Cell type-specific transcriptomics identifies neddylation as a novel therapeutic target in multiple sclerosis

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Multiple sclerosis is an autoimmune disease of the CNS in which both genetic and environmental factors are involved. Genome-wide association studies revealed more than 200 risk loci, most of which harbour genes primarily expressed in immune cells. However, whether genetic differences are translated into cell-specific gene expression profiles and to what extent these are altered in patients with multiple sclerosis are still open questions in the field. To assess cell type-specific gene expression in a large cohort of patients with multiple sclerosis, we sequenced the whole transcriptome of fluorescence-activated cell sorted T cells (CD4+ and CD8+) and CD14+ monocytes from treatment-naïve patients with multiple sclerosis ($n = 106$) and healthy subjects ($n = 22$). We identified 479 differentially expressed genes in CD4+ T cells, 435 in monocytes, and 54 in CD8+ T cells. Importantly, in CD4+ T cells, we discovered upregulated transcripts from the *NAE1* gene, a critical subunit of the NEDD8 activating enzyme, which activates the neddylation pathway, a post-translational modification analogous to ubiquitination. Finally, we demonstrated that inhibition of NEDD8 activating enzyme using the specific inhibitor pevonedistat (MLN4924) significantly ameliorated disease severity in murine experimental autoimmune encephalomyelitis. Our findings provide novel insights into multiple sclerosis-associated gene regulation unravelling neddylation as a crucial pathway in multiple sclerosis pathogenesis with implications for the development of tailored disease-modifying agents.

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Abbreviations: DEGs = differentially expressed genes; EAE = experimental autoimmune encephalomyelitis; NAE = NEDD8 activating enzyme; PPI = protein-protein interaction

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Introduction

Multiple sclerosis is a chronic autoimmune condition of the CNS characterized by demyelination and neurodegeneration leading to permanent clinical disability (Hauser and Oksenberg, 2006). While it is clear that both genetic and environmental factors contribute to multiple sclerosis pathogenesis, its aetiology remains elusive. Except for anti-B-cell therapies, current immunomodulatory therapies are only partially effective (Baecher-Allan *et al.*, 2018).

Over the past decade, genome-wide association studies (GWAS) identified 233 independent risk loci in multiple sclerosis (International Multiple Sclerosis Genetics Consortium, 2013, 2019), most of which harbour genes primarily expressed in immune cells such as T cells and monocytes. Mounting evidence suggests that post-translational modification pathways such as the ubiquitin-proteasome system (UPS) play important roles in various cellular functions. In that regard, neddylation—a ubiquitin-like post-translational modification pathway—has been implicated as a key regulator in T-cell function (Saha and Deshaies, 2008; Jin *et al.*, 2013; Mathewson *et al.*, 2016). The most well-known substrate of neddylation pathway is the Cullin protein family, in particular cullin-RING E3 ubiquitin ligases (CRLs), which are important in UPS (Duda *et al.*, 2008; Soucy *et al.*, 2009). The inhibition of neddylation using a small molecule inhibitor (pevonedistat) has shown promising results in clinical trials in lymphoproliferative disorders (Czuczman *et al.*, 2016). While previous studies found that the UPS activity is related to the regulation of myelin protein degradation and IFN-beta-1b treatment in multiple sclerosis (Giordana *et al.*, 2002; Minagar *et al.*, 2012; Belogurov *et al.*, 2014), the role of neddylation in immune cell function in general, and T-cell function specifically, has not been studied in multiple sclerosis.

Previous gene expression studies used whole blood or peripheral blood mononuclear cells (PBMCs) mostly searching for dysregulated multiple sclerosis pathogenic pathways and biomarkers of disease progression (Gandhi *et al.*, 2010; Ottoboni *et al.*, 2012; Nickles *et al.*, 2013). However, this approach only detects the strongest signals due to highly variable gene expression across the complex mixture of cell types present in each sample. Here, we performed cell type-specific RNA-seq from fluorescence-activated cell sorting (FACS)-sorted specific immune cell populations, thus enhancing the signal-to-noise ratio.

Materials and methods

Study design and subjects

We collected blood from 153 patients with multiple sclerosis participating in the UCSF multiple sclerosis EPIC/ORIGINS studies and 22 healthy subjects. We isolated PBMCs from the blood by Ficoll method using a Vacutainer® CPT™ tube (BD Biosciences). For this particular analysis, we excluded those

treated by disease-modifying therapy and those with unknown disease status, totalling 106 patients (Fig. 1). The characteristics of the patients are described in Table 1. All patients were free of steroids within at least 30 days prior to sampling. This study was approved by the Institutional Review Board at the University of California San Francisco (IRB #10-00104). All participants signed appropriate informed consent before providing samples.

Cell sorting and RNA sequencing

PBMCs were sorted into three different cell subsets (CD4+, CD8+, and CD14+ cells) using a MoFlo Astrios cell sorter (Beckman Coulter). CD4+ (helper) T cells were defined as CD3+ CD19–CD4+, CD8+ (cytotoxic) T cells as CD3+ CD19–CD8+, and CD14+ monocytes as CD14+. Each cell subset was directly sorted into RLT buffer (Qiagen) and stored at –80°C. Total RNA was isolated using RNeasy® Mini or Micro kit (Qiagen). The RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was stored at –80°C until used for sequencing library preparation. We prepared strand-specific whole transcriptome RNA-seq libraries for each cell subset using 5 ng to 900 ng of total RNA as a template using the NEBNext® Ultra II Directional RNA library prep kit (NEB) and 96 index primers according to the manufacturer's instructions. We performed cytoplasmic and mitochondrial ribosomal RNA depletion using the NEBNext® rRNA depletion kit (NEB) followed by library construction. RNA-seq libraries were pooled with 36 or 37 samples of equal amount and sequenced by 150 nt paired-end on the NovaSeq instrument (Illumina).

Sequencing read mapping, quantification and quality control

We assessed quality control of raw fastq files using FastQC v0.11.7 (Wingett and Andrews, 2018). Sequencing read depth was average 88 M reads (minimum 60 M to maximum 153 M reads). Adapter sequence and low-quality bases were trimmed using BBDuk of BBTools v38.05 (<https://jgi.doe.gov/data-and-tools/bbtools/>). Trimmed sequence reads were mapped into the primary assembly of human genome reference (GRCh38.p12) with Gencode annotation (release 28) using STAR v2.6.0c with 2-pass mode (Dobin *et al.*, 2013). QualiMap was used to assess the quality of mapped reads (Okonechnikov *et al.*, 2016). On average, 94.6% of reads mapped to CD4+ and CD8+ cells, and 96.3% mapped to CD14+ cells. The uniquely mapped rate was 75.8%, 75.3%, and 86.5% for CD4+, CD8+, and CD14+ cells, respectively. We quantified gene and transcript isoform expression abundance using RSEM v1.3.1 (Li and Dewey, 2011). Based on the PCA and sample-to-sample distance heat map, we corrected five mislabelled cell types and removed four outlier samples (data not shown). We selected only baseline samples from multiple time-point subjects for further analysis.

Differential gene expression analysis

Transcript and gene expression abundance was imported into R object by the tximport package (Soneson *et al.*, 2015). We

Figure 1 Schematic overview of the study and dataset quality control. (A) The diagram shows a schematic overview of the sample collection, experiments, and data analysis in this study. **(B)** PCA plot for overall gene expression shows that each cell subset was separated by gene expression, but not clustered by disease status. CIS = clinically isolated syndrome; HC = healthy control subjects; MS = multiple sclerosis; RMS = relapsing multiple sclerosis.

performed a gene-level differential expression analysis between treatment-naïve patients with multiple sclerosis and healthy subjects, between different disease courses (clinically isolated syndrome versus relapsing multiple sclerosis) using DESeq2 v1.20.0 (Love *et al.*, 2014). From the DESeq2 results, we filtered low-expressed genes by $\text{baseMean} > 5$, and significant genes were selected with 5% of false discovery rate (FDR) threshold. Differential expression analyses were conducted in R v3.5.1 and Bioconductor v3.7.

Protein-protein interaction network analysis

We performed a protein-protein interaction (PPI) network analysis to find the module network and prioritize genes. Cytoscape v3.7.1 and stringApp v1.4.0 were used to retrieve a known PPI network from the STRING database with significant protein-coding genes selected by $\text{FDR} < 0.1$ cut-offs (Shannon *et al.*, 2003; Szklarczyk *et al.*, 2017; Doncheva *et al.*, 2019).

Table 1 Characteristics of multiple sclerosis patients and healthy subjects in this study

Disease status and condition	
Treatment naive patients (n = 106)	
Female/male (%)	74 (67.9) / 35 (32.1)
Mean age at examination, years (range)	34.5 (19–58)
Disease subtypes (%)	
CIS	40 (37.7)
RRMS	66 (62.3)
Median disease duration, years (range)	0.0 (0–19)
Median EDSS (range)	2.0 (0–6.0)
Healthy subjects (n = 22)	
Female/male (%)	10 (45.5) / 12 (54.5)
Mean age at examination, years (range)	29.9 (23–48)

CIS = clinically isolated syndrome; EDSS = Expanded Disability Status Scale; RRMS = relapsing-remitting multiple sclerosis.

Experimental autoimmune encephalomyelitis induction and neddylation inhibition in mice

Four-week-old female C57BL/6 mice were purchased from Jackson Laboratory and maintained at the UCSF specific pathogen-free animal facility. All animal protocols were approved by and in accordance with the guidelines established by the Institutional Animal Care and Use Committee and Laboratory Animal Resource Centre. All mice were housed in closed caging systems and provided with standard irradiated chow diet, acidified water *ad libitum* and housed under a 12-h light cycle.

Pevonedistat (MLN4924) (Chemietek) was dissolved in DMSO and further diluted in 30% of PEG300 (Sigma-Aldrich) and 5% of TWEEN[®] 80 (all Sigma-Aldrich) in ddH₂O at a concentration of 40 mg/ml and stored at −20°C. Seven to eight-week-old mice were treated daily with 20 mg/kg pevonedistat or vehicle (same concentration of buffer without pevonedistat) starting at Day −1 (Fig. 5A). Mice were immunized subcutaneously on Day 0 with 100 µg MOG35-55 (Anaspec) emulsified in incomplete Freund's adjuvant (Becton Dickinson) supplemented with *Mycobacterium tuberculosis* (H37Ra strain, Difco) followed by two intraperitoneal injections on Day 0 and Day 2 with 300 ng pertussis (MilliporeSigma) each. Mice were scored daily on a 10-point scale (0.5-unit increments) in a blinded fashion as follows: 0, no deficit; 1, limp tail only; 2, limp tail and hind limb weakness; 3, complete hind limb paralysis; 4, complete hind limb paralysis and partial/complete forelimb paralysis; 5, death.

Immunohistochemistry

Mice were deeply anaesthetized with CO₂ and transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde (PFA) and subsequently post-fixed in PFA for 1 h. After post-fixation, samples were cryoprotected in 30% sucrose in PBS for 48 h at 4°C and embedded in optimal cutting temperature compound (Tissue-Tek). Cryosections (16 µm) were collected on superfrost slides (VWR) using a CM3050S cryostat (Leica) and blocked in 0.1 M PBS/0.1% Triton[™] X-100/10% goat sera for 1 h at room temperature. Primary antibody incubations were

carried out overnight at 4°C. After washing in 0.1 M PBS, cryosections were incubated with secondary antibodies diluted in 0.1 M PBS/0.1% Triton[™] X-100 for 2 h at room temperature. For immunofluorescence, anti-Iba1 (rabbit polyclonal; 1:500; Wako), anti-CD3 (rat monoclonal; clone KT3; 1:100; Thermo Fisher), anti-MBP (rat monoclonal; clone 12; 1:200; Merck), anti-GFAP (rat monoclonal; clone 2.2B10; 1:200; Thermo Fisher), anti-CD45R (B220) (rat monoclonal; clone RA3-6B2; 1:200; Thermo Fisher), and anti-SMI 312 (mouse monoclonal; clone SMI 312; 1:1000; BioLegend) antibodies were used for primary antibody detection. The diameter range for counting SMI312+ axons was set from 0.2 µm² to 6.0 µm² in Fiji.

Slides with fluorescent antibodies were mounted with DAPI Fluoromount-G[™] (Thermo Fisher). Negative control sections without primary antibodies were processed in parallel. All immunohistochemistry analysis was carried out blinded.

Image acquisition and analysis

Images were taken with a DMi8 widefield (equipped with Leica DFC7000 GT camera) microscope with 20× objective; fluorescent pictures are z-stack images, unless stated otherwise. Images were processed using Fiji ImageJ (v2.0) and exported to vector-based software (Adobe Illustrator and Affinity Designer) for figure generation.

Statistical analysis

For DEG analysis, we used DESeq2 v1.20.0 (Love *et al.*, 2014) in R v3.5.1 and Bioconductor v3.7. DESeq2 estimates size factor and dispersion, then test differential expression by fitting a negative binomial generalized linear model. The significance of general linear model (GLM) coefficient was tested by the Wald test for each gene. Sex and age at exam were used as covariates in the model. The likelihood ratio test with a reduced model was used to retrieve genes affected by each covariate. We obtained log 2-fold change and *P*-values. *P*-values from differential gene expression were corrected by the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

For the experimental autoimmune encephalomyelitis (EAE) study, two-way ANOVA with the Sidak test was used for the comparison between the daily diseases score in the pevonedistat and placebo-treated groups. Statistical analysis of the histological quantifications in the two groups was performed using parametric (Student's *t*-test) or non-parametric (Mann-Whitney U) tests. Data are presented as mean ± standard error of the mean (SEM). All tests were performed using two-tailed analysis unless stated otherwise. The significance cut-off was set at *P* < 0.05. *P*-values were designated as follows: **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001, *****P* ≤ 0.0001. Statistical analyses were performed using GraphPad Prism software version 7.0.

Data availability

The cell type-specific RNA-seq dataset in this publication has been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO Series accession number GSE137143. The code used for the RNA-seq analysis can be found on the GitHub page.

Results

Quality of cell type-specific transcriptome dataset

We sequenced total RNA of FACS-sorted CD4⁺ T cells, CD8⁺ T cells, and CD14⁺ monocytes from 106 patients with multiple sclerosis and 22 healthy subjects. Most of the patients were newly diagnosed with multiple sclerosis or at an early stage of the disease (Table 1) highlighted by the short median disease duration (1 year) and median Expanded Disability Status Scale (EDSS) score (2.0). The experimental and analytical workflows are shown in Fig. 1A. A principal component analysis plot using all transcripts that passed quality control in all samples showed that the gene expression was primarily clustered by cell types but not by disease status (Fig. 1B). T cells and monocytes were separated by the first principal component (PC1), and CD4⁺ and CD8⁺ T cells were mostly separated by second (PC2) and third components (PC3). A total of 377 samples from 128 subjects were used for further analysis (Table 1).

Differentially expressed transcripts in patients with multiple sclerosis

We next searched for differentially expressed genes (DEGs) (FDR 5%) between treatment-naïve patients with multiple sclerosis and healthy subjects in each cell subset, and identified 479 DEGs in CD4⁺, 54 in CD8⁺, and 435 in CD14⁺ cells (Fig. 2A and Supplementary Table 1). No gene expression differences across disease subtypes were observed. On average, 39% of total DEGs were non-coding RNA and pseudogenes (ncRNA), and remarkably, 91.9% of the ncRNAs were downregulated in patients with multiple sclerosis. On the other hand, on average, 63% of protein-coding genes were upregulated in CD4⁺ and CD14⁺ cells (32% in CD8⁺ cells). Figure 2B shows the comparison of DEGs between different cell subsets for all multiple sclerosis cases and between disease subset and healthy. Eighty-two per cent of DEGs in CD4⁺ cells ($n = 395$), 31% in CD8⁺ ($n = 17$), and 85% in CD14⁺ ($n = 370$) were significant only within each cell subset. Thirty-three per cent of DEGs overlapped in T cells and ~40% of genes were unique in each patient with clinically isolated syndrome and relapsing multiple sclerosis. On the other hand, only 10% of differential genes overlapped both disease subtypes in CD14⁺ cells. Major differential genes in CD14⁺ cells were uniquely significant only in patients with relapsing multiple sclerosis. This suggests that different pathways might operate in each of these immune cell types during multiple sclerosis pathogenesis. We found the largest number of DEGs in CD4⁺ cells between patients with multiple sclerosis and healthy subjects ($n = 479$) (Fig. 2C and Supplementary Tables 2–4). One of the most significantly upregulated transcripts in CD4⁺ T cells from patients with multiple sclerosis was *NAE1* (NEDD8 activating enzyme E1 subunit 1) (Fig. 2D). *NAE1*

encodes a subunit of the NEDD8 activating enzyme (NAE), which forms a heterodimer with UBA3 (Walden *et al.*, 2003) and can activate the NEDD8 complex (Fig. 3). Of interest, expression of *OMG* (oligodendrocyte myelin glycoprotein), whose expression is typically restricted to the CNS (Fagerberg *et al.*, 2014), was detected among controls but strongly downregulated in multiple sclerosis cases. This gene was also downregulated in CD8⁺ and CD14⁺ cells. The transcript found here is one of four described for this gene and contains a retained intron which results in a non-protein-coding sequence with a potential regulatory role, as seen in other ncRNA families. A disease course comparison with treatment-naïve patients identified only a small number of DEGs (Supplementary Tables 11 and 12). Of note, T cells (in particular CD8⁺) showed the largest effect by sex and age at examination, while CD14⁺ monocytes displayed the smallest effect (Supplementary Table 1).

Most DEGs in CD8⁺ cells were downregulated in multiple sclerosis (Fig. 2A and Supplementary Tables 5–7). Meanwhile, we found 435 significant DEGs in CD14⁺ cells (Fig. 2A, Supplementary Fig. 1 and Supplementary Tables 8–10). Specifically, *SOCS3* was also significantly upregulated. *SOCS3* has been described as upregulated in the M1-like macrophage, an inflammatory monocyte/macrophage state (Jiang *et al.*, 2014; Nicholson and Murray, 2014; Derlindati *et al.*, 2015; Xuan *et al.*, 2015). We also found upregulation of other inflammatory state-associated genes such as *IL1B* and *CSF2RB*. Taken together, our data suggest that monocytes in patients with multiple sclerosis are more polarized towards an inflammatory state.

We found 35 significant DEGs whose expression overlapped between both CD4⁺ and CD8⁺ T cells, and only 11 transcripts were differentially expressed in all cell subsets (Fig. 2B).

To evaluate if gene expression was correlated with genetic susceptibility variants, we compared significant DEGs with the proximal gene list of International Multiple Sclerosis Genetics Consortium (IMSGC) genome-wide effect region. We report only one such gene in CD4⁺ T cells (*CD37*) and two in CD8⁺ T cells (*CD37*, *VANGL2*), while 10 genes (*CD6*, *IL7R*, *NCF4*, *CD37*, *CCR4*, *CSF2RB*, *CD28*, *LEF1*, *TCF7*, *EPPK1*) met this criterion in CD14⁺ cells (Supplementary Table 13) (International Multiple Sclerosis Genetics Consortium, 2019). Although many genes from the IMSGC study are associated with T-cell pathways, we found more IMSGC proximal genes from CD14⁺ monocytes. Interestingly, those genes are associated with cell surface receptor signalling pathways including T-cell receptor and immune response.

Protein-protein interaction analysis identified post-translational modification pathways

To prioritize genes and identify related pathways, we performed PPI network analysis. We generated a PPI network

Figure 2 Results for differential expression analysis. (A) A number of significant DEGs for protein-coding and non-coding RNA in each cell subset in a comparison between multiple sclerosis (MS) and healthy control subjects (HC). DEGs indicates the most significant RNA genes from CD4+ cells. We observed that ~64% of protein-coding genes were upregulated but the majority of ncRNAs (~91%) were downregulated in multiple sclerosis. Protein-coding includes protein-coding, immunoglobulin, and T-cell receptor genes. Non-coding includes lincRNA, antisense, pseudogenes, and all other types of transcripts. (B) Venn diagram shows overlapping DEGs across cell subsets in a comparison between multiple sclerosis [clinically isolated syndrome (CIS) + relapsing multiple sclerosis (RMS)] and healthy controls, and across different comparisons in each cell subset. (C) Heat map displays significant protein-coding DEGs between multiple sclerosis and healthy controls in CD4+ cells. Colour scheme indicates gene expression level (red = high; blue = low). (D) Box plot indicates the difference in strongly significant DEG. *NAE1* was upregulated in patients with multiple sclerosis.

of DEGs using the STRING database in each cell subset (Supplementary Figs 2–4). In CD4+ cells, we identified the main PPI network containing 446 genes (Fig. 4 and Table 2). This network is significantly enriched in genes from ‘Acetylation’ and ‘Ubl conjugation (conjugation of ubiquitin-like protein)’ pathways. These terms are closely

related to the protein modification function (post-translational modification). Of note, *NAE1* itself was included in the main network. Furthermore, we found E3 enzyme subunits that associated with *NAE1* such as *ASB7* (ankyrin repeat and SOCS box containing 7), *LRR41* (leucine rich repeat containing 41), *WDTC1* (WD and tetratricopeptide

Figure 3 Scheme for neddylation pathway and effect in CD4⁺ T cells. In addition to *NAE1*, we found dysregulation of neddylation pathway associated genes such as *UBE2F*. The most well-known substrate of the neddylation pathway is the Cullin protein family, which is a subunit of CRLs. The neddylation controls CRLs activity, and the CRLs regulate degradation of numerous proteins including Tob1, NFκB, and SOCS1/3 as an E3 ligase of the ubiquitin-proteasome system.

repeats 1), and *FBXL22* (F-box and leucine rich repeat protein 22).

Given the prominent dysregulation of pathways involved in post-translational modifications and, in particular, the increased expression of *NAE1* in multiple sclerosis, we decided to explore the neddylation pathway in more detail. Previous studies have shown that neddylation is required for T-cell receptor (TCR)-mediated T-cell functions (Jin *et al.*, 2013; Mathewson *et al.*, 2016; Cheng *et al.*, 2018). Interestingly, we also found dysregulation of other genes in this pathway including *UBE2F* (ubiquitin conjugating enzyme E2 F) and known substrate proteins of the neddylation pathway: *VHL* (von Hippel-Lindau tumour suppressor), *AKIP1* (A-kinase interacting protein 1), and *SMURF1* (SMAD specific E3 ubiquitin protein ligase 1) (Fig. 3) (Zhou *et al.*, 2019). *UBE2F* encodes the NEDD8-conjugating enzyme E2, which catalyses the transfer of NEDD8 from NAE to a substrate protein in the neddylation pathway. Based on the above information, we hypothesized that neddylation plays an important role in multiple sclerosis pathogenesis, and its pharmacological manipulation might open a new therapeutic venue for this disease.

Inhibition of neddylation reduces experimental autoimmune encephalomyelitis

As described above, we identified the upregulation of *NAE1*, a subunit of NAE, which is essential for neddylation. We also found that the ‘post-translational modification pathway’ was significantly enriched in samples from multiple sclerosis subjects. This result suggests the importance of neddylation in multiple sclerosis pathogenesis. Given the reported effect of NAE on T-cell proliferation, we hypothesized that *NAE1* inhibition could be a promising therapeutic

approach in multiple sclerosis. To test our hypothesis, we used pevonedistat (MLN4924), which is a small molecule and first-in-class inhibitor of NAE (Soucy *et al.*, 2009), in EAE, an established multiple sclerosis mouse model driven by a strong CD4⁺ T-cell response (Robinson *et al.*, 2014) (Fig. 5A). Although pevonedistat was originally tested as a cancer therapy, recent studies proposed this agent as a potential therapeutic target for immune-related diseases due to the important role of neddylation in immune cell functions (Mathewson *et al.*, 2013, 2016; Jin *et al.*, 2013; Cheng *et al.*, 2018).

Treatment with pevonedistat significantly reduced EAE severity compared to the placebo-treated group (Fig. 5B). This difference in disease severity was also reflected by steady weight in the pevonedistat-treated animals during the disease course compared to a significant weight loss at peak of disease in the placebo group (Fig. 5C). Histological assessment of the CNS tissue at peak disease confirmed a significant reduction of demyelination (Fig. 5D). Further, multifocal inflammatory infiltrates were observed in the EAE spinal cords of placebo-treated animals, while treatment with pevonedistat resulted in a dramatic reduction in spinal cord inflammation in particular Iba1⁺ myeloid cells and CD3⁺ T cells (Fig. 5D). Notably, the reduction was paralleled by a marked reduction of axonal loss (Fig. 5E) suggesting an additional neuroprotective effect of pevonedistat during autoimmune neuroinflammation. Further assessment of other immune cell infiltrates revealed that B cells (Fig. 5D), but not astrocytes, (Supplementary Fig. 5) were markedly decreased in the pevonedistat group corresponding to the expression profile of *NAE1* (Kim *et al.*, 2020). These data confirm a strong functional effect of NAE1 on pathogenic T-cell proliferation in multiple sclerosis and further point towards a potential more pleiotropic effect of pevonedistat also on other immune subsets including B and myeloid cell but not astrocytes, as suggested in previous studies (Milhollen *et al.*, 2010; Chang *et al.*, 2012; Mathewson *et al.*, 2013; Song *et al.*, 2016). Future studies will have to further assess the potential of pevonedistat as an interventional treatment. Taken together, our data show neddylation inhibition dampens EAE severity through the reduction of inflammation in CNS and an additional neuroprotective effect on axonal integrity, strongly suggesting that neddylation is an important pathway in multiple sclerosis pathogenesis.

Discussion

Immune cells, especially T cells, play an important role in multiple sclerosis pathogenesis (Gourraud *et al.*, 2012; Dendrou *et al.*, 2015). In the early stages of multiple sclerosis, autoreactive T cells are thought to traffic to the CNS from peripheral blood where they trigger demyelination and cause neuro-axonal injury (Dendrou *et al.*, 2015). Notably, T cells and macrophages are enriched in the brain and CSF of patients with multiple sclerosis (Safford *et al.*, 2005; Legroux and Arbour, 2015). In this work, we explored the

Table 2 PPI network and related functions using DEGs in CD4+ cells

Category	Description	Background genes, <i>n</i>	Genes, <i>n</i>	FDR value	Term name
UniProt keywords	Phosphoprotein	8066	297	1.20×10^{-4}	KW-0597
GO component	Nucleoplasm	3446	148	2.90×10^{-4}	GO.0005654
GO component	Intracellular membrane-bounded organelle	10 365	364	2.90×10^{-4}	GO.0043231
GO component	Nucleoplasm part	1073	56	0.0026	GO.0044451
GO component	Organelle subcompartment	1616	76	0.0029	GO.0031984
GO component	Golgi apparatus	1474	68	0.009	GO.0005794
UniProt keywords	Acetylation	3335	135	0.0104	KW-0007
GO component	Phagocytic vesicle membrane	71	9	0.0152	GO.0030670
GO component	Integral component of Golgi membrane	48	7	0.0255	GO.0030173
GO component	Protein-containing complex	4792	174	0.028	GO.0032991
GO component	Intrinsic component of organelle membrane	330	21	0.0313	GO.0031300
GO component	Nuclear speck	381	23	0.0335	GO.0016607
GO component	Cytosol	4958	178	0.0339	GO.0005829
GO component	Endosome	876	42	0.0363	GO.0005768
GO component	Integral component of luminal side of endoplasmic reticulum membrane	26	5	0.0363	GO.0071556
UniProt keywords	Ubl conjugation	2380	98	0.0386	KW-0832

See also Fig. 4.

Figure 5 Peponedistat treatment dampens EAE. **(A)** Treatment trial design. C57BL/6 mice were treated daily beginning on Day -1 with either peponedistat (20 mg/kg) (blue) or placebo (red) ($n = 10$ /group). EAE was induced through active immunization with myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 and pertussis following standard protocols (triangles). Weights were taken on Days -1, 7 and 14 (dots). Animals were scored daily. Mice were sacrificed and brain and spinal cords were harvested on Day 16 at peak disease ($n = 4$ /group) (square). **(B)** and **(C)** EAE severity and weights. Diseases were assessed blinded using a 10-point scale from 0 to 5 (0 = no symptoms, 5 = death). Data shown

(continued)

cell type-specific transcriptional landscape of CD4+ and CD8+ T cells and CD14+ monocytes in treatment-naïve patients with multiple sclerosis. The cell type-specific transcriptome results indicated that CD4+ T cells were the most dysregulated in multiple sclerosis among the three different immune cells (Fig. 2).

Most notably, we found upregulation of *NAE1* in CD4+ T cells and demonstrated that NAE inhibition attenuates disease severity in EAE (Fig. 5). Similar to ubiquitination, neddylation is a cascade of three enzymatic processes (E1–E3) (Fig. 3) that is activated by NAE (an E1 enzyme) by NEDD8 conjugation (Soucy *et al.*, 2009). Neddylation regulates protein function including CRLs activity (Jin *et al.*, 2013; Mathewson *et al.*, 2016). CRLs in turn, associate with Skp2, forming the SCFskp2 complex, which mediates degradation of numerous proteins by the UPS (Hiramatsu *et al.*, 2006). One of the targets of the CRLs complex, which itself is regulated by neddylation, is Tob1, a tumour suppressor gene with activity in T-cell proliferation, EAE, and potentially multiple sclerosis (Corvol *et al.*, 2008; Schulze-Toppoff *et al.*, 2013; Baranzini, 2014). We also identified several E3 enzyme subunits that can be associated with more specific pathways following neddylation (Fig. 4 and Table 2). These E3 enzyme subunits may be important in a specific CD4+ T-cell function of multiple sclerosis pathogenesis. Inhibition of neddylation in CD4+ T cells suppresses T-cell proliferation and cytokine production by inhibiting the NF- κ B pathway and increasing *SOCS1* and *SOCS3* expression, which in turn suppresses T-cell function (Jin *et al.*, 2013; Mathewson *et al.*, 2016; Cheng *et al.*, 2018). The NF- κ B pathway is important in the immune cells' pro-inflammatory response. The neddylation pathway activates NF- κ B via degradation of NF- κ B inhibitor (I κ B) by the UPS and/or neddylation of TRAF6 (TNF receptor associated factor 6) protein (Hinz and Scheidereit, 2014; Liu *et al.*, 2019). Therefore, inhibition of neddylation also suppresses NF- κ B activation. In the EAE model, CD4+ T cells activated against myelin protein infiltrate the CNS and cause neuroinflammation. We showed that the NAE inhibitor pevonedistat (MLN4924) significantly reduces EAE severity with a dramatic reduction in spinal cord inflammation. These murine results indicate that the neddylation pathway plays a critical role in T-cell activation and/or proliferation during EAE and potentially also other immune subsets, and combined with our human RNA-seq data, this pathway is potentially also implicated in human multiple sclerosis pathogenesis.

We acknowledge our study has some limitations that warrant further examination. First, gene expression profiles in

this study only represent changes in peripheral blood but not in the CNS, and even immune cell subsets are very heterogeneous. In addition, we deemed our sample size relatively small to cover the diverse genetic variation in human populations. Thus, further studies with larger sample sizes are required to fully elucidate the relationship between genes, genetic variants, and disease. Finally, while our proof-of-concept experiment shows that pevonedistat can ameliorate EAE in a preventive fashion, further studies need to be carried out to show its therapeutic potential. Despite these limitations, through cell type-specific transcriptomics, we were able to identify the importance of the neddylation pathway in multiple sclerosis.

In conclusion, our findings provide important new insights into gene regulation related to multiple sclerosis pathogenesis and a new potential therapeutic target for multiple sclerosis. Currently, the best available multiple sclerosis therapies mostly target immune cells to reduce disease progression. Our study revealed for the first time that inhibition of neddylation using pevonedistat reduces disease severity in EAE. Furthermore, a recent study showed that pevonedistat can enhance rituximab activity in lymphoma (Czuczman *et al.*, 2016). Therefore, neddylation may be a new therapeutic target for multiple sclerosis and amenable to be used in combination with other disease-modifying drugs.

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Competing interests

K.K., A.-K.P. and S.E.B. have filed a patent for the use of pevonedistat (MLN4924) as a treatment for neuroinflammation. K.K., A.-K.P., R.B., L.M., J.D., R.L., E.L.E., S.S., S.J.C., L.S. have nothing to disclose.

Figure 5 Continued

here are from one representative of three experiments. **** $P < 0.0001$, two-way ANOVA with Sidak test. (D and E) Immunohistochemistry of the cervical spinal cord shows demyelination of the posterior funiculus white matter (WM) with increased infiltration of Iba1 + myeloid, as well as CD3+ T cells and B220+ B cells in mice treated with placebo (top) as compared to pevonedistat (bottom). Note, significant preservation of the number of SMI312+ axons in the pevonedistat-treated group. Shown is a representative experiment of four animals/group. * $P < 0.05$, ** $P < 0.01$, Student's *t*-test. Data are presented as mean $\pm$ SEM.

Supplementary material

Supplementary material is available at *Brain* online.

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