Clinical relevance and functional consequences of the *TNFRSF1A* multiple sclerosis locus

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ABSTRACT

Objective: We set out to characterize the clinical impact and functional consequences of rs1800693^G, the multiple sclerosis (MS) susceptibility allele found in the *TNFRSF1A* locus.

Methods: We analyzed prospectively collected data on patients with MS to assess the role of the *TNFRSF1A* locus on disease course and treatment response. Using archival serum samples and freshly isolated monocytes from patients with MS and healthy subjects, we evaluated the effects of rs1800693^G and a second risk allele, R92Q, on immune function.

Results: In 772 patients with MS, we see no evidence that rs1800693^G strongly influences clinical or radiographic indices of disease course and treatment response; thus, rs1800693^G appears to be primarily involved in the onset of MS. At the molecular level, this validated susceptibility allele generates an RNA isoform, *TNFRSF1A* Δ6, that lacks the transmembrane and cytoplasmic domains. While there was no measurable effect on serum levels of soluble TNFRSF1A, rs1800693^G appears to alter the state of monocytes, which demonstrate a more robust transcriptional response of *CXCL10* and other genes in response to tumor necrosis factor (TNF)-α. We also report that activation of the TNF-α pathway results in altered expression of 6 other MS susceptibility genes, including T-cell activation rho GTPase activating protein (*TAGAP*) and regulator of G-protein signaling 1 (*RGS1*), which are not previously known to be responsive to TNF-α.

Conclusions: The MS rs1800693^G susceptibility allele affects the magnitude of monocyte responses to TNF-α stimulation, and the TNF pathway may be one network in which the effect of multiple MS genes becomes integrated. *Neurology*® 2013;81:1891-1899

GLOSSARY

AA = amino acids; **BWH** = Brigham and Women's Hospital; **CI** = confidence interval; **GA** = glatiramer acetate; **IFN-β** = interferon-β; **MAPK** = mitogen-activated protein kinase; **MS** = multiple sclerosis; **MSSS** = Multiple Sclerosis Severity Scale; **NF** = nuclear factor; **PBMC** = peripheral blood mononuclear cell; **sTNFRSF1A** = soluble TNFRSF1A; **TNF** = tumor necrosis factor; **TRAPS** = TNF receptor-associated periodic syndrome.

The tumor necrosis factor (TNF) pathway has long been implicated in inflammatory diseases, and anti-TNF-α agents have proven to be successful in the treatment of several of these diseases.¹ However, multiple sclerosis (MS) responds differently: treatment with anti-TNF-α therapies results in new episodes of inflammation.² Inflammatory demyelination is also seen in TNF receptor-associated periodic syndrome (TRAPS), which is due primarily to rare mutations in *TNFRSF1A*.^{3,4} These observations suggest that perturbations of TNF-α signaling may be important in triggering inflammatory events in MS.

The *TNFRSF1A* variant rs1800693 is associated with MS susceptibility,⁵ and this finding has been well replicated.⁶⁻⁸ Recent reports^{9,10} describe some of the functional consequences associated with the risk allele, including its production of a novel isoform of TNFRSF1A. However, little is known about the role of this variant in MS disease course and treatment response as well as the mechanisms by which the rs1800693 variant influences MS susceptibility.

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TNFRSF1A engagement by its cognate ligand TNF- α leads to activation of different signaling pathways, such as the nuclear factor (NF)- κ B or mitogen-activated protein kinase (MAPK) pathways, and the regulation of interferon-regulatory factors in myeloid cells.¹¹ There is evidence for a signaling loop between TNF- α and type I interferon genes,^{11,12} which links 2 pathways associated with MS susceptibility.^{5,7} Here, we explore in greater detail the role of the TNF- α pathway in MS susceptibility by investigating 1) the functional and clinical consequences of the rs1800693 variant and another MS-associated variant, rs4149584 (R92Q), on TNFRSF1A function and 2) the effect of the TNF- α pathway on other MS susceptibility genes.

METHODS Detailed descriptions of our experimental methods are presented in e-Methods on the *Neurology*[®] Web site at www.neurology.org. In brief, samples from subjects with MS and healthy control subjects were obtained from the Comprehensive Longitudinal Investigation of Multiple Sclerosis at Brigham and Women's Hospital (CLIMB) study and the Brigham and Women's Hospital (BWH) PhenoGenetic Project.^{13,14} Genotypes were available from a prior study⁵ or obtained using the Sequenom Mass Array platform (Sequenom, San Diego, CA). We used commercially available and custom-designed Taqman assays (Applied Biosystems, Grand Island, NY) to quantify *TNFRSF1A* RNA isoforms. Sandwich ELISA was used to measure soluble TNFRSF1A in serum samples. Flow cytometry was used to measure surface expression of TNFRSF1A. Our in vitro model system was based on the culture of peripheral blood monocytes purified using Miltenyi isolation beads (Miltenyi Biotec, Auburn, CA) from selected healthy individuals. We stimulated these cells in culture with TNF- α for 48 hours to measure secreted CXCL10 by ELISA and gene expression using a custom Nanostring nCounter codeset (NanoString Technologies, Seattle, WA).

Standard protocol approvals, registrations, and patient consents. All subjects were consented using protocols approved by the Institutional Review Board of Partners Healthcare.

RESULTS **Assessing the role of the *TNFRSF1A* locus in disease course.** Since blocking TNF- α is associated with MS relapses,² we assessed whether rs1800693^G has an effect on MS clinical and radiographic outcomes in up to 772 subjects from the Partners MS Center in Boston (table e-1). We see no effect of this variant on the following: 1) the Multiple Sclerosis Severity Scale (MSSS), a measure of clinical disability adjusted for disease duration ($n = 692$; β [95% confidence interval (CI)] = 0.012 [−0.261, 0.285]; $p = 0.93$), which is consistent with results in a recent genome-wide study of MSSS^{15,16}; 2) age at symptom onset ($n = 771$; β [95% CI] = 0.525 [−0.465, 1.515]; $p = 0.30$); 3) T2 hyperintense lesion volume in brain MRI ($n = 668$; β [95% CI] = −0.023 [−0.104, 0.059];

$p = 0.59$); 4) brain parenchymal fraction, a measure of brain volume ($n = 669$; β [95% CI] = −0.001 [−0.0054, 0.0034]; $p = 0.74$); or 5) time to an inflammatory event after starting first-line disease-modifying treatment agents with either glatiramer acetate (GA) or an interferon- β (IFN- β) therapy ($n = 448$; hazard ratio [95% CI] = 1.04 [0.88–1.24], Cox proportion hazard test $p = 0.64$). Treatment-specific analyses using proportional hazard models did not show any association of genotype with time to a new demyelinating event (data not shown). With these sample sizes, we can exclude the possibility that rs1800693 has a strong effect on these clinical measures: given our sample size and an $\alpha = 0.05$, we have >96% power in excluding the possibility that rs1800693 explains 2% or more of the variance in these traits. Thus, while rs1800693 has a validated effect on MS susceptibility, it does not appear to have a strong effect on available outcomes of disease course in MS or of response to first-line disease-modifying therapy, after adjusting for sex, age at symptom onset, and disease duration.

The rs1800693 variant alters splicing of *TNFRSF1A*. rs1800693 is located within a splice acceptor site in intron 6 of *TNFRSF1A* (figure 1A). Using a custom TaqMan assay, we found that the rs1800693^G risk allele generates a novel isoform of *TNFRSF1A* that lacks exon 6, TNFRSF1A $\Delta 6$, which is consistent with a recent report (figure 1B).⁹ In RNA from peripheral blood mononuclear cells (PBMCs) of healthy control subjects, we see a dose-dependent increase of TNFRSF1A $\Delta 6$ RNA but not full-length TNFRSF1A with each rs1800693^G allele (figure 1, C and D). In these healthy PBMC samples, the *TNFRSF1A* $\Delta 6$ isoform represents a mean of 27.9% of the total quantity of transcript in the 20 rs1800693^{GG} homozygotes and 17.9% in the 17 rs1800693^{GA} heterozygotes that were tested.

In vitro expression of TNFRSF1A $\Delta 6$. We have cloned and sequenced both the full-length and $\Delta 6$ isoforms and confirmed the predicted sequence created by joining exons 5 and 7 in the $\Delta 6$ isoform (data not shown). The protein translated from the TNFRSF1A $\Delta 6$ construct is predicted to consist of the 183 N-terminal amino acids (AA) and 45 new AA introduced by the frameshift in the $\Delta 6$ mRNA; this protein isoform therefore lacks the transmembrane and the cytoplasmic domains that are encoded by exon 7 and distal exons. In HEK293T cells transfected with the full-length TNFRSF1A isoform, we detect high levels of TNFRSF1A expression both on the cell surface and intracellularly (figure 1E). However, we detect only intracellular TNFRSF1A after transfection with the $\Delta 6$ isoform (figure 1E). The $\Delta 6$ isoform is also detected in the culture supernatant of transfected cells, suggesting that it may be secreted (figure e-1). This is consistent with a prior report.⁹ Other studies

Figure 1 The rs1800693^a risk allele creates a new *TNFRSF1A* RNA isoform

(A) Conservation plot of single nucleotide polymorphism rs1800693 in the *TNFRSF1A* gene. The position of the variant is outlined in the black box and is 10 base pairs from the intron/exon boundary at the 3' end of exon 6. The sequence of the chromosomal segment of interest is presented from the reference human sequence as well as from 7 vertebrate species to illustrate the sequence conservation at this single nucleotide polymorphism. Sequences were obtained from the UCSC Genome Browser, hg19, February 2009 Assembly. (B) Gel representation of PCR products using the primer design described in Methods. RNA was extracted from peripheral blood mononuclear cells (PBMC) of genotyped healthy donors (PhenoGenetic project), $n = 5$ for each genotypic category GG, GA, and AA. Synthesized cDNA was used for amplification of transcribed RNA species from ex vivo unstimulated PBMC. The product of lower molecular weight (109 bp) corresponds to the one predicted by alternative splicing (*TNFRSF1A* Δ6), while the product of higher molecular weight (183 bp) is consistent with the amplified full-length variant (*TNFRSF1A*). Density quantification of the bands is reported in the upper panel. Independent real-time quantitative PCR assays for the full-length (C) and the Δ6 (D) isoforms were performed and expression is reported relative to the housekeeping reference gene $\beta 2M$. Values shown are the average of duplicate determinants. The mean value in each genotype category is illustrated using a black line. GG $n = 20$; GA $n = 17$; AA $n = 23$. (E) Cytometric characterization of HEK293T cells transfected with the full-length and Δ6 *TNFRSF1A* cDNA cells stained on (left) the cell surface or (right) intracellularly for an extracellular epitope of *TNFRSF1A*. Each graph represents a histogram illustrating the distribution of cells based on staining with the listed monoclonal antibody; the x-axis is the geometric mean fluorescence intensity for the tested antibody. A key is presented to the right of the figure summarizing the 5 experimental conditions that are tested. In short, the black filled profile corresponds to nontransfected HEK293T cells stained with the isotype control monoclonal antibody (mAb); the dark gray profile corresponds to nontransfected HEK293T cells stained with the anti-*TNFRSF1A* mAb; and the light gray profile, blue profile, and black solid line profile report the distribution of HEK293T cells transfected with, respectively, an empty vector, full-length (FL) *TNFRSF1A*, and *TNFRSF1A* Δ6 that are stained with the anti-*TNFRSF1A* mAb.

have shown that deletions of certain domains of *TNFRSF1A* could result in altered subcellular distribution of the protein or in alteration of intracellular activation.^{17–19}

Ex vivo expression level of soluble and transmembrane *TNFRSF1A*. Using sera from a cohort of healthy control subjects ($n = 47$) and subjects with MS ($n = 215$) (table e-2), we find evidence that circulating levels of

soluble *TNFRSF1A* (s*TNFRSF1A*) are reduced, relative to subjects with untreated MS, only in subjects treated with GA ($p = 0.01$) (figure 2A). We went on to test the genotypic association relative to rs1800693 genotype in both healthy subjects ($n = 326$) and subjects with MS ($n = 189$) (table e-2). In the collection of healthy subjects, we see no correlation after accounting for age and sex ($p = 0.44$, figure 2B). The same is true in our analysis of all subjects with MS (untreated,

Figure 2 Soluble TNFRSF1A level in healthy control and multiple sclerosis serum samples

(A) Distribution of the level of soluble TNFRSF1A (sTNFRSF1A) comparing healthy control (HC) subjects ($n = 47$), untreated (UNTR) individuals with multiple sclerosis (MS) ($n = 42$), and subjects with MS upon treatment with glatiramer acetate (GA) ($n = 74$) or interferon- β (IFN β) ($n = 99$). The mean value is highlighted with a horizontal red bar. The result of the significant pairwise comparison (adjusting for batch, age, and sex) is presented. Overall analysis of variance p value = 0.04. (B, C) The distribution of sTNFRSF1A levels measured in (B) HC subjects ($n = 326$) and (C) subjects with MS ($n = 189$) is presented, partitioned by genotype category. Differences among groups were tested using a linear model assuming an additive effect of the rs1800693^G risk allele and adjusting for batch effect, age at serum sampling, and sex. The HC individuals consist of 51 GG, 158 GA, and 117 AA subjects. The subjects with MS consist of 40 GG, 93 GA, and 56 AA subjects. Demographic details for the 2 sets of subjects are presented in table e-2. Reported data correspond to values normalized across batches. A red straight line illustrates the mean.

GA-treated, or IFN- β -treated), accounting for age, sex, and treatment effect ($p = 0.80$, figure 2C). Thus, the level of sTNFRSF1A measured using commercially available antibodies is not significantly altered by the rs1800693^G risk allele. At this time, the antibody specific for the $\Delta 6$ isoform is not available, so we cannot measure that isoform separately and address whether it is found in serum samples, as previously reported.⁹

In parallel, we assessed whether the rs1800693 variant modulates TNFRSF1A surface expression by measuring receptor surface staining in monocytes from healthy subjects selected by genotype (table e-3). We see no difference in surface expression of TNFRSF1A in total CD14⁺ monocytes (figure e-2, A and C) or in the proinflammatory subset of CD14⁺CD16⁺ monocytes (figure e-2, B and D), confirming a previous report.⁹

Evaluating the effect of the R92Q *TNFRSF1A* variant.

In addition to rs1800693, rs4149584 (a coding *TNFRSF1A* variant) has also been associated with MS susceptibility: it is an arginine-to-glutamine substitution at position 92 (R92Q) in exon 4. While less frequent (0.5%–4%) in populations of European descent, the risk-associated rs4149584^T (92Q) allele has a stronger effect on MS susceptibility^{5,20,21} and is also associated with TRAPS (<http://fmf.igh.cnrs.fr/ISSAID/infevers/search.php?n=2>). rs4149584 and rs1800693 were genotyped in 3 collections of individuals with European ancestry: healthy control subjects from the BWH PhenoGenetic Project collection ($n = 1,078$), as well as healthy control subjects ($n = 397$) and subjects with MS ($n = 1,708$) in the BWH collection (table e-4). In these subjects, the 2 variants

are in complete linkage disequilibrium: the rs4149584^T (92Q) risk allele only exists on a haplotype that also contains the rs1800693^G risk allele (frequency 1.4%, figure e-3A). This intriguing observation prevents us from testing whether rs4149584^T and rs1800693^G interact statistically.

The 92Q TNFRSF1A allele is reported to have the same ligand binding and trafficking properties as the common R92 allele.¹⁸ However, several TRAPS variants affect TNFRSF1A shedding from the cell surface.^{22,23} To test shedding, we purified monocytes from healthy subjects of each of 3 classes: 11 92Q subjects (7 rs1800693^{GG} and 4 rs1800693^{GA}), 25 rs1800693^{GG}/R92 subjects, and 29 rs1800693^{AA}/R92 subjects (table e-5). There is no significant difference in the level of TNFRSF1A expression on the cell surface at baseline among the 3 groups of subjects ($p = 0.39$, analysis of variance, Kruskal-Wallis test) (figure e-3B). We then stimulated receptor shedding using 5 different concentrations of phorbol-12-myristate-13-acetate (PMA), to activate the ADAM17 protease that releases sTNFRSF1A from the cell surface.^{24,25} As seen in figure e-3C, there is no difference in the shedding of TNFRSF1A among the 3 genotypic groups ($p = 0.68$). This is consistent with our serum data: 8 of the healthy control subjects have the 92Q allele, and we see no difference in their mean soluble TNFRSF1A level when compared to 317 subjects with the R92 allele ($p = 0.13$).

Effect of TNF- α stimulation on the expression profile of monocytes. We then assessed whether the rs1800693 variant modulates the state of cells that express TNFRSF1A, hypothesizing that the intracellular accumulation of the $\Delta 6$ isoform could lead to altered TNFRSF1A activation (as previously seen with other truncated and mutated constructs^{18,19}). We focused on peripheral blood monocytes that have a high baseline of TNFRSF1A expression, and measured 3 complementary outcomes: 1) the secretion of CXCL10, a key TNF- α -responsive chemokine¹¹; 2) the expression of genes previously described as being induced by TNF- α or type 1 interferon^{11,12}; and 3) the expression of genes found within validated MS susceptibility loci (table e-6).

In purified monocytes stimulated with TNF- α , we find a significantly greater CXCL10 secretion into the culture supernatant of cells from subjects homozygous for the risk allele (rs1800693^{GG}) than in those for the protective allele (rs1800693^{AA}) in a joint analysis of 2 batches of subjects ($p = 0.012$) (figure 3A). We then used the same samples to explore whether this difference in CXCL10 secretion reflects a broad difference in the expression of TNF- α pathway and downstream type I IFN-responsive genes. Specifically, we examined the expression of 33 representative genes (figure 3B). At the RNA level, we see that

CXCL10 RNA expression is also significantly greater in rs1800693^{GG} monocytes following TNF- α stimulation ($p = 8.9 \times 10^{-4}$), as are the expression of *CCL5* ($p = 1.4 \times 10^{-4}$) and *NCF1* ($p = 4.4 \times 10^{-4}$) (Bonferroni threshold of $p < 0.0015$ for significance). In addition, 17 of 21 IFN genes demonstrate suggestive evidence of being more highly expressed in rs1800693^{GG} monocytes ($p < 0.05$). Thus, it appears that subjects bearing the rs1800693^G risk-associated allele may have a more robust IFN response to TNF- α stimulation than subjects homozygous for the protective allele. In secondary analyses, we compared the 10 rs1800693^{GG} subjects who also carry the 92Q variant to 43 individuals with a rs1800693^{GG} R92 genotype and find no difference in gene expression (data not shown).

Examining the RNA expression of MS susceptibility genes, we note a significant upregulation of *CD58*, *CYP27B1*, *IL7R*, and *TAGAP* as well as downregulation of *IRF8* and *RGS1* upon TNF- α stimulation (table 1). These interesting results tie, for the first time, MS susceptibility genes such as *RGS1* and *TAGAP* with the TNF- α pathway. The observations at *CD58*, *CYP27B1*, *IL7R*, and *IRF8* in human monocytes confirm earlier work relating expression of these genes to TNF- α stimulation in different experimental systems.^{26–29} Moreover, assessing the genotypic effect of rs1800693 on the expression of these MS genes, we find a greater number of suggestive effects ($p < 0.05$) following TNF- α stimulation when compared to baseline (table 2). For example, *CD40* gene expression is enhanced following TNF- α stimulation in rs1800693^{GG} subjects relative to rs1800693^{AA} subjects ($p = 0.007$). These results demonstrate that the expression of MS susceptibility genes in monocytes is altered following TNF- α stimulation, and they suggest that the *TNFRSF1A* risk allele may further enhance this effect.

DISCUSSION We explored the functional consequences of 2 different alleles found in the *TNFRSF1A* locus that are associated with MS susceptibility: for MS susceptibility, the odds ratio for the rs1800693^G allele is 1.14⁸ and is 1.58 for the R92Q allele.⁵ Unlike 2 other studies,^{10,19} we have not detected an effect of the 92Q allele in either our ex vivo data or our in vitro models. On the other hand, we did find that the rs1800693^G allele produces a new RNA isoform, *TNFRSF1A* $\Delta 6$, consistent with recent reports.^{9,10} This $\Delta 6$ isoform represents on average 27% of the total TNFRSF1A transcript; nonetheless, it does not significantly alter the surface expression of TNFRSF1A in ex vivo monocytes (figure e-2). It also does not appear to influence the level of sTNFRSF1A seen in the serum of healthy subjects or subjects with MS (figure 2, B and C), consistent with an earlier

Figure 3 The rs1800693^G risk allele effect on monocyte response to tumor necrosis factor- α

(A) CXCL10 protein level was measured by ELISA in supernatant of monocytes purified from peripheral blood mononuclear cells of genotyped healthy control individuals and cultured for 48 hours with or without 10 ng/mL of tumor necrosis factor (TNF)- α (1.5×10^6 /mL). Combined results from the 2 sample sets, batch effect corrected, are depicted. The y-axis reports the difference in CXCL10 secretion between the stimulated and unstimulated samples of each individual. Unlike the rs1800693^{AA} subjects, the rs1800693^{GG} subjects have a substantial number of outliers in terms of response to TNF- α . Our dataset had 57% statistical power to find this effect as well as the effect on RNA expression found in B. (B) Heatmap of the differentially expressed genes (y-axis) that are nominally significant ($p < 0.05$) when comparing rs1800693^{GG} and rs1800693^{AA} healthy subjects (x-axis). The RNA expression data captured from cultured monocytes after 48 hours of culture with TNF- α stimulation were generated in the same experiment that yielded the culture supernatants assayed in A. Red and blue corresponds to high and low standardized relative expression ($[\text{x-row.mean}]/\text{row.standard.deviation}$), GENE-E analytical tool (www.broadinstitute.org/cancer/software/GENE-E/). The heatmap represents the results of the combined datasets after batch correction. Nominal p values of the linear regression analysis for the comparison of GG and AA subjects are reported on the right side of the heatmap. Five of 111 individuals profiled in the experiment carry the 92Q variant and none belong to the 17 individuals with an exaggerated response to TNF- α . R92Q information is not available for 3 of the individuals in these analyses.

report.¹⁰ However, reagents that specifically measure the translated product of the $\Delta 6$ isoform were not available and, therefore, we cannot assess its presence directly at the protein level.⁹ From a clinical point of view, rs1800693 does not have a strong effect on either the course of MS or disease activity, which is consistent with the results of other studies.^{10,15,16}

We explored the hypothesis that some of the effects of rs1800693^G on MS susceptibility may occur through an alteration of a cell's state, which leads to differential response to TNFRSF1A engagement by its cognate ligand TNF- α . Prior studies suggest that alterations of TNFRSF1A sequence can alter baseline cellular function in TRAPS.^{18,19} Further, in mice, the accumulation of mutant TNFRSF1A proteins that have a cytoplasmic domain in the endoplasmic reticulum leads to elevated baselines of c-Jun NH2-terminal kinase (*JNK*) and p38 MAPK phosphorylation and exaggerated transcriptional responses to lipopolysaccharide and TNF- α .¹⁹ Our results provide evidence that, in a subset of individuals, carrying the rs1800693^G allele is associated with increased RNA expression and secretion of the proinflammatory soluble chemokine CXCL10, following TNF- α stimulation (figure 3, A and B). CXCL10 has previously been reported to be expressed in macrophages and astrocytes found in actively demyelinating MS plaques,³⁰ and diminished CXCL10 expression appears to mediate a reduction of monocyte and lymphocyte migration across an artificial blood-brain barrier

Table 1 Multiple sclerosis susceptibility genes whose RNA expression is altered following tumor necrosis factor- α stimulation (each gene meets a false discovery rate <5% in 2 independent sample sets)

	With tumor necrosis factor- α stimulation	Sample set 1, fold change	Sample set 2, fold change
<i>IRF8</i>	↓	2.36	2.23
<i>TAGAP</i>	↓	1.44	2.10
<i>RGS1</i>	↓	1.98	2.68
<i>CD58</i>	↑	2.14	1.36
<i>CYP27B1</i>	↑	11.32	3.40
<i>IL7R</i>	↑	31.01	12.85

The direction of the arrow denotes the change in RNA expression: ↓ decreased expression, ↑ increased expression. The direction is consistent for each gene in the 2 sets of samples.

Table 2 Effect of the rs1800693^G risk allele on the expression of multiple sclerosis susceptibility genes before (t = 0) and after (t = 48 hours) tumor necrosis factor (TNF)- α stimulation

Gene	t = 0		t = 48 h after TNF- α	
	t stat ^a	p Value	t stat ^a	p Value
<i>CD40</i>	1.2380	0.2184	2.7658	0.0067
<i>CYP27B1</i>	1.3181	0.1902	2.3367	0.0213
<i>TAGAP</i>	1.1042	0.2719	2.0874	0.0392
<i>CD58</i>	0.6431	0.5215	2.0689	0.0409
<i>STAT3</i>	0.9307	0.3541	1.8679	0.0645
<i>PTGER4</i>	1.4063	0.1625	1.1227	0.2640
<i>CLEC16A</i>	1.7223	0.0879	1.1191	0.2655
<i>MPHOSPH9</i>	1.2068	0.2301	1.0191	0.3104
<i>IL2RA</i>	1.0191	0.3104	0.8879	0.3766
<i>IL7R</i>	-0.1752	0.8612	-0.6054	0.5462
<i>RGS1</i>	1.4640	0.1461	0.1288	0.8977
<i>IRF8</i>	0.8984	0.3710	0.0246	0.9804

^a t stat = parameter generated from the analysis performed using the MatrixEQTL R package. It provides a measure of the reference allele's effect size that incorporates the ordinary least square estimator (slope) in the additive linear regression model and the standard error of the slope. The sign reflects the orientation of the slope relative to the reference allele, with positive value meaning upregulation and negative value downregulation with increased number of susceptible alleles.

model system.³¹ Thus, enhanced CXCL10 secretion could, in vivo, yield a more robust chemoattractant effect and contribute to an inflammatory demyelinating process.

Moreover, CXCL10 expression is part of the secondary type I IFN response that follows TNF- α stimulation, and our data suggest that this response is more robust in subjects who bear the rs1800693^G allele (figure 3B). Thus, the state of monocytes in these subjects may be different: they appear to be primed to respond more vigorously to TNF- α . We focused our analysis on homozygous subjects and therefore cannot resolve whether the CXCL10 and other transcriptional traits follow a dominant or an additive model. Interestingly, a subset of the rs1800693^{GG} subjects displays large responses to TNF- α (figure 3, A and B), suggesting that other genetic or environmental factors might contribute to the phenotype of exaggerated response. The exact proportion of rs1800693's contribution to this phenotype cannot be ascertained in the current data. Mechanistically, at this time, it is not clear how the formation of the TNFRSF1A Δ 6 molecule and its intracellular presence⁹ contribute to this alteration of the state of monocyte activation. Prior in vitro work delineates altered NF- κ B pathway and other cellular responses in the context of truncated forms of murine TNFRSF1A that, unlike the Δ 6 molecule, have a cytoplasmic domain.¹⁹ Constructs without a cytoplasmic domain did not alter the outcome measures in this

in vitro experiment. We speculate that the Δ 6 molecule could also enhance the activation of the NF- κ B pathway in response to stimuli such as TNF- α , perhaps through a different mechanism. This is a hypothesis that needs to be tested in future work, but this working model might help to explain our observed alterations of *CD40*, *CYP27B1*, and *CD58* transcription in relation to the rs1800693^G allele (table 2) since these genes harbor NF- κ B binding sites in their promoter (<http://www.bu.edu/nf-kb/gene-resources/target-genes/> and reference 26).

Following TNF- α stimulation, we find that 6 genes implicated in MS susceptibility are differentially expressed, suggesting one manner in which these different susceptibility genes could converge on a single pathway to increase the probability of an inflammatory demyelinating process. This hypothesis is also supported by the results of the genotypic analysis: in the context of TNF- α stimulation (but not at baseline), we see suggestive evidence that the presence of *TNFRSF1A* risk allele leads to the greater expression of several other MS susceptibility genes, such as *CD40*. These results, while intriguing, remain to be validated in additional samples, but they begin the process of assembling independent susceptibility alleles into what may ultimately be coherent networks that can have a strong effect on immune responses and trigger an inflammatory process. Several testable hypotheses emerge from these results, which join an emerging literature of altered cytokine signaling (*IL7R*, *IL2R*),^{32,33} altered lymphocyte costimulation (*CD58*, *CD6*),^{34,35} and altered vitamin D response³⁶ that is beginning to map the components of immune dysregulation leading to the onset of MS.

AUTHOR CONTRIBUTIONS

L.O. contributed to the conception, design, and coordination of the study, performed and analyzed all experiments, and drafted the manuscript. B.C.A. and B.T.K. were involved in statistical analysis of the experimental data. I.W. and M.L. were responsible for healthy control blood samples recruitment. T.C., C.R.G., S.J.K., Z.X., and H.L.W. contributed to the design and execution of the CLIMB study that collected the outcome measures on patients with MS. P.L.D. participated in the conception, design, analysis, interpretation, and coordination of the study. D.A.H. participated in the conception and interpretation of the study. All authors contributed to the drafting of the manuscript.

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