Dynamic Response Genes in CD4+ T Cells Reveal a Network of Interactive Proteins that Classifies Disease Activity in Multiple Sclerosis

Graphical Abstract

Highlights
- Dynamic CD4+ T cell responses in vitro revealed a dysregulated network module in MS
- This module was highly enriched for GWAS genes and translated into protein expression
- A combined protein score readily predicted disease activity and treatment response
- Integrating GWASs with dynamic expression profiling disclosed personalized biomarkers

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In Brief
Hellberg et al. have constructed a highly connected gene module of dysregulated genes in multiple sclerosis patients in which the gene products can collectively be used to classify patients and predict disease activity and response to treatment. This set of key proteins holds promise as clinically useful biomarkers in personalized MS treatment.

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Dynamic Response Genes in CD4+ T Cells Reveal a Network of Interactive Proteins that Classifies Disease Activity in Multiple Sclerosis

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SUMMARY

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS and has a varying disease course as well as variable response to treatment. Biomarkers may therefore aid personalized treatment. We tested whether in vitro activation of MS patient-derived CD4+ T cells could reveal potential biomarkers. The dynamic gene expression response to activation was dysregulated in patient-derived CD4+ T cells. By integrating our findings with genome-wide association studies, we constructed a highly connected MS gene module, disclosing cell activation and chemotaxis as central components. Changes in several module genes were associated with differences in protein levels, which were measurable in cerebrospinal fluid and were used to classify patients from control individuals. In addition, these measurements could predict disease activity after 2 years and distinguish low and high responders to treatment in two additional, independent cohorts. While further validation is needed in larger cohorts prior to clinical implementation, we have uncovered a set of potentially promising biomarkers.

INTRODUCTION

Multiple sclerosis (MS), a chronic inflammatory disease of the CNS, is a common cause of neurological disability in young persons (Dendrou et al., 2015). The introduction of new treatments has improved the situation for many patients, yet some patients do not respond to a given treatment and treatments are costly and associated with side effects, sometimes severe. Thus, biomarkers for personalized treatment would be highly beneficial (Derfuss, 2012). The development of biomarkers is hampered by the heterogeneity and complexity of the disease process in MS, in which the underlying disease mechanisms reflect the interplay between a large number of genes and their downstream targets. Accordingly, genome-wide association studies (GWASs) have revealed a growing list of genes associated with MS (Beecham et al., 2013). While subsequent genome-wide expression profiling of immune cells has increased our functional understanding of disease mechanisms in MS, these studies have so far not resulted in clinically useful biomarkers (Comabella and Montalban, 2014; Kemppinen et al., 2011).

Previous gene expression profiling studies in MS primarily examined unstimulated cells, i.e., a “snapshot” of how the cells appear in vivo, where most cells are not stimulated. We hypothesized that the dynamic response to activation would reveal disease-associated pathways that were not found during conventional profiling of blood cells. We therefore examined gene expression in unstimulated as well as in stimulated cells, with the goal of identifying relevant genes that might translate into clinically valuable biomarkers. We focused our attention on CD4+ T cells because (1) they are early and important regulators of adaptive immunity (Sallusto, 2016), (2) substantial evidence implicates peripherally activated CD4+ T cells in MS pathogenesis both in humans (Dendrou et al., 2015; Fletcher et al., 2010; Okuda et al., 2005; Putheti et al., 2003) and in animal models (Fletcher et al., 2010), and (3) the association of MS with the CD4+ T cell-specific antigen-presenting molecule HLA Class II (DRB1*15:01) is well established, as well as the more recent identification of
several other T cell-associated genes in large GWASs of MS, such as the interleukin (IL)-2 receptor alpha chain, the IL-12 receptor, and IL-7 (Sawcer et al., 2011; Patsopoulos et al., 2011).

By combining traditional analysis of differential gene expression in stimulated CD4+ T cells with a network-based modular approach, we found that aberrant dynamic response (DR) genes, i.e. genes behaving differently upon stimulation in patients than in control individuals, were highly enriched for MS genes, as defined by GWAS data (Sawcer et al., 2011). The aberrantly expressed genes were associated with immune activation and chemotaxis and a set of them encoded secreted proteins (chemokine [C-X-C motif] ligand 1-3 [CXCL1-3], CXCL10, chemokine [C-C motif] ligand 2 [CCL2], and osteopontin [OPN]). A combination of these proteins, as measured in cerebrospinal fluid (CSF), readily classified patients and controls and, in two new cohorts, predicted 2-year disease activity in early MS as well as response to treatment, respectively.

**RESULTS**

**Stimulation of CD4+ T Cells Reveals Differently Expressed Genes Enriched for MS-Associated GWAS Genes**

The principal workflow of the study was as follows: identifying genes that were dysregulated in isolated and activated CD4+ T cells from MS patients (Figure 1A); investigating whether the identified genes were known to be associated with MS (Figure 1B); identifying functional relationships of the dysregulated genes (Figures 1C and 1D); and testing whether this information might be useful for identifying biomarkers in new cohorts of MS patients (Figure 1E). To assess gene expression dynamics in CD4+ T cells, we used an in vitro model, in which isolated CD4+ T cells were cultured unstimulated or with commonly used combined activation through the TCR (anti-CD3) and co-stimulatory CD28 receptor (anti-CD28) (Sharpe and Freeman, 2002). We used a moderate level of stimulation, as measured...
by CD69 surface expression (Figure S1), a marker of global T cell co-stimulation and activation. Gene expression profiling (22,610 genes) was performed on unstimulated and stimulated CD4+ T cells from a cohort of patients (n = 14; Table 1) with relapsing-remitting MS, without treatment prior to the sampling occasion, as well as cells from matched control subjects (n = 14; Table 1). In addition, to assess the dynamic response to stimulation, we calculated the changes in gene expression. This was done by subtracting expression in unstimulated cells from expression in the stimulated cells in both patient and control cells (unlogged data were used). The groups were thereafter compared statistically, i.e. the change in gene expression upon activation was compared between patients and controls. We refer to these genes as dynamic response (DR) genes. When comparing patients and controls using an unpaired t test (unadjusted double-sided p < 0.05), we identified nominally differentially expressed genes in unstimulated CD4+ T cells (n = 2,075), stimulated CD4+ T cells (n = 1,250), and DR genes (stimulated – (unstimulated), n = 932)). However, when adjusting for genome-wide testing (at 20% Benjamin Hochberg false discovery rate), no genes were differentially expressed in any of the three gene lists (unstimulated, stimulated, and DR). We thus displaced the single gene analysis and proceeded by analyzing groups of genes, starting by testing whether any of the lists were enriched for genes relevant to MS. By assessing original GWAS data from the International MS Genetic Consortium (Sawcer et al., 2011) based on ~25,000 patients and controls, and by using a moderate stringency cut off (p < 10^-5), we identified 760 MS-associated SNPs that mapped, through the closest transcription start site, to 216 unique genes (hereafter referred to as MS genes; Table S1). To investigate whether our three gene lists with differently expressed genes (unstimulated, stimulated, and DR) actually contained genes that were associated with MS (as defined by previous GWASs), we overapped the three gene lists with the 216 MS genes and computed statistical enrichment using Fisher’s exact test (Figures 1B and 3). The highest fold enrichment (FE) of MS genes was found for the DR genes (FE = 2.08, n = 19, p = 2.0 x 10^-3; Figure 2). These 19 genes are referred to as MS-DR genes. Unstimulated CD4+ T cells showed a significant, but smaller, fold enrichment (FE = 1.16, n = 44, p = 5.3 x 10^-4; Figure 2), while the stimulated gene set showed no enrichment (FE = 0.93, n = 14, p = 0.648; Figure 2) of GWAS genes (Table S1). While the unstimulated gene set also included a high proportion of GWAS genes, we decided to perform subsequent analyses using the DR genes, since the primary objective of the study was to investigate dys-regulated genes upon activation of CD4+ T cells from MS patients. To rule out the possibility that the GWAS genes found among the DR genes were not generally associated with autoimmune disease, but specific to MS, we calculated the FE of GWAS genes from two other autoimmune diseases, rheumatoid arthritis (RA) and type 1 diabetes (T1D). For RA, we analyzed around 2,500,000 SNPs (Stahl et al., 2010) and found 81 genes at a moderate stringency cut off (p < 10^-5). Only four of these RA-associated genes were found among the DR genes (FE = 1.16, p = 0.46). For T1D, about 500,000 SNPs were analyzed (Barrett et al., 2009), of which 109 genes were considered to be associated with this disease (p < 10^-5). Only two of these T1D-associated genes were found among the DR genes (FE = 0.43, p = 0.95). Taken together, genes that were differentially expressed during in vitro activation of CD4+ T cells from MS patients, representing the dynamic response to stimulation, were highly associated with MS-relevant genes, as defined by GWASs.

Table 1. Characteristics of the MS Cohorts

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Controls</th>
<th>MS</th>
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<tbody>
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<td><strong>Study Cohort</strong></td>
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<td></td>
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<tr>
<td>n</td>
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<td>16</td>
</tr>
<tr>
<td>Relapsing remitting (n)</td>
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<td></td>
</tr>
<tr>
<td>Median age, years (range)</td>
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<tr>
<td>Sex (M/F)</td>
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<td>0/16</td>
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<tr>
<td>Median disease duration, years (range)</td>
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<tr>
<td>Annual relapse rate (mean)</td>
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<td>Median EDSS (range)</td>
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<td>Median MSSS (range)</td>
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<tr>
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<td>Possible MS</td>
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<td>Median age, years (range)</td>
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<td>Median disease duration, months (range)</td>
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<tr>
<td>No previous treatment</td>
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<tr>
<td><strong>Response to Treatment Cohort</strong></td>
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<tr>
<td>Median age, years (range)</td>
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</tr>
<tr>
<td>Sex (M/F)</td>
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<td></td>
</tr>
</tbody>
</table>

EDSS, Expanded Disability Status Scale; MS, multiple sclerosis; MSSS, MS severity score. M, male; F, female.

*a*Controls in both cohorts consisted of blood donors or employees at Linköping University Hospital. All controls were free from disease, and medication and the controls in the early MS cohort had normal CSF findings and no evidence of neurological disease.

*b*Calculated over 2 years prior to sampling.

*c*None of the patients had received immunomodulatory or immunosuppressive treatment for at least 2 months prior to sampling, with the exception of one patient who received intravenous immunoglobulin that was stopped 15 days before sampling.

*d*Consecutively included when first seeking medical attention at the Department of Neurology, Linköping University Hospital, and fulfilling criteria of clinical isolated syndrome (CIS) or relapsing-remitting (RR) MS according to the revised MacDonald criteria from 2010 (Polman et al., 2011).

*e*This cohort was described previously (Gustafsson et al., 2014; Mellerård et al., 2010, 2013).
Dynamic Response Genes Co-localize in the PPI Network

It is known that proteins encoded by MS-associated genes are more likely to be connected in the protein-protein interaction (PPI) network (International Multiple Sclerosis Genetics Consortium, 2013), thereby suggesting a functional relationship. We therefore examined whether the 19 MS-DR genes identified herein were also functionally related (Figure 1C).

To test this we used all high confidence human protein interactions (n = 413,896) from among 14,543 proteins (STRING database v.10 [Szklarczyk et al., 2015]). Analyzing this network showed that most of the MS-DR genes were co-localized in a neighborhood with a significantly shorter distance, compared to randomly equally sized sets of proteins (harmonic average \( d < 1.0 \geq 2.9 \) versus \( d_{\text{rand}} \geq 5.2 \); permutation test \( p = 2.0 \times 10^{-3} \)), where distance is defined as the smallest number of interacting proteins between two given genes. This analysis suggests a functional relationship between the MS-DR genes.

Module Proteins in Cell Culture Supernatants Are Able to Classify Patients versus Controls

Since proteins measurable in fluids are most suitable as biomarkers, we investigated whether the observed differences in gene expression between patients and controls could be associated with differences in protein expression. A total of six proteins were selected from the two main submodules (Figure 3) based on whether the protein was (1) known to be a secreted product, (2) associated with CD4+ T cells, and (3) possible to measure by commercial ELISA or multiplex bead technology. Next, we measured the selected proteins (CCL2, CXCL1-3, CXCL10, OPN, neurotrophin [NT]-3, and soluble tumor necrosis factor receptor 1 [sTNFR1]; Table S3) in supernatants collected from unstimulated and stimulated CD4+ T cells from MS patients (n = 16) and healthy controls (n = 16).
were obtained from the same primary cohort in which gene expression was analyzed. As with the dynamic gene expression data, protein levels were defined as the difference in concentration between stimulated and unstimulated cells (referred to as DR protein levels; Figure S2). In line with the gene expression data, there was a significant increase in the levels of OPN (Mann-Whitney test, p = 0.007) and a tendency toward an increase in CXCL1-3 (p = 0.09) and CXCL10 (p = 0.08) in patient samples, while no difference was observed in the levels of CCL2 (p = 0.39). NT-3 and sTNFR1 were undetectable in all samples.

To see if the secreted protein levels after stimulation of CD4+ T cells could distinguish patients from controls in our primary study cohort, we used the DR protein levels and tested the ability of a single protein or the combination of the four proteins to classify patients and controls. As a combined disease score, the protein levels of OPN, CXCL1-3, and CXCL10 were added, while the CCL2 protein level was subtracted. We found that the sum of all four proteins could discriminate patients and controls with high accuracy (area under the curve [AUC] = 0.84, Mann-Whitney test p = 0.0013; Figure 4A), whereas using a single protein or combinations of two or three proteins resulted in poorer discrimination (Table S2).
S4), as did mRNA levels obtained from the expression data (AUC = 0.71, p = 0.063). As a control classifier we constructed a similar combined score by measuring eight other secreted proteins in the supernatants (granulocyte macrophage colony-stimulating factor [GM-CSF], interferon [IFN]-γ, IL-10, IL-13, IL-17, CCL17, macrophage colony-stimulating factor [M-CSF], and CCL20; Table S3) that were not part of the module, but are of immunological relevance and known to be affected by stimulation. The 1,000 random combined scores created from the DR protein levels from this set of 8 control proteins (mean AUC = 0.54) were outperformed by the combined score from our selected module proteins (p = 4.38 × 10⁻⁶, FE = 1.55). Taken together, the increase in gene expression of the selected module genes was associated with an increase in protein secretion, and by considering the secreted levels of a combination of proteins in the supernatants we were able to classify patients from healthy controls.

**Module Proteins in Cerebrospinal Fluid Are Able to Predict Disease Activity after 2 Years**

Encouraged by the possibility of classifying patients from controls based on protein levels after stimulation of CD4+ T cells, we asked if a combined score of proteins could serve as a biomarker in MS (Figure 1E). To become a useful biomarker, it would be advantageous if the proteins could be measured in vivo, i.e. as soluble proteins in plasma or CSF. Of note, these levels would indirectly reflect the dynamic response in vivo, as an increase is probably caused by cell activation. This is particularly pertinent to CSF, where disease-associated, activated T cells are highly enriched. To test biomarker potential in a clinical setting, we used a second, independent cohort of early MS patients (Table 1) and measured the four proteins in CSF and plasma samples obtained when patients first sought medical attention. Since CSF cells could not be activated, as performed for CD4+ T cells, and a dynamic response could therefore not be formed, we inferred their relations in CSF. We performed logistic regression of the four proteins in CSF from patients (n = 41) versus healthy controls (n = 11) and estimated the predictive value by computing the probability by leave-one-out cross-validation in order to assess the prediction error (Table S5). Interestingly, the relationships among the four proteins was again inferred as previously, i.e. higher levels of OPN, CXCL1-3, and CXCL10 added to the combined disease score, while higher...
levels of CCL2 decreased the score. We found that the combined regression score for proteins in CSF separated patients and controls with a high accuracy (AUC = 0.79, p = 3.8 \times 10^{-14}; Figure 4B).

A critical factor in clinical management is prediction of disease activity. At 2-year follow-up, 12 patients had no evidence of disease activity (NEDA) (Rolstein et al., 2015), while 27 had signs of it (non-NEDA). We then tested if our combined score of these four proteins, as measured in CSF (Table S5) at baseline, was able to distinguish patients with or without disease activity by displaying the disease probability scores. We found that the combined score in CSF at baseline was able to predict NEDA after 2 years with high accuracy (AUC = 0.84, p = 9.6 \times 10^{-5}; Figure 4C).

**Module Proteins Can Distinguish Low and High Responders to Treatment**

Another aspect of personalized treatment is the possibility of predicting response to treatment. We therefore tested, in a third cohort of patients, whether our combined score in CSF (Table S5) could also be used to distinguish subsequent high (n = 6) and low (n = 9) responders to natalizumab, a commonly used drug for MS, which targets \( \alpha 4 \)-integrin and blocks entry of lymphocytes into the CNS (Mellergård et al., 2013). Since natalizumab is one of the most effective MS drugs in use (Tramacere et al., 2015), the low responders constitute a minor group (Gustafsson et al., 2014; Mellergård et al., 2010) that was defined by having one (n = 7) or two (n = 1) relapses during the first year of treatment or having two relapses during 3 years of treatment (n = 1). Although choosing this treatment is based on several considerations, we reasoned that this cohort (Gustafsson et al., 2014; Mellergård et al., 2010), while very small, could indicate the potential for predicting responses to treatment. For these samples we lacked CXCL1-3 values so we re-trained our original model without this protein on the 41 patients with early MS. Remarkably, we found that the combined score obtained in CSF before start of treatment was able to separate high and low responders with high accuracy (AUC = 0.89, p = 0.012; Figure 4D). AUC values for both the second cohort (disease activity) and the third cohort (response to treatment) were not affected by the difference in group size, which we tested by performing 1 million random balanced subsets. In contrast to CSF, the combined protein score in plasma (Table S5) did not distinguish any of the tested clinical groups (disease versus non-disease in the primary and the second cohort, NEDA versus non-NEDA in the second cohort, or high versus low response to treatment in the third cohort).

**The Module Proteins Can Classify Patients and Controls in Other Autoimmune Diseases, but They Cannot Predict Disease Activity**

Lastly, we asked if the ability of the four proteins to predict the presence of disease and disease activity was limited to MS or if it was relevant in other autoimmune diseases involving CD4+ T cells. To test this we measured the four proteins in serum from patients diagnosed with early RA (n = 40) and systemic lupus erythematosus (SLE, n = 43), as well as a new cohort of healthy donors (n = 40). Similarly to the previous analyses in the MS cohorts, we performed logistic regression and assessed the predicted probabilities with cross-validation (combined scores are shown in Table S6). Interestingly, we found the same relationship among the proteins as for the MS patients (positive for CXCL1-3, CXCL10, and OPN, but negative for CCL2) and a significant separation for both RA (AUC = 0.89, \( p = 1.6 \times 10^{-5} \)) and SLE (AUC = 0.77, \( p = 2.0 \times 10^{-5} \)) with respect to controls. However, when we tested the ability of the combined proteins score to predict disease activity (defined as DAS28 in RA and mSLEDAI in SLE; Table S5) at 2-year follow-up, we found no significant correlation between the combined protein score and DAS28 at 2-year follow-up (Spearman correlation = –0.27, \( p = 0.13 \)) for RA patients, nor between mSLEDAI scores at 2-year follow-up and combined protein score (Spearman correlation = 0.006, \( p = 0.97 \)) in SLE patients. Furthermore, there was no correlation between the combined protein score and disease activity at inclusion in any of the two cohorts (data not shown). In summary, this analysis suggests the general relevance of using the identified combined biomarker score for autoimmune diseases, but shows that the correlation to disease activity seems to be limited to MS.

**DISCUSSION**

In the present study we demonstrated an aberrant response to activation in CD4+ T cells from MS patients and found that a set of differentially activated proteins can act as biomarkers in MS. This was achieved by transcriptomic profiling of the dynamic response to activation in CD4+ T cells, which is in contrast to previous studies based on transcriptomic profiling of unstimulated cells. Using a combined protein score based on CSF levels, we were able, in two MS cohorts, to (1) classify patients and controls, (2) predict evidence or no evidence of disease activity after 2 years, and (3) classify low and high responders to treatment. Our results confirm that activation of CD4+ T cells is a central process in MS pathogenesis, as suggested by previous GWAS findings (Sawcer et al., 2011). Our findings also suggest that potential biomarkers might be identifiable for personalized treatment, which is highly needed given variable disease course and responses to given treatments for MS.

In order to validate our approach, we confirmed that each step increased the concordance with GWAS data, thereby performing an a posteriori validation using previous evidence supporting MS relevance. Indeed, MS-GWAS genes, in contrast to GWAS genes from two other autoimmune diseases, RA and T1D, were highly enriched in the DR gene set. Next, based on colocalization of the DR genes, we took a network-based approach and identified functionally interconnected genes in a disease module that was again highly enriched with MS genes. Our finding that chemotaxis forms a prominent submodule was not suggested by previous GWAS analyses, but could potentially be regarded as an event downstream of cell activation. The role of chemokines in MS is supported by several gene expression profiling studies of T cells (Corvol et al., 2008; Mayne et al., 2004; Satoh et al., 2006). In addition, chemotaxis is a prerequisite for entry of CD4+ T cells to the CNS. From a clinical perspective, the dysregulated chemokine response can be utilized for biomarker identification since chemokines are often...
measurable in plasma and CSF, in general at higher concentrations than cytokines (Mellergård et al., 2010).

The general TCR-mediated stimulation, affecting all CD4+ T cells, was chosen because myelin-specific T cells are scarce in circulation (Lünnemann et al., 2004). Furthermore, the most relevant specificities of myelin-specific CD4+ T cells may still be unknown, although recent progress may address this issue (Ayoglu et al., 2016). We used both naive and memory CD4+ T cells, since the memory fraction may contain disease-associated memory cells and the naive fraction facilitates the finding of activation-induced dysregulation. In addition, we and others (Okuda et al., 2005) found no difference between patients and controls in the distribution of naïve and memory cells.

Of the six proteins selected for biomarker studies, two (sTNFR-1 and NT-3) were not secreted by CD4+ T cells in vitro, probably because the model was designed for detection of gene expression and not protein secretion and levels may have been below the detection limit. However, four proteins could be measured as secreted products from CD4+ T cells, and when combined, their secreted levels could be used to distinguish patients from controls. Notably, three of the proteins were chemokines. CXCL1 is downstream of Th17 and involved in recruitment of granulocytes, a process that was recently highlighted in experimental autoimmune encephalomyelitis (EAE) and MS, including a potential role as a biomarker (Rumble et al., 2015). CXCL10 is induced by IFN-γ and involved in the recruitment of CXCR3+ Th1 cells. Although pertinent to Th1-associated mechanisms, as well as findings of increased CSF levels (Mellergård et al., 2010; Sørensen et al., 2002), the role of CXCL10 as a biomarker in MS has not been fully explored (Vázquez-Astudillo et al., 2014). CCL2 contributed to the classifier, but was associated with lower levels in patients compared with controls, in patients with disease activity and in patients not responding to natalizumab treatment. The levels of CCL2 are in line with other reports in MS (Mahad et al., 2006; Sørensen et al., 1999) and have a suggested beneficial role in neuroinflammation (Kwon et al., 2015). Furthermore, a beneficial role of CCL2 is supported by its role in (1) the recruitment and activation of Th2 cells (Ip et al., 2006), (2) M2-associated fetal tolerance in pregnancy (Gustafsson et al., 2008; Svensson et al., 2011), and (3) reduced macrophage production of proinflammatory cytokines (Sierra-Filardi et al., 2014). The fourth protein, OPN (SPP1), is a well-known driver of Th1-and Th17-responses, which is associated with MS through increased levels in both CSF (Börnsen et al., 2011) and plasma (Shimizu et al., 2013).

A key finding is that a combined protein score of CXCL1-3, CXCL10, CCL2, and OPN, measured in CSF, was able not only to discriminate early MS from controls in a new cohort but also to predict evidence of disease activity after 2 years, a most valuable finding when deciding treatment strategies. Other proteins in CSF, such as CXCL13, have been suggested as prognostic tools to predict disease activity (Khademi et al., 2011), but show lower AUC (0.64) when identifying patients with clinically isolated syndrome (CIS) developing definite MS within 2 years (Brettaschneider et al., 2010). Of note, the combined score of proteins being able to predict disease activity was also able to predict low or high response to treatment, which suggests that proteins relevant to the disease process are also important for the response to treatment, further strengthening the potential use of the combined score in personalized treatment. It should also be noted that the vast majority of MS patients do respond to natalizumab and that other considerations, such as JC-virus status, decide treatment preference. Combinations of biomarkers to predict response to treatment have been evaluated previously, for example the ability of certain gene triplets predicted response to interferon beta in MS patients with an AUC ranging between 0.63 and 0.80 (Baranzini et al., 2015). Nevertheless, the present results clearly show a potential of the combined disease score in predicting response to treatment. While all clear biomarker results were based on protein levels in CSF, there were no such findings for plasma levels of proteins. Returning to the dysregulated dynamic response in CD4+ T cells, it is logical that CSF displays an altered protein profile, since activated disease-associated T cells travel to and are enriched in the CNS/CSF compartment, thus representing the dynamic response in vivo. It would have been of interest to study the dynamic response in CD4+ T cells from both our response to treatment cohort and early MS cohort, but this lies beyond the scope of the current study. There has been an extensive search for biomarkers in MS (Comabella and Montalban, 2014; Raphael et al., 2015), although they were mainly tested to detect the presence of disease or ongoing disease activity rather than to predict disease activity (Raphael et al., 2015). Our combination of proteins performed well in relation to previous studies of biomarkers, showing a unique potential to predict both disease activity and response to treatment. Although we tested performance in new independent cohorts, it is important to conduct further testing in prospective studies before clinical implementation.

One potential limitation of the protein module is that we selected proteins that could be measured by commercial products. Thus, other gene products in the module could further contribute to the performance as a biomarker. Indeed, our study clearly shows the principle that aberrations in the dynamic response can be used to identify relevant pathways and biomarkers. The identified biomarkers showed some general relevance by distinguishing other autoimmune disease (RA and SLE) from controls, while disease activity was not reflected. Thus, the general principle of aberrant dynamic response should first be specifically applied to each disease. It should be emphasized that although our combination of four proteins showed excellent performance, it cannot be excluded that other biomarkers, in particular reflecting the neurodegenerative component of the disease, might add to the biomarker potential (Modvig et al., 2015).

In summary, we showed a dysregulated dynamic gene response in the activation process of CD4+ T cells in MS. The dysregulated genes formed an interconnected gene module with cell activation and chemotaxis as central components. From this gene module, we could identify a set of proteins that can be measured in CSF, and by using the combined information from this protein set, we were able to classify patients from controls, predict disease activity after 2 years, and distinguish low and high responders to treatment. We conclude that our study shows a useful principle for detecting aberrant dynamic responses to cell activation. The combined score of proteins holds promise as a clinically useful biomarker in personalized MS.
treatment, and the proposed translational strategy is generally applicable and could be used to identify biomarkers for other diseases in which CD4+ T cells play an active role.

**EXPERIMENTAL PROCEDURES**

**Patients and Controls**

CD4+ T cells were isolated from 16 women diagnosed with definite relapsing-remitting MS according to the McDonald criteria (Polman et al., 2011). None of the patients had experienced a relapse within 3 months prior to blood sampling or had received immunomodulatory or immunosuppressive treatment for at least 2 months (one exception was intravenous immunoglobulin treatment given 15 days before treatment in one patient). 16 age-matched, healthy control women were recruited among blood donors (Table 1). To validate the clinical classifying capacity at the protein level, plasma and CSF from a second cohort of 41 patients with early MS was used (Table 1). These patients were followed for 2 years and classified into NEDA (Rotstein et al., 2015) or not. CSF was also collected from 11 matched healthy control subjects (Table 1). A third cohort was used to evaluate if biomarkers were able to predict response to natalizumab; pre-treatment CSF samples were selected from nine patients that did not fully respond to treatment. The high responders (n = 6) were matched according to age and disability (Table 1). This study cohort has been described elsewhere (Gustafsson et al., 2014; Meiergärd et al., 2010, 2013). To investigate the specificity of the findings to MS, patients diagnosed with two other autoimmune diseases (RA and SLE) were also included. 43 cases classified according to the 1982 American College of Rheumatology classification criteria (Tan et al., 1982) and the 2012 SLICC criteria (Petri et al., 2012), were selected from the cohort Clinical Lupus Register in Northernmost Gothia (KLURING), previously described in detail (Ighe et al., 2012), and were included and followed for 2 years. Disease activity was recorded using the SLE Disease Activity Index 2000 (SLEDAI-2K), which was also modified (mSLEDAI) by the exclusion of laboratory items for hypocomplementemia and anti-dsDNA antibody binding (Gladsøm et al., 2002). The RA cohort consisted of 40 patients with early RA (first joint swelling < 12 months ago), fulfilling the 1987 American College of Rheumatology classification criteria for RA (Amett et al., 1988), who were recruited to “the Swedish TIRA project” 2006–2009 (Švárd et al., 2015). Serum samples were drawn at the inclusion visit (prior to institution of disease-modifying anti-rheumatic drugs) and stored at −70°C. 29 (73%) tested positive for agglutinating rheumatoid factor, and 31 (78%) had antibodies against cyclic citrullinated peptides (anti-CCP2). Disease activity was assessed according to the original “28-joint disease activity score” (DAS28) (Prevoo et al., 1995). At inclusion, DAS28 was available from 39 of the 40 cases. At the 2-year follow-up, DAS28-scores were reported in 31 cases. A cohort of 40 healthy blood donors was also included as control. All patients and controls were recruited at the Linköping University Hospital, and the studies were approved by the regional ethics committee in Linköping, Sweden. See Supplemental Experimental Procedures for further details.

**Sample Preparation**

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density centrifugation (Lymphoprep, Axis-Shield), placed in a freezing container at −70°C overnight, and subsequently transferred and stored in liquid nitrogen until use. After thawing, CD4+ T cells were isolated by immuno-magnetic positive selection according to the instructions provided by the manufacturer (Miltenyi Biotec). The purity of the isolated CD4+ T cells was > 96%, based on flow cytometry (Figure S1). See Supplemental Experimental Procedures for details.

**Cell Culture**

Isolated CD4+ T cells were stimulated with 0.03 μg/mL anti-CD3 and anti-CD28 antibodies or cultured unstimulated at a density of 1 million cells/ml for 24 h at 37°C. Culture supernatants were collected and the cells were used for flow cytometry or homogenized and lysed in buffer RLT Plus (QIAGEN) and stored at −70°C. See Supplemental Experimental Procedures for details.

**Flow Cytometry Staining and Analysis**

CD4+ T cells were labeled with mouse anti-human CD4, CD45RA, CCR7/CD197, and CD69 (all from BD Biosciences) prior to flow cytometry analysis. To assess the viability, CD4+ T cells were stained with Annexin V and 7AAD (BD Biosciences). Patients and controls responded similarly to anti-CD3/CD28 stimulation, based on CD69 expression (Figure S1). Further, the proportion of naive (CCR7+CD45RA+) and memory (CD45RA−) CD4+ T cells did not differ between patients and controls (Figure S1). See Supplemental Experimental Procedures for details.

**RNA Extraction and Microarray Analysis**

Total RNA from CD4+ T cells was extracted using the Allprep DNA/RNA Micro kit (QIAGEN), amplified and labeled using the Low Input Quick Amp Labeling Kit (Agilent Technologies), and purified with RNeasy Mini Kit (QIAGEN) before hybridization onto Agilent Sureprint G3 Human Gene Expression 8x60k arrays (Agilent Technologies) using the Gene Expression Hybridization Kit (Agilent Technologies). The arrays were scanned on an Agilent Microarray Scanner (Agilent Technologies). Data extraction and quality control fulfillment was performed with the Feature Extraction software v10.7.3.1 (Agilent Technologies). The accession number for the datasets reported in this paper is GEO: GSE78244. See Supplemental Experimental Procedures for details.

**Module Identification**

The module was constructed using a modified, more robust and CD4+ cell-specific version of a previously described method (Barrenäs et al., 2012; Gustafsson et al., 2014), by identifying a disease module from the median over an ensemble of modules. We used the human PPI database STRING (v.10 [Szklarczyk et al., 2015]) and considered the interactions with confidence scores (p s) above 0.7 between all included 14,543 proteins of the database. We calculated an interaction weight w ij by the geometric mean of p C and (1 − p C), where p C is the double-sided Pearson correlation p value between gene i and gene j expressions in all samples. We then repeated the following steps 100 times. (1) Realize a perturbed network by drawing a random matrix r with matrix elements uniformly in [0,1] and let each interaction be included in the network if w > r ij. Then, preceding with steps similar as in Barrenäs et al. (2012) to (2) identify all maximal cliques of the network with size 3 or more, (3) calculate enrichment of each cliques for differentially expressed genes (unedited limma test p < 0.05) by Fisher’s exact test for each clique. (4) Select all cliques with p < 0.01 as differentially expressed, and (5) extract the set of genes and interactions present in these differentially expressed cliques as one perturbed module. From the 100 modules we inferred the robust module as the genes present in more than half of the runs and removed the genes with less than twice as many interactions as a randomization module created with shuffled input data. Lastly, we removed the singleton from the disease module, i.e. genes with no connections.

**Analysis of Cytokines and Chemokines**

Quantification of NT-3 was performed using DuoSet ELISA reagents from R&D Systems (DY267), as described elsewhere (Boij et al., 2012). Multiplex Bead Technology (MILLIPLEX MAP Kit, Catalog No. HCYTOMAG-60K-11, HSCRMAG-32K, and HBNMAG-51K, Millipore) was used to measure all other cytokines and chemokines in the supernatant and CSF samples according to the manufacturer’s description. See Supplemental Experimental Procedures for details.

**Classification of Patients and Controls**

To investigate if the measured mRNA and proteins, selected from the module, could be used to separate patients from controls in culture supernatants, we used the following combined score: (OPNStim − OPNUnstim) + CXCL1−3Stim − CXCL1−3Unstim) + (CXCL10Stim − CXCL10Unstim) − (CCL2Stim − CCL2Unstim). Area under ROC curve (AUC) value was calculated using the perfcurve MATLAB function; p value was calculated using double-sided Wilcoxon test. Bootstrap p values were calculated by performing 1,000 random selections of 3 positive and 1 negative proteins among a set of 8 control proteins (GM-CSF, IFN-γ, IL-10, IL-13, IL-17, CCL17, M-CSF, and CCL20). The p values were estimated from the bootstrap distribution of AUC values obtained for the random classifier. For the CSF we had no baseline to subtract.
and instead for each of the subjects we performed a leave-one-out regression score by performing logistic regression of patient probability estimation without that subject in the training. For the high and low responders to natalizumab treatment, we lacked CXCL1-3 values and therefore retrained the logistic regression on the original 41 patients without this protein. Moreover, as three subjects also lacked CXCL10 values, we imputed their values using knnimpute MATLAB function with default settings.

Statistical Analysis
Gene expression microarray data were quantile normalized, and p values for differential expression were calculated using t test from the LIMMA package in R. The p values for protein measurements were calculated using Mann-Whitney test. For set enrichment analysis of GWAS genes, p values were calculated using one-sided Fisher’s exact test, using all genes measured on the gene expression array with at least one assayed SNP mapped as closest as background (n = 16,799). Analysis of correlation between disease activity and combined proteins score in SLE and RA patients was performed using Spearman rank correlation.

ACCESSION NUMBERS
The accession number for the datasets reported in this paper is GEO: GSE78244.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, two figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.08.036.

AUTHOR CONTRIBUTIONS
M.G., D.R.G., and M.K. conceived and performed bioinformatics analyses; S.H., D.E., M.V., I.H., T.S., A.K. and C.S. collected and analyzed clinical materials; D.E., S.H., D.R.G., M.K., and C.E.N. performed and analyzed microarray experiments; D.E. and S.H. performed cell culture experiments, flow cytometry, and protein analyses; M.C.J., M.B., I.K., and T.O. contributed to the study design and bioinformatics analyses; and M.G. and J.E. jointly supervised the study and should be regarded as shared senior authors.

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