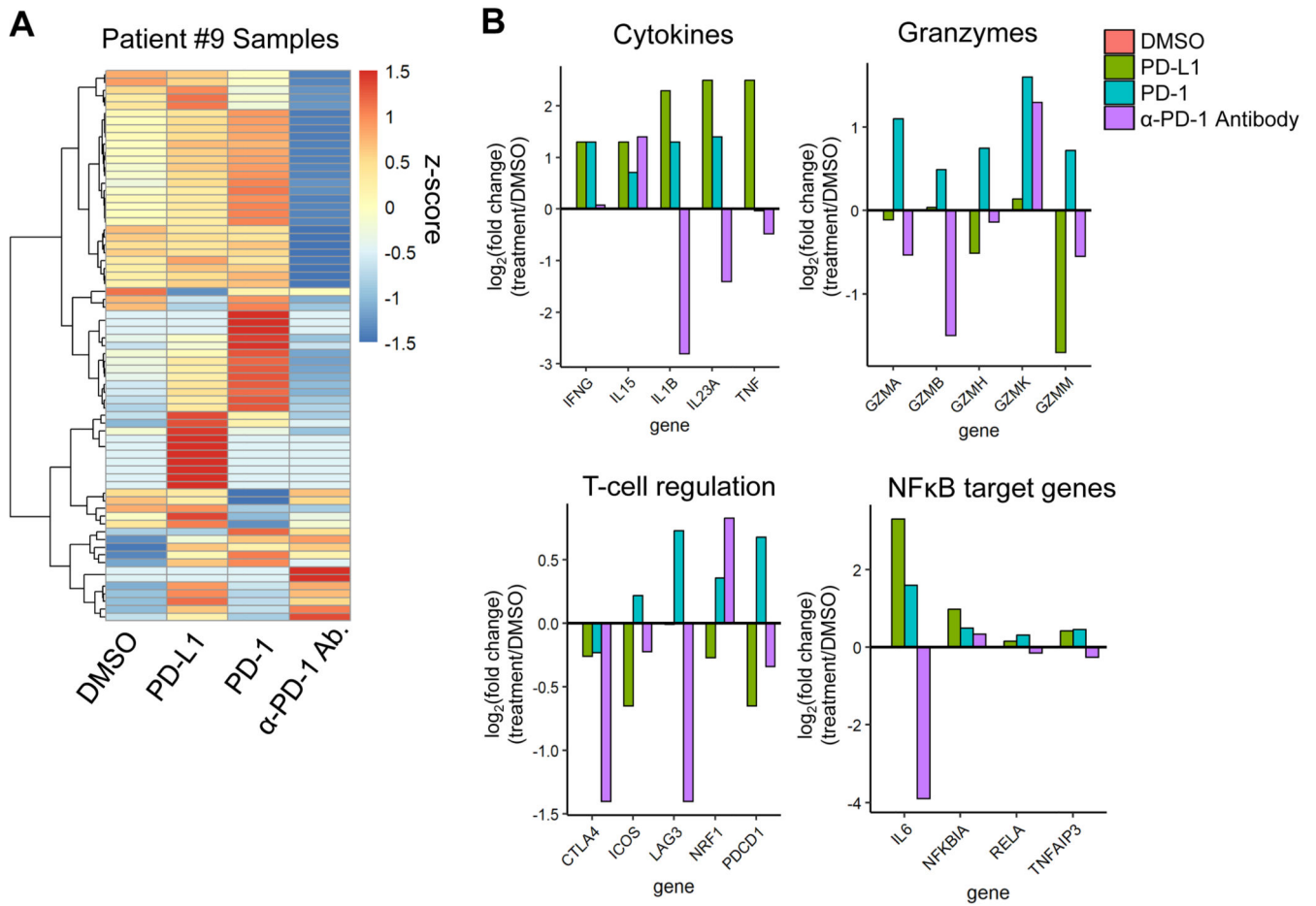
**Fig. 5. Aberrant paradoxical effect of the checkpoint blockade modulation in ALS macrophages.**
PDL1 and PD1 are pro-inflammatory, while DMF and PD1 antibody are anti-inflammatory.
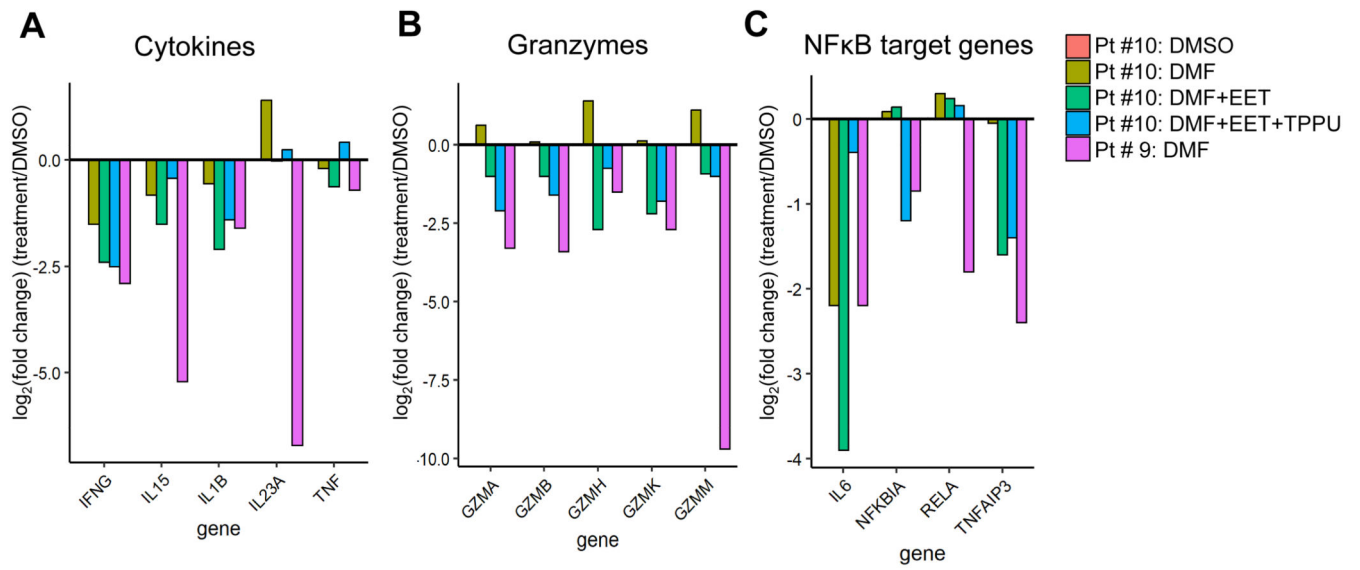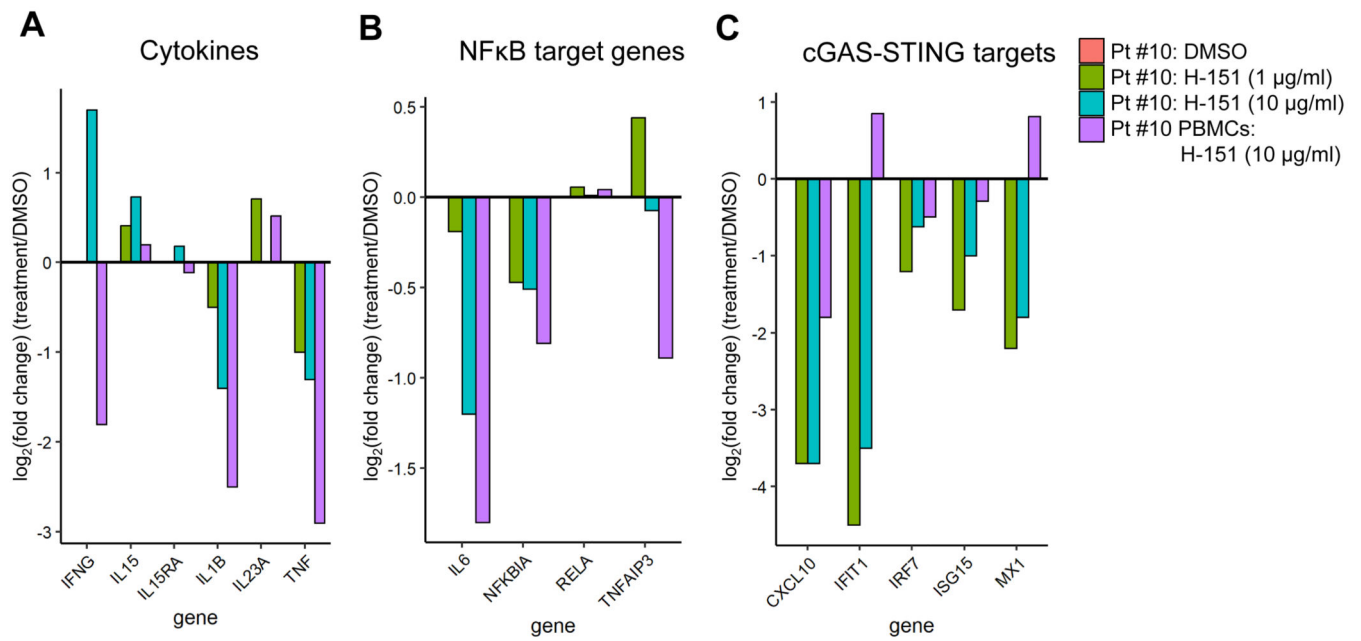
**Fig. 6.**

Dimethyl fumarate (DMF) down-regulates inflammatory cytokines (except *TNF*), granzymes, and *NFκB* target genes in macrophages of both ALS patients

**Fig. 7.**
The STING inhibitor H-151 down-regulates in a concentration-dependent fashion the inflammatory cytokines, granzymes, and the transcription factor *NFκB* targets in ALS macrophages of the slowly progressing patient #10.
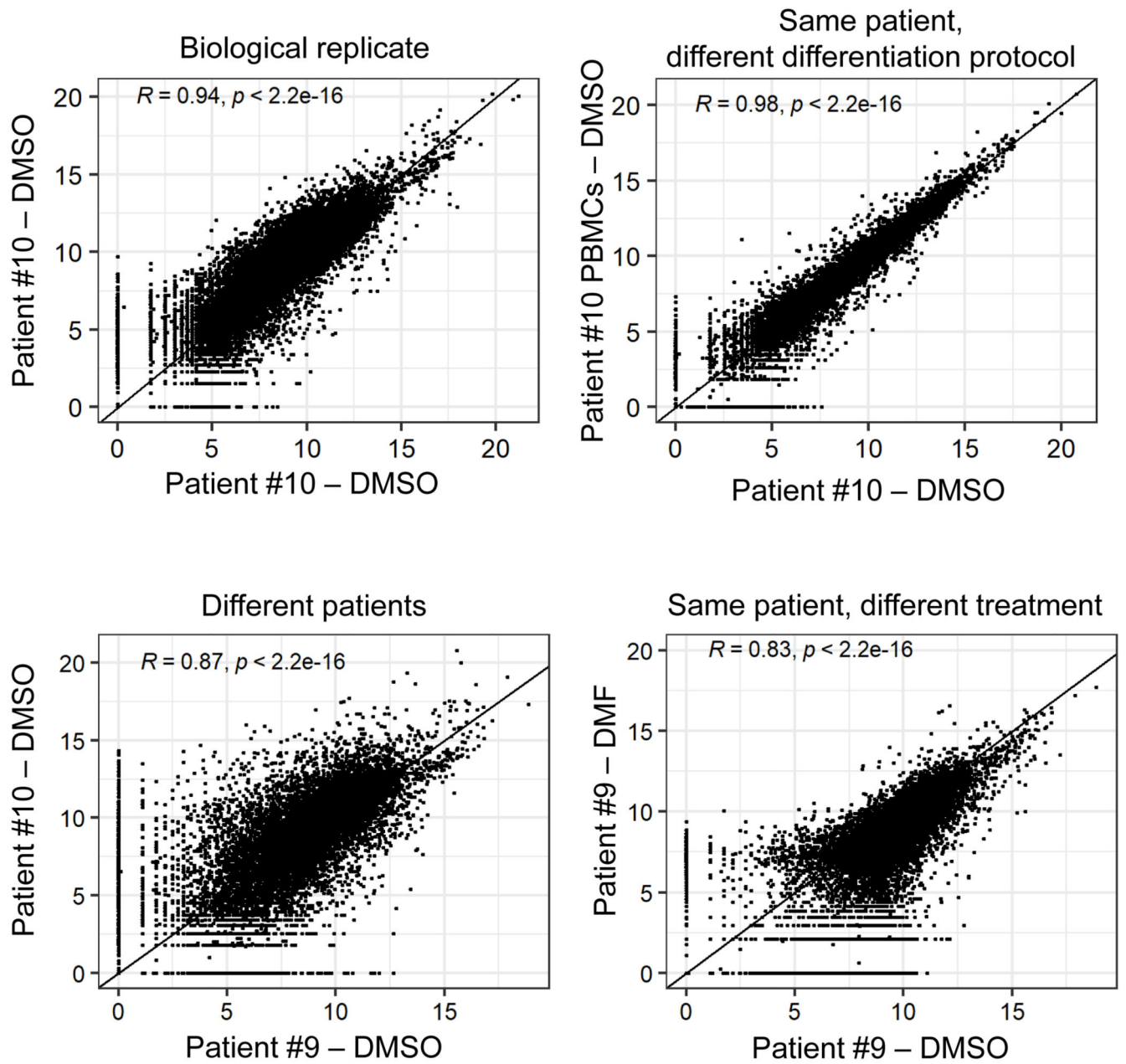
**Fig. 8.**
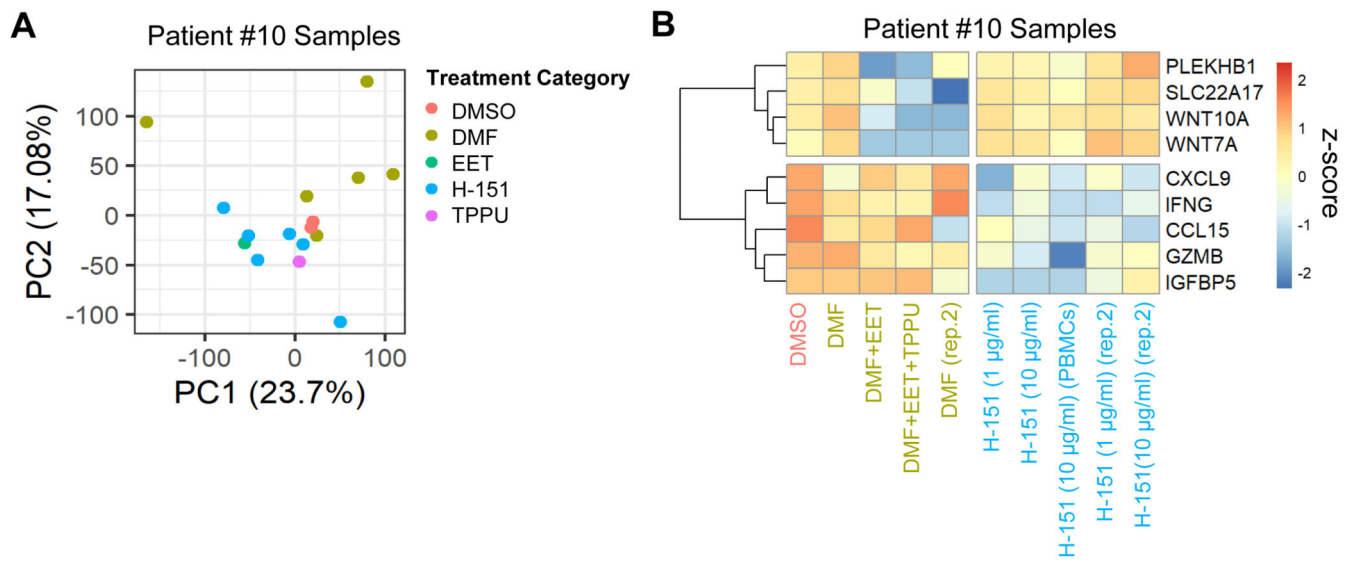Biological and treatment replicates

**Fig. 9. PCA of DMF and H-151 effects:**
The unsupervised analysis shows stronger down regulation of inflammatory chemokines (CXCL9 and CCL15), inflammatory cytokine (IFN), granzymes, and IGFBP5 by H-151 in comparison to DMF.

**TABLE 1.**

sALS patients demographics, clinical data, functional rating score loss, and list of medications.

| Patient number | Age (years) | Sex | FRS score change (over 20 months) | Date of onset | Sample collection date | Medications |
|---|---|---|---|---|---|---|
| 1 | 35 | M | −0.56 | 7/15/2011 | 11/5/2014 12/1/2014 3/5/2015 4/2/2015 5/13/2015 | Rituximab, IVIG, vitamin D, vitamin B12, curcumin, ubiquinol, MitoQ, vitamin C, resveratrol, L-serine, acetyl-l-carn, zinc, lions mane, N-acetylcysteine vitamin E, riluzole, ashwagandha, inosine, and omega-3 |
| 2 | 41 | M | −0.22 | 2/15/2013 | 3/3/2014 | Riluzole, omega-3, and vitamin D3 |
| 3 | 72 | M | −0.80 | 2/15/2012 | 1/14/2014 3/13/2014 3/9/2015 | Neudexta, omega-3, vitamin E, tocilizumab, vitamin D3, N-acetylcysteine, and riluzole |
| 4 | 62 | M | −1.03 | 7/15/2013 | 3/25/2014 | Vitamin D3, vitamin B12, levothyroxine, liothyronine, citalopram, alprazolam, omega-3, riluzole, Dextromethorphan-quinidine |
| 5 | 65 | M | −1.00 | 1/15/2014 | 12/16/2014 | Riluzole, actemra, nuedexta, mavik, crestor, vitamin E, vitamin C, vitamin D3, omega-3, folic acid, and melatonin |
| 6 | 59 | F | −0.57 | 9/15/2011 | 3/24/2014 | Riluzole and effexor |
| 7 | 65 | M | −0.41 | 2/15/2014 | 4/6/2015 | Omega-3, vitamin D3, and riluzole |
| 8 | 68 | M | −0.85 | 1/15/2011 | 9/19/2012 | Riluzole, Lexapro, neupro patch, imitrex, xarelto, valium, and ambien |