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Chiara Cappelletti

Oslo Metropolitan University: OsloMet - storbyuniversitetet

Anna Maria Eriksson

University of Oslo: Universitetet i Oslo

Ina Skaara Brorson

Oslo University Hospital: Oslo Universitetssykehus

Ingvild S. Leikfoss

Oslo University Hospital: Oslo Universitetssykehus

Oda Glomstad Kråbøl

Oslo University Hospital: Oslo Universitetssykehus

Einar August Høgestøl

Oslo University Hospital: Oslo Universitetssykehus

Valeria Vitelli

University of Oslo: Universitetet i Oslo

Olav Mjaavatten

University of Bergen: Universitetet i Bergen

Hanne F. Harbo

Oslo University Hospital: Oslo Universitetssykehus

Frode Steingrimsen Berven

University of Bergen: Universitetet i Bergen

Steffan D. Bos

University of Oslo: Universitetet i Oslo

Tone Berge (✉ tone.berge@oslomet.no)

OsloMet - storbyuniversitetet <https://orcid.org/0000-0002-5297-2034>

Research

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Quantitative proteomics reveals protein dysregulation during T cell activation in multiple sclerosis patients compared to healthy controls

Chiara Cappelletti,¹ Anna Eriksson,³ Ina Skaara Brorson,^{3,4} Ingvild S. Leikfoss,^{3,4} Oda Kråbøl,² Einar August Høgestøl,^{3,4,5} Valeria Vitelli,⁶ Olav Mjaavatten,⁷ Hanne F. Harbo,^{3,4} Frode Berven,⁷ Steffan D. Bos,⁴ and Tone Berge^{1,2,*}

¹Department of Mechanical, Electronics and Chemical Engineering, Faculty of Technology, Art and Design, OsloMet - Oslo Metropolitan University, Postboks 4, St. Olavs Plass, 0130 Oslo, Norway.

²Neuroscience Research Unit, Department of Research, Innovation and Education, Oslo University Hospital, Oslo, Norway

³Institute of Clinical Medicine, Medical Faculty, University of Oslo, Oslo, Norway.

⁴Department of Neurology, Oslo University Hospital, Ullevål, Postboks 4950, 0424 Nydalen, Oslo, Norway.

⁵Department of Psychology, Faculty of Social Sciences, University of Oslo, Oslo, Norway

⁶Oslo Centre for Biostatistics and Epidemiology, Department of Biostatistics, University of Oslo, Oslo, Norway.

⁷Proteomics Unit at University of Bergen (PROBE), Department of Biomedicine, University of Bergen, Postboks 7804, 5020 Bergen, Norway.

*Correspondence: tone.berge@oslomet.no

Abstract

Background: Multiple sclerosis (MS) is an autoimmune, neurodegenerative disorder with a strong genetic component that acts in a complex interaction with environmental factors for disease development. CD4⁺ T cells are pivotal players in MS pathogenesis, where peripherally activated T cells migrate to the central nervous system leading to demyelination and axonal degeneration. Through a proteomic approach, we aim at identifying dysregulated pathways in activated T cells from MS patients as compared to healthy controls.

Methods: CD4⁺ T cells were purified from peripheral blood from MS patients and healthy controls by magnetic separation. Cells were left unstimulated or stimulated *in vitro* through the TCR and costimulatory CD28 receptor for 24 hours prior to sampling. Electrospray liquid chromatography-tandem mass spectrometry was used to measure protein abundances.

Results: Upon T cell activation the abundance of 1,801 proteins was changed. Among these proteins, we observed an enrichment of proteins expressed by MS-susceptibility genes. When comparing protein abundances in T cell samples from healthy controls and MS patients, 18 and 33 proteins were differentially expressed in unstimulated and stimulated CD4⁺ T cells, respectively. Moreover, 353 and 304 proteins were identified as proteins exclusively induced upon T cell activation in healthy controls and MS patients, respectively and dysregulation of the Nur77 pathway was observed only in samples from MS patients.

Conclusions: Our study highlights the importance of CD4⁺ T cell activation for MS, as proteins that change in abundance upon T cell activation are enriched for proteins encoded by MS susceptibility genes. The results provide evidence for proteomic disturbances in T cell activation in MS, and pinpoint to dysregulation of the Nur77 pathway, a biological pathway known to limit aberrant effector T cell responses.

Keywords

Autoimmunity, multiple sclerosis, T cell activation, proteomics, disease susceptibility genes

Background

Multiple sclerosis (MS) is a complex autoimmune disorder with a significant health and societal burden (1, 2). It is a chronic inflammatory, demyelinating disorder of the central nervous system (CNS) that leads to both cognitive and physical deficits (1, 3). Introduction of disease modifying treatments has ameliorated the conditions of many patients (4), but development of personalized health care is partly precluded due to poor understanding of the biological processes underlying the disease. In addition to major genetic risk variants located in the HLA-gene region, genome-wide association studies (GWAS) have identified additional 200 autosomal MS-associated single nucleotide polymorphisms (SNPs). These findings combined with gene expression profiles have highlighted the importance of several peripheral immune cell types for MS onset, including both the innate and the adaptive immune response (5-7). CD4⁺ T cells are important regulators of the adaptive immune system and have long been considered to play pivotal roles in MS pathogenesis (8), in which peripheral activation results in migration of these cells into the CNS, leading to demyelination and axonal degeneration (9). Genome-wide studies on epigenetic modifications (e.g. DNA methylation) and gene expression of whole blood, peripheral blood mononuclear cells (PBMCs) and immune cell subtypes have been conducted to investigate potential immune dysregulation in MS. With few exceptions, no overlap was observed between the studies (10-23). Moreover, it is becoming increasingly clear that the correlation between mRNA and protein copy numbers varies widely (24, 25), and proteomic studies are therefore needed to complement and confirm findings at the epigenetic or gene expression level. Quantitative high-resolution mass spectrometry-based proteomics enables system-wide studies at the protein level; however, such studies are scarce in samples from individuals with complex diseases such as MS. We have recently performed this approach on CD4⁺ and CD8⁺ T cells freshly purified from blood in a small cohort of MS patients and healthy controls (HCs) (26). Importantly, our proteomic study, as well as other studies at the epigenetic and gene expression level, were performed on unstimulated cells and represents an image of the state of the cells at the time of harvesting. Novel disease-associated

70 pathways could be identified if cells were activated prior to proteomic profiling, as illustrated at the
71 RNA level for MS and coeliac disease, by Hellberg et al. (27) and Quinn et al. (28), respectively.
72 Using liquid chromatography combined with tandem mass spectrometry, we performed quantitative
73 proteomics of CD4⁺ T cells from relapsing-remitting MS (RRMS) patients and HCs. Cells were left
74 unstimulated or stimulated through the T cell receptor (TCR) *in vitro* allowing us to disentangle potential
75 CD4⁺ T cell specific differences induced by T cell activation, providing novel insights into disease
76 mechanisms of MS.

Materials and Methods

MS patients and healthy controls

Blood samples were collected from 20 untreated female RRMS patients (mean age 36.7 years, range 21-63 years) with median extended disability status scale (EDSS) score of 1.5 (range 0-5.5) and mean disease duration of 8 years (range 0.5-38). For one of the patients, the EDSS score was assessed by inspection of their medical journals. HC samples were collected from 20 age- and sex-matched individuals (mean age 37.0 years, range 23-50 years). All participants were of self-declared Nordic ancestry, and the HCs reported no MS in close family members. MS patients were recruited from the MS out-patient clinic at Oslo University Hospital, Norway, and the HCs from the patients' social networks and among hospital employees. All MS patients fulfilled the updated McDonald criteria for MS at their time of diagnosis (29). At the time of sample collection, the included individuals did not have any ongoing infection, and the MS patients had not experienced a relapse, or received steroids for at least three months prior to enrollment. The Regional Committee for Medical and Health Research Ethics South East, Norway approved the study. Written informed consent was obtained from all study participants.

Isolation of human CD4⁺ T cells

Peripheral blood mononuclear cells were isolated from whole blood using density gradient centrifugation with Lymphoprep™ (Axis Shield, Dundee, Scotland), before negative selection of CD4⁺ T cells with EasySep™ Human CD4⁺ T Cell Isolation Kit (STEMCELL Technologies, Vancouver, Canada). Cell purity was measured by flow cytometry (Attune Acoustic Focusing Flow Cytometer, Life Technologies, Carlsbad, CA, USA or FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA) using the fluorescein isothiocyanate-conjugated (FITC) mouse anti-human CD4 antibody (clone RTF-4g) and mouse IgG1 isotype control (15H6) (both from Southern Biotech, Birmingham, AL, USA). Aliquots of CD4⁺ T cells were subsequently frozen with dimethyl sulfoxide (DMSO) (Sigma-Aldrich®, Darmstadt, Germany) and stored in liquid nitrogen until usage.

103

104 *T cell activation*

105 Live CD4⁺ T cells stored in liquid nitrogen were thawed and left unstimulated in X-VIVO 15 medium
106 (Lonza, Basel, Switzerland) or stimulated in 96-well plates coated with 5 µg/ml anti-CD3 (mouse anti-
107 human CD3, Clone OKT3, eBioscience™ by Thermo Fisher Scientific, San Diego, CA, USA) in X-VIVO 15
108 medium supplemented with 2 µg/ml anti-CD28 (purified NA/LE mouse anti-human CD28, BD
109 Biosciences). Cells were cultured at a starting density of 1 million cells/ml for 24 h at 37°C and 5 % CO₂.
110 Cell pellets of 200 000 cells from each sample were kept at -80°C until preparation for mass
111 spectrometry analyses. An aliquot of unstimulated and stimulated CD4⁺ T cells were stained with FITC-
112 conjugated mouse anti-human CD69 antibody or mouse IgG1 isotype control (both from ImmunoTools,
113 Friesoythe, Germany) prior to staining with the LIVE/DEAD™ Fixable Far Red Dead Stain Kit (Invitrogen,
114 by Thermo Fisher Scientific, Carlsbad, CA, USA) for flow cytometry analysis using FACS Canto II flow
115 cytometer (BD Biosciences) to evaluate cell activation and viability. Analysis of flow cytometry data was
116 performed with FCS Express 6 Flow Cytometry Software 2.1 (De Novo Software, Glendale, CA, USA).

117

118 *Sample preparation and protein digestion*

119 The frozen cell pellets were solubilized in 40 µl ice-cold RIPA buffer, containing 1 % NP40, 50 mM TrisHCl
120 pH 7.6, 0.5 % sodium deoxycholate, 0.1 % SDS, 150mM NaCl, and 1x cOmplete™ EDTA-free protease
121 inhibitor (cat. No. 11873580001, Roche). Samples were homogenized on ice for 15 min followed by
122 four cycles of ultra-sonification in ice-cold water with 30 sec on and 30 sec off, followed by another 15
123 min on ice. After centrifugation for 10 min at 16200×g at 4°C, supernatants were collected. Protein
124 concentrations in the lysates were measured by Pierce BCA protein assay (Thermo Fisher Scientific,
125 Rockford, IL, USA) and the absorbance values at 562 nm were obtained by Multiscan FC 3.1 ELISA reader
126 (Thermo Fisher Scientific, Rockford, IL, USA). Subsequently, 2 µl 150 mM dithiothreitol (DTT) were
127 added to 6 µg protein in 25 µl RIPA buffer for cysteine reduction and incubated for 1 hour at RT.
128 Cysteines were alkylated after addition of 3 µl 300 mM iodoacetamide (1 hour, at room temperature

protected from light). Digestion of proteins was accomplished using the SP3 protocol (30) with a few modifications: 2 μ l (65 μ g) magnetic beads (Sera-Mag SpeedBeads, GE Healthcare, cat. no. 45152105050250 and 65152105050250) were added to the sample, and the protein binding/aggregation with the beads was accomplished by adding ethanol to 70 % final concentration. After thorough washing in 80 % ethanol, the protein/beads pellet was digested with trypsin (sequencing grade-modified trypsin from Promega, GmbH, Mannheim, Germany) dissolved in 50 μ l 100 mM ammonium bicarbonate with a trypsin-to-protein ratio of 1:25. Samples were incubated at 37°C for 16 hours at 1000 rpm. Tryptic peptides were collected, and beads washed once with 50 μ l 0.5 M NaCl. Sample cleanup was performed using a reverse-phase OasisR HLB μ Elution Plate 30 μ m (2 mg HLB sorbent, Waters, Milford, MA). After lyophilization, the dried peptides were suspended in 12 μ l of 0.5 % formic acid containing 2 % acetonitrile. Two μ l were used for protein quantification based on absorbance at 280 nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA), and 0.6 μ g of the mixture were analyzed with mass spectrometry.

Liquid chromatography-mass spectrometry/mass spectrometry analysis

Peptides were analyzed by electrospray liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a quadrupole–orbitrap instrument (QExactive HF, Thermo Fisher Scientific, Carlsbad, CA, USA). The LC run length of 3 hours was performed on a 50 cm analytical column (PepMap RSLC, 50 cm x 75 μ m ID EASY-spray column, packed with 2 μ m C18 beads (Thermo Fisher Scientific, Carlsbad, CA, USA)). Peptides were loaded and desalted on a pre-column (Acclaim PepMap 100, 2 cm x 75 μ m ID nanoViper column, packed with 3 μ m C18 beads (Thermo Fisher Scientific, Carlsbad, CA, USA)) with 0.1 % (v/v) trifluoroacetic acid, and eluted with a gradient composition as follows: 5 % B during trapping (5 min) followed by 5–8 % B for 0.5 min, 8–24 % B for the next 109.5 min, 24–35 % B over 25 min, and 35–80 % B over 15 min. Elution of very hydrophobic peptides and conditioning of the column were performed during 15 min isocratic elution with 80 % B and 20 min isocratic elution with 5 % B respectively. Mobile phases A and B contained 0.1 % formic acid (vol/vol) in water and 100 %

acetonitrile, respectively, and the flow rate was 200 nl per min. A full scan in the mass area (m/z) of 375–1500 was performed in the Orbitrap. For each full scan performed at a resolution of 120 000 (m/z 200), the 12 most intense ions above an intensity threshold of 50 000 counts, and charge states 2 to 5 were sequentially isolated and fragmented in the Higher-Energy Collision Dissociation (HCD) cell. Fragmentation was performed with a normalized collision energy (NCE) of 28 %, and fragments were detected in the Orbitrap at a resolution of 30 000 (m/z 200), with first mass fixed at m/z 100. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 25 sec with “exclude isotopes” on. Lock-mass internal calibration (m/z 445.12003) was used.

Mass spectrometry data analysis

Mass spectrometry (mass spec) raw files were analyzed by the Proteome Discoverer™ software (Thermo Fisher Scientific, Carlsbad, CA, USA, version 2.4), and peak lists were searched against the human SwissProt FASTA database (version May 2020), and a common contaminants database by Sequest HT and MS Amanda 2.0 search engines. Methionine oxidation and acetylation on protein N-terminus were added as variable modifications, while cysteine carbamidomethylation was used as fixed modification. False discovery rate (Percolator, <http://percolator.ms/>) was set to 0.01 for proteins and peptides (minimum length of six amino acids) and was determined by searching the reversed database. Trypsin was set as digestion protease, and a maximum of two missed cleavages were allowed in the database search. Mass recalibration was performed prior to peptide identification using precursor and fragment mass deviation of 20 ppm and 0.5 Da respectively. The main search was then conducted with an allowed mass spec and mass spec/mass spec mass deviation tolerance of 10 ppm and 0.02 Da respectively. Retention time alignment and detection of precursor features across samples were done using the Minora Feature Detector node in Proteome Discoverer™.

Data processing

A total of 6,687 proteins were identified by the Proteome Discoverer™ 2.4 Software (Thermo Fisher Scientific, Carlsbad, CA, USA). Of these, 178 protein signals were marked as contaminants and therefore removed from further analysis. In Perseus (Perseus Software, version 1.5.6.0), the normalized abundances from Proteome Discoverer™ were log2 transformed and the normal distributions were controlled by plotting the histograms. Proteins with valid values in at least 70 % of the samples in at least one of the four groups (HC: unstimulated, HC: stimulated, MS: unstimulated and MS: stimulated) were used for analysis. The missing protein abundances were imputed from the normal distribution using default settings in Perseus.

Statistical analyses

All analyses presented were performed using the R software version 4.0.4. Differences in protein abundances upon T cell activation were assessed using a paired two-tailed Student's *t*-test. When comparing protein abundance between samples from MS patients and HCs, a Welch's test (for unequal variances) was used. Principal component analysis (PCA) plots were generated using protein intensities of differentially expressed proteins as variables. For validation analysis, 100 discovery cohorts were simulated by randomly selecting ten MS samples and ten HC samples and the differentially expressed proteins identified in these simulated cohorts were used as input for performing PCA in the remaining samples.

Within each analysis stratum, the Benjamini-Hochberg (B-H) procedure was used to correct for multiple testing and adjusted p-values considered significant are indicated in the results section.

Ingenuity pathway analysis

QIAGEN's Ingenuity® pathway Analysis (IPA® QIAGEN, version 52912811, date: 2020-09-07) was used for functional interpretation of significantly expressed proteins. The default settings were used, species was set to "all" and "T lymphocytes", "Immune cell lines", "CCRF-CEM", "Jurkat" and "MOLT-4" were

205 selected among the tissues and cell lines. A Benjamini-Hochberg (B-H) multiple testing correction was
206 used, and a value below 0.05 ($-\log(\text{B-H p-value}) > 1.3$) was considered significant.

Results

Protein dysregulation is observed in CD4⁺ T cells from MS patients

In this study, we examined the differences at the proteomic level of CD4⁺ T cells from RRMS patients (n=20) and HCs (n=20). CD4⁺ T cells were left unstimulated or stimulated through the TCR (anti-CD3; OKT3) and costimulatory CD28 receptor (anti-CD28) for 24 hours prior to sampling (Figure 1.A). T cell activation was verified by measuring the cell surface expression of the T cell activation marker CD69 by flow cytometry (Figure 2.A). Using a label-free proteomics approach, we were able to identify and quantify a total of 5,704 proteins. Of these proteins, the abundance of 1,801 was changed upon T cell activation (adjusted $p \leq 0.01$) (Figure 1.B).

When comparing protein abundances in the T cell samples from HCs and MS patients, 18 and 33 proteins were differentially expressed (adjusted $p \leq 0.05$) in unstimulated (Table 1, Figure 1.C) and stimulated CD4⁺ T cells (Table 2, Figure 1.D), respectively, with two proteins; diphthamide synthetase, encoded by *DPH6*, and enhancer of polycomb homolog 1, encoded by *EPC1*, being significant in both conditions. Diphthamide synthetase expression was higher in unstimulated cells from MS patients (log2 fold change = 3.30), whereas its expression was lower in stimulated cells from MS patients (log2 fold change = -1.91), compared to HC. Enhancer of polycomb homolog 1 showed higher fold change between MS and HC samples in stimulated samples (log2 fold change = 3.47) compared to unstimulated samples (log2 fold change = 2.34). The principal component analysis (PCA) plots of significant proteins in each analysis show separated clusters of samples from MS patients and HCs in unstimulated (Figure 3.A) and stimulated (Figure 3.B) CD4⁺ T cells. Close to 50 % of the total variation in the dataset, which captures the separation between MS and HCs, was explained by the first component, whereas the second component captured 11-12 % of the variance in each analysis.

Validation of protein dysregulation in CD4⁺ T cells from MS patients by resampling

To validate the protein dysregulation observed in CD4⁺ T cells from MS patients, we simulated 100 discovery cohorts by randomly selecting ten MS samples and ten HC samples ($n_{MS} = 10$, $n_{HC} = 10$) for

each simulated dataset. For both conditions (unstimulated and stimulated), we performed differential expression analysis in each of the 100 simulated discovery cohorts. We carried out PCA analysis based on the differentially expressed proteins (adjusted $p \leq 0.05$) in each corresponding replication cohorts, consisting of the remaining samples ($n_{MS} = 10$, $n_{HC} = 10$). The number of significant proteins in the main analysis and the median number of significant proteins obtained from the validation analysis for each condition are listed in Table 3. The lower number of significant proteins found in the validation analysis is due to the lower power to detect differentially expressed proteins in smaller datasets ($n=10$ versus $n=20$). In the validation analysis, we found that the scores of the first principal components were statistically different ($p \leq 0.05$) between MS and HC samples in 82 % of the iterations for the unstimulated samples and in 61 % of the iterations in the stimulated samples. Of note, in two out of 100 iterations in the unstimulated samples, no significant proteins were found, whereas in the validation analysis of the stimulated samples, significant proteins were found in all iterations. These analyses confirmed that most of the variance present in our samples captured by the first principal component was due to protein dysregulation in CD4⁺ T cells between MS patients and HCs.

When comparing the differentially expressed proteins in samples from MS patients and HCs identified in the iteration analyses, we discovered that diphthamide synthetase, encoded by *DPH6*, was found in 98 iterations of the unstimulated samples, while Grb2-related adapter protein and enhancer of polycomb homolog, encoded by *GRAP* and *EPC1*, respectively, were found in all 100 iterations from stimulated samples.

Proteins differentially expressed upon T cell activation are enriched for proteins expressed by MS-susceptibility genes

To test for enrichment of proteins encoded by MS susceptibility genes among the 1,801 proteins whose abundance is changed upon T cell activation (Figure 1.B), the IDs of 285 most proximal genes were extracted from the list of 200 autosomal, non-HLA MS-associated SNPs (7). For intergenic MS-associated SNPs, we extracted the most proximal genes both upstream and downstream of the SNPs.

Out of these, 34 gene IDs corresponded to non-coding RNAs and were removed from the analysis. Not all MS susceptibility genes are expressed in T cells, and in our samples, we detected 97 proteins encoded by MS susceptibility genes that were expressed either in the unstimulated or stimulated samples. Of these, 43 proteins were among the 1,801 differentially expressed upon T cell activation regardless of the disease status. A Person's Chi-squared test showed that there was a significant enrichment for proteins encoded by MS susceptibility genes among the 1,801 proteins that were changed upon T cell activation ($p = 0.0089$; Table 4), highlighting the importance of this process in MS.

Ingenuity pathway analysis of differentially expressed proteins exclusively induced upon T cell activation in MS patients or in healthy controls

To elaborate on the differences in the T cell activation process in CD4⁺ T cells from MS patients and HCs, we specifically analyzed proteins that displayed a significant change in abundance upon T cell activation in HC and MS (Figure 1.E). We discovered 990 differentially expressed proteins (adjusted $p \leq 0.01$) between unstimulated and stimulated CD4⁺ T cells in HCs and 941 differentially expressed proteins in MS patients. Of these proteins, 637 were differentially expressed in both HC and MS samples, whereas 353 and 304 proteins were exclusively differentially expressed upon CD4⁺ T cell activation in HCs and in MS patients, respectively (Figure 1.E). Of the 637 proteins differentially expressed in both groups, all proteins, except for pyruvate dehydrogenase and Late Endosomal/Lysosomal Adaptor, MAPK and MTOR activator 5, encoded by the *PDH6* and *LAMTOR5* genes, showed a change in expression in the same direction across the groups.

The IPA software was used for network analyses of proteins whose expression was affected by T cell activation exclusively in samples from MS patients or HCs. We identified enrichment in ten biological processes (Figure 4.A; $-\log(\text{B-H } p\text{-value}) > 1.3$) among the proteins exclusively changed upon stimulation of CD4⁺ T cells from MS patients, whereas among the proteins exclusively changed upon activation of CD4⁺ T cells from HCs, we identified one biological process (Figure 4.B; $-\log(\text{B-H } p\text{-value}) > 1.3$). The top four pathways (eIF2 signaling, regulation of eIF4 and p70S6K signaling, Coronavirus pathogenesis

285 pathway, and mTOR signaling) identified among the proteins exclusively changed in MS patients
286 corresponded to the top four pathways identified among the proteins whose expression were changed
287 upon T cell activation in both groups (Table 5). Of note, the Nur77 signaling pathway identified among
288 the proteins exclusively changed upon activation of CD4⁺ T cells from HCs has been shown to be a key
289 regulator of T cell function by restricting activation, cell cycle progression, and proliferation (31).

Discussion

Genome-wide association studies have revealed 230 risk loci for MS, mostly located within or close to genes expressed in immune cells (7). However, it remains to be analyzed whether genetic differences are translated into cell-specific expression profiles in samples from MS patients and HCs. Previous transcriptomic analyses of CD14⁺ monocytes, CD4⁺ and CD8⁺ T cells, indicated that CD4⁺ T cells were the most dysregulated cell type in MS among these three immune cells (32). Transcriptomic profiling is frequently performed to identify genes and pathways of relevance for complex diseases such as MS. Given the lack of complete correlation between mRNA and protein copy numbers (24, 25), proteomic profiling enables an alternative or complementary approach for identification of disease relevant pathways. To our knowledge, we were the first to perform proteomic profiling of purified immune-cell subsets from MS patients. Using electrospray liquid chromatography-tandem mass spectrometry, we were able to identify aberrant protein expression in freshly purified T cells, i.e. CD4⁺ and CD8⁺ T cells, from MS patients as compared to HCs (26). In the current study, we used the same technique for proteomic profiling of CD4⁺ T cell samples left unstimulated or stimulated for 24 hours *in vitro* through the TCR, to analyze protein dysregulation during T cell activation in MS. We identified novel proteins that were differentially expressed in response to activation in samples from MS patients as compared to HCs. Furthermore, we found that the proteins, whose expression was changed upon T cell activation, were enriched for proteins encoded by MS susceptibility genes. These findings confirmed the importance of CD4⁺ T cell activation for MS pathogenesis. As the included patients already had developed MS, it remains to be shown whether this aberrant response contributes to developing MS or rather is a consequence of the ongoing disease. Of note, all included MS patients were untreated and clinically stable at the time of sample collection, excluding the possibility for disease modifying treatment having affected the T cells used in this study.

There is little overlap between the findings from this study and Berge et al., 2019 (26), but both studies were relatively low powered due to the small sample sizes. To rule out findings attributable to low sample size, a validation analysis was performed in the current study and confirmed the protein

dysregulation observed in MS patients. Furthermore, even though eight samples (four MS and four HCs) were obtained from the same donors as included in (26), the experimental set ups were different between the two studies. In our previous study (26), the CD4⁺ T cells were prepared for mass spectrometry directly after cell purification to investigate their status in MS patients. On the contrary, for this study, live cells were stored on liquid nitrogen prior to thawing and cell cultivation for 24 hours in the presence or absence of stimulating antibodies to investigate T cell behavior upon activation. All samples included in this study were treated equally, and there was no difference between the two groups in cell viability or cell activation (Figure 2), as measured by cell surface expression of CD69 using flow cytometry. Using our stimulation protocol, cells were triggered through the TCR and CD28 co-receptor. Therefore, all T cells in the culture, independent of specificity and binding strength, were likely to be activated, ruling out the possibility of a different TCR repertoire in the MS population. Through proteomic profiling of stimulated cells, we identified MS-associated proteins that were hitherto not identified with the current available approaches, i.e. global DNA methylation analyses or RNA sequencing, performed in untreated immune cell subsets, full blood or in PBMCs (10-23).

There were only two proteins differentially expressed between MS patients and HCs in both the unstimulated and stimulated samples, i.e. diphthine:ammonia ligase (also called diphthamide synthetase) encoded by *DPH6* and enhancer of polycomb homolog 1 encoded by *EPC1*. Diphthamide synthetase catalyzes the conversion of histidine to diphthamide for regulation of the translation factor EEF2 (33), which controls neurological processes (34), but with hitherto no known role in autoimmunity. Enhancer of polycomb homolog 1 is a transcriptional regulator (35) with no known function in T cells and was also one of two proteins differentially expressed in all the 100 iterations performed with the stimulated samples. Another protein differentially expressed in all the 100 iterations and the top hit of the main analysis in the stimulated samples (log2 fold change = 5.35), was Grb2-related adapter protein encoded by *GRAP*. Of note, Grb2-related adapter protein 2 encoded by the MS susceptibility gene *GRAP2* was expressed at higher levels in CD4⁺ T cells from MS patients as compared to HCs in our

previously published proteomic analyses (26). The Grb2 family of adapter proteins has been shown to interact with the activated T cell costimulatory receptor CD28 (36) and to be involved in Erk-MAP kinase activation in human B cells (37). Moreover, the *GRAP* gene is primarily expressed in human thymus and spleen (38), and it negatively regulates TCR-elicited proliferation and interleukin-2 induction in murine lymphocytes (39). Identification of these adapters in our proteomic approaches suggests further investigation of the Grb2 family of adaptor proteins in MS.

Among the differentially expressed proteins between MS patients and HCs, three proteins have previously been suggested to play a role in MS pathogenesis: (1) tyrosine kinase 2 (TYK2), (2) protein tyrosine phosphatase non-receptor type 2 (PTPN2), and (3) DNA polymerase subunit gamma-1 (POLG). In our data set, TYK2 was slightly upregulated in unstimulated samples from MS patients (log2 fold change = 1.14). An exonic *TYK2* variant (rs34536443) has been shown to associate with increased MS risk (7), and the presence of the protective allele at rs3453443 resulted in reduced TYK2 kinase activity in T cells and a shift in the cytokine secretion profile favoring Th2 development, but did not modify TYK2 expression when measured by Western blotting (40). With a minor allele frequency of 0.01423 (www.snpedia.com) for the MS associated rs34536443 SNP in *TYK2* and the limited sample size in the presented study, it is unlikely that the genotype of this SNP underlies the difference in TYK2 expression between the two groups. PTPN2 has previously been linked to MS as a microRNA, i.e. miR-448, that was upregulated in PBMC and cerebrospinal fluid (CSF) from MS patients, promoted IL-17 production directly through PTPN2, thereby contributing to development of an autoinflammatory immune environment. However, being a direct target of miR-448, *PTPN2* expression was reduced in PBMC and CSF from MS patients (41), whereas we observed a small increase in stimulated CD4⁺ T cells from MS patients (log2 fold change = 0.85). Nevertheless, the experimental set-up and the biological materials were different in the two studies. In our analyses, we were able to detect cell-specific differences, which could be convoluted when analyzing heterogeneous samples such as PBMCs or CSF. POLG expression was reduced in stimulated CD4⁺ T cells from MS patients (log2 fold change = -1.35) as compared to HC

samples. Genetic variants in the *POLG* gene have been associated with familial MS (42). In a smaller genetic study, *POLG* was suggested as an MS susceptibility gene (43), but it did not reach genome-wide significance in the latest MS GWAS (7).

As MS is an autoimmune disease, it is not a surprise that proteins expressed from MS susceptibility genes are enriched among the proteins that change expression upon T cell activation, highlighting the importance of this process in MS. Findings from our previous proteomic study (26) also pointed to the importance of T cell activation, as the differentially expressed proteins between CD4⁺ T cells from MS patients and HCs were enriched in pathways related to T cell activation. In the current study, most proteins that were induced or inhibited upon CD4⁺ T cell stimulation were overlapping in samples from MS patients and HCs. However, there were sets of proteins that were selectively regulated in one group only. Pathway analyses showed that proteins with changes in expression upon T cell activation in the MS group only correspond to pathways also identified among the proteins changed upon T cell activation in both groups, including pathways of translation initiation and immune response (eIF2 and eIF4) and cell survival and proliferation (mTOR). Interestingly, pathway analysis showed that proteins with changes in expression upon T cell activation in the HC group only were enriched for the Nur77 pathway. This signaling pathway limits aberrant effector T cell responses and impedes the development of T cell-mediated inflammatory diseases such as autoimmune disorders (31). Our data suggest that the Nur77 pathway is not induced upon T cell activation in MS patients as in HCs, and more thorough functional analyses are needed to understand its role for development of MS.

In the current study, we examined one immune cell subtype from blood, CD4⁺ T cells, which provided a detailed insight into one specific immune cell subtype with a likely role in MS. However, it should be noted that CD4⁺ T cells can be further divided into subclasses and consequently differences in subtypes of CD4⁺ T cells, such as Th17 or regulatory T cells, might not be detected, as these signals may be concealed by signals from the more abundant CD4⁺ T cells subtypes. Although we have identified novel

proteins of potential importance for MS, further studies are needed to validate and verify the biological impact of selected proteins and pathways in T cells.

Conclusions

In summary, using electrospray liquid chromatography-tandem mass spectrometry for analyses of *in vitro* stimulated CD4⁺ T cells from MS patients and HCs, we were able to identify aberrant regulation of novel proteins in MS, hitherto not identified through other approaches. Proteins encoded by MS susceptibility genes are enriched among proteins that change in abundance upon T cell activation, and through pathway analyses, we have identified enrichment of induced proteins from the Nur77 pathway in HC samples only.

List of abbreviations

MS: Multiple sclerosis

HC: Healthy control

CNS: Central nervous system

GWAS: Genome wide association study

SNP: Single nucleotide polymorphism

PBMC: Peripheral blood mononuclear cell

RRMS: Relapsing-remitting multiple sclerosis

TCR: T cell receptor

EDSS: Extended disability status scale

FITC: Fluorescein isothiocyanate-conjugated

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

LC-MS/MS: Electrospray liquid chromatography-tandem mass spectrometry

HCD: Higher-energy collision dissociation

419 **NCE:** Normalized collision energy

420 **Mass spec:** Mass spectrometry

421 **PCA:** Principal component analysis

422 **B-H:** Benjamini-Hochberg

423 **IPA:** Ingenuity pathway analysis

424 **TYK2:** Tyrosine kinase 2

425 **PTPN2:** protein tyrosine phosphatase non-receptor 2

426 **POLG:** DNA polymerase subunit gamma-1

427 **CSF:** Cerebral spinal fluid

428

429 **Declarations**

430 *Ethics approval and consent to participate*

431 The Regional Committee for Medical and Health Research Ethics South East, Norway approved the
432 study. Written informed consent was obtained from all study participants.

433

434 *Consent for publication*

435 Not applicable.

436

437 *Availability of data and materials*

438 The datasets generated in the study are currently being uploaded into the PRoteomics IDentifications
439 (PRIDE) database and will be publically available.

440

441 *Competing interests*

442 TB received unrestricted research grants from Biogen Idec and Sanofi-Genzyme, EAH received
443 honoraria for lecturing and advisory board activity from Biogen, Merck and Sanofi-Genzyme and
444 unrestricted research grant from Merck, ISL received unrestricted research grant from Novartis and

HFH has received travel support, honoraria for advice or lecturing from Biogen, Merck, Sanofi-Genzyme, Roche and an unrestricted research grant from Merck. The other authors declare that they have no competing interests.

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Authors' contributions

TB and AE conceived the idea. CC, TB and FB planned the study. AE, ISB, ISL, EAH, HFH, SDB and TB recruited MS patients and healthy controls. CC, AE, ISB, ISL, OK, SDB and TB collected samples. OM carried out mass spectrometry. CC, AE, ISB, OK, VV, SDB, OM, FB and TB analyzed and interpreted the data. CC and TB wrote the manuscript. All authors revised the manuscript and approved the final manuscript.

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Figure Legends

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606

Figures

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