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Original research

Genomic and functional evaluation of *TNFSF14* in multiple sclerosis susceptibility

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ABSTRACT

Among multiple sclerosis (MS) susceptibility genes, the strongest non-human leukocyte antigen (HLA) signal in the Italian population maps to the *TNFSF14* gene encoding LIGHT, a glycoprotein involved in dendritic cell (DC) maturation. Through fine-mapping in a large Italian dataset (4,198 patients with MS and 3,903 controls), we show that the *TNFSF14* intronic SNP rs1077667 is the primarily MS-associated variant in the region. Expression quantitative trait locus (eQTL) analysis indicates that the MS risk allele is significantly associated with reduced *TNFSF14* messenger RNA levels in blood cells, which is consistent with the allelic imbalance in RNA-Seq reads ($P < 0.0001$). The MS risk allele is associated with reduced levels of *TNFSF14* gene expression ($P < 0.01$) in blood cells from 84 Italian patients with MS and 80 healthy controls (HCs). Interestingly, patients with MS are lower expressors of *TNFSF14* compared to HC ($P < 0.007$).

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LIGHT
Fine-mapping analysis
SNV

Individuals homozygous for the MS risk allele display an increased percentage of LIGHT-positive peripheral blood myeloid DCs ($CD11c^+$, $P = 0.035$) in 37 HCs, as well as in *in vitro* monocyte-derived DCs from 22 HCs ($P = 0.04$). Our findings suggest that the intronic variant rs1077667 alters the expression of *TNFSF14* in immune cells, which may play a role in MS pathogenesis.

Introduction

Multiple sclerosis (MS, OMIM:#126200) is a common autoimmune disorder of the central nervous system (CNS) characterized by multifactorial etiology and polygenic heritability. Recent genome-wide association studies (GWAS) have succeeded in uncovering multiple genetic variants influencing MS susceptibility. In this regard, a total of 32 human leukocyte antigen (HLA) and 201 non-HLA genetic risk factors for MS have been identified (ANZgene, 2009; Patsopoulos et al., 2011; Sawcer et al., 2011; Beecham et al., 2013; Moutsianas et al., 2015; International Multiple Sclerosis Genetics Consortium, 2019). Although several signals are near genes involved in immunologic processes, the effector mechanisms for most associations remain unknown.

To date, there have been only a few fine-mapping analyses of MS susceptibility GWAS loci aimed to identify the primary causal variant or gene. These efforts are particularly relevant to characterize the molecular mechanism underlying the association signal. For instance, a recent GWAS on the Sardinian population has reported a variant in the *TNFSF13B* locus (BAFF-var), primarily associated with the regulation of BAFF transcription. This variant creates an alternative polyadenylation signal that generates a shorter 3′ UTR transcript lacking the microRNA binding site associated with substantial changes in humoral immunity because of increased levels of soluble BAFF, higher B cell number, and enhanced immunoglobulin production, which is likely responsible for increased risk of developing autoimmune diseases (Steri et al., 2017). In another study, a single-nucleotide polymorphism (SNP) in the tumor necrosis factor receptor superfamily member 1A (*TNFRSF1A*) gene, encoding TNFR1 protein, was shown to hamper tumor necrosis factor signaling through the expression of a truncated soluble form of TNFR1 (Gregory et al., 2012).

The present study was aimed to characterize MS loci through a fine-mapping approach. To this end, we conducted a GWAS in a large cohort of Italian patients with MS and healthy controls (HCs) and identified a known MS locus in the tumor necrosis factor (ligand) superfamily member 14 (*TNFSF14*) region as the strongest MS-associated signal. *TNFSF14* encodes LIGHT (Lymphotoxin/Inducible/Glycoprotein/Herpesvirus/T lymphocytes), a transmembrane glycoprotein expressed by various immune cell types and involved in dendritic cells (DCs) maturation. Accordingly, our aim was to perform a fine-mapping of this locus and to functionally characterize the primarily associated variant. In this study, we provide evidence suggesting that an intronic *TNFSF14* variant may contribute to MS pathogenesis.

Results

Identification of the most associated risk locus for MS in the Italian population

In the framework of a fine-mapping study of MS loci in the Italian population, we selected the region showing the strongest association among all known non-HLA MS loci (Sawcer et al., 2011; Beecham et al., 2013) from a large Italian dataset comprising 1,711 patients with MS and 2,234 HCs. This dataset represents a meta-analysis of two sample sets genotyped at genome-wide level with either the Human610-Quad (750 MS and 1,272 HCs) or Immunochip (961 MS, 962 HCs) platform. As shown in Fig. 1, the marker with the strongest association was rs1077667 ($P = 1.73 \times 10^{-8}$), mapping to intron 1 of *TNFSF14* (chromosome 19).

Sequencing of the *TNFSF14* region in the Italian population

To identify all the variants in the *TNFSF14* region specifically present in the Italian population and patients with MS, we performed next-generation sequencing (NGS) of the whole genomic region encompassing the *TNFSF14* gene in a sample set of 588 patients with MS and 408 HCs (sequencing dataset 1; Table S1), pooled in groups of 12 individuals. According to our previous data, this approach can provide an accurate estimate of the allele frequencies in the pool and was therefore deemed suitable to carry out a preliminary association analysis of the variants identified in the sequencing experiment (Anand et al., 2016). After quality control (QC), we identified 112 variants in the *TNFSF14* locus. Among these, 6 variants were in the coding region, and 38 had a minor allele frequency (MAF) > 1%. Only 11 variants, nine of which with a MAF > 1%, were already present in the genotyping array platforms (Sawcer et al., 2011; Beecham et al., 2013; International Multiple Sclerosis Genetics Consortium, 2018; International Multiple Sclerosis Genetics Consortium, 2019), whereas 43 were not present in public population databases (i.e., Database of Single Nucleotide Polymorphisms; Sherry et al., 2001), of which only two had an MAF > 1%. Comparison of the allele frequencies estimated in the pools of patients with MS *vs*. HCs showed a statistically significant association with MS for 15 variants ($P < 0.05$; Table S2), 13 of which were present in public databases. The *TNFSF14* intronic variant (rs1077667) showed one of the strongest signals ($P = 1.47 \times 10^{-5}$; Fig. 2A). We also identified an exonic synonymous variant (rs2291668) in linkage disequilibrium (LD) with rs1077667 ($r^2 = 0.808$), not present in the genotyping array platforms, with a similar association signal ($P = 5.3 \times 10^{-5}$). Intriguingly, this SNP is located in exon 1, near the site involved in the alternative splicing that generates the ΔTM transcript isoform encoding a *TNFSF14* protein lacking the transmembrane domain (Granger et al., 2001).

Analysis of the SNPs selected from the *TNFSF14* sequencing in an independent cohort to identify the most significantly MS-associated variant

To perform fine-mapping on the *TNFSF14* locus using an independent sample set, we individually genotyped 867 patients with MS and 878 HCs (after QC, target genotyping platform, Table S1) for 42 variations, including the following: (1) any variant significantly associated with MS ($P < 0.05$) as well as all the coding variants identified in the aforementioned NGS cohort sequencing dataset 1; (2) LD-pruned ($r^2 > 0.9$) common variants (MAF > 1%); and (3) LD-pruned ($r^2 > 0.9$) variants reported in the 1000 Genomes database not detected in the NGS experiment. We observed a significant MS association for six variants ($P < 0.05$; Fig. 2B), confirming five of the associations

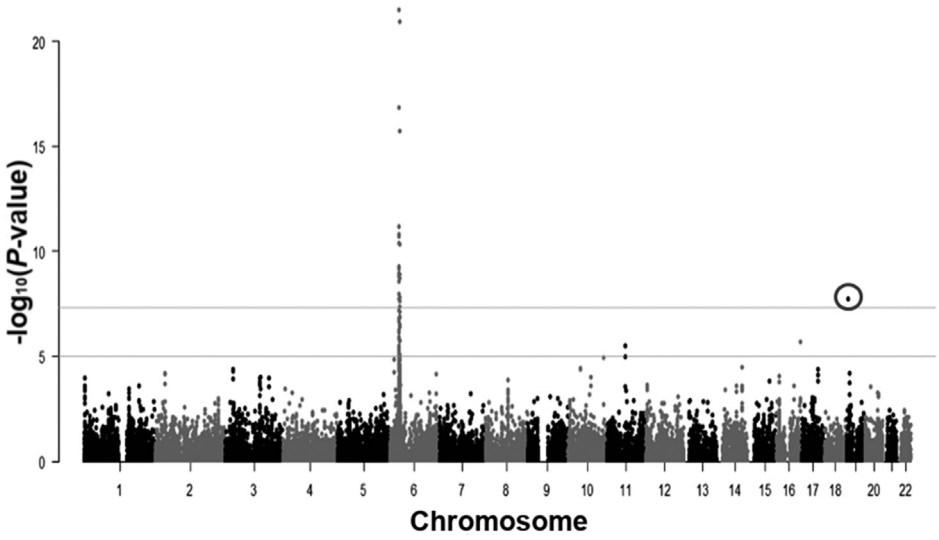

Fig. 1. Manhattan plot depicting the genome-wide risk loci for MS in the Italian population. The results of meta-analysis of GWAS dataset 1 and Immunochip dataset (Table S1) are displayed. The signal corresponding to the intronic variant (rs1077667) in the *TNFSF14* gene is indicated with a circle (chromosome 19). The two horizontal lines indicate the thresholds of statistical significance corresponding to genome-wide significant association ($P = 5 \times 10^{-8}$, upper line) and suggestive association ($P = 1 \times 10^{-5}$, lower line).

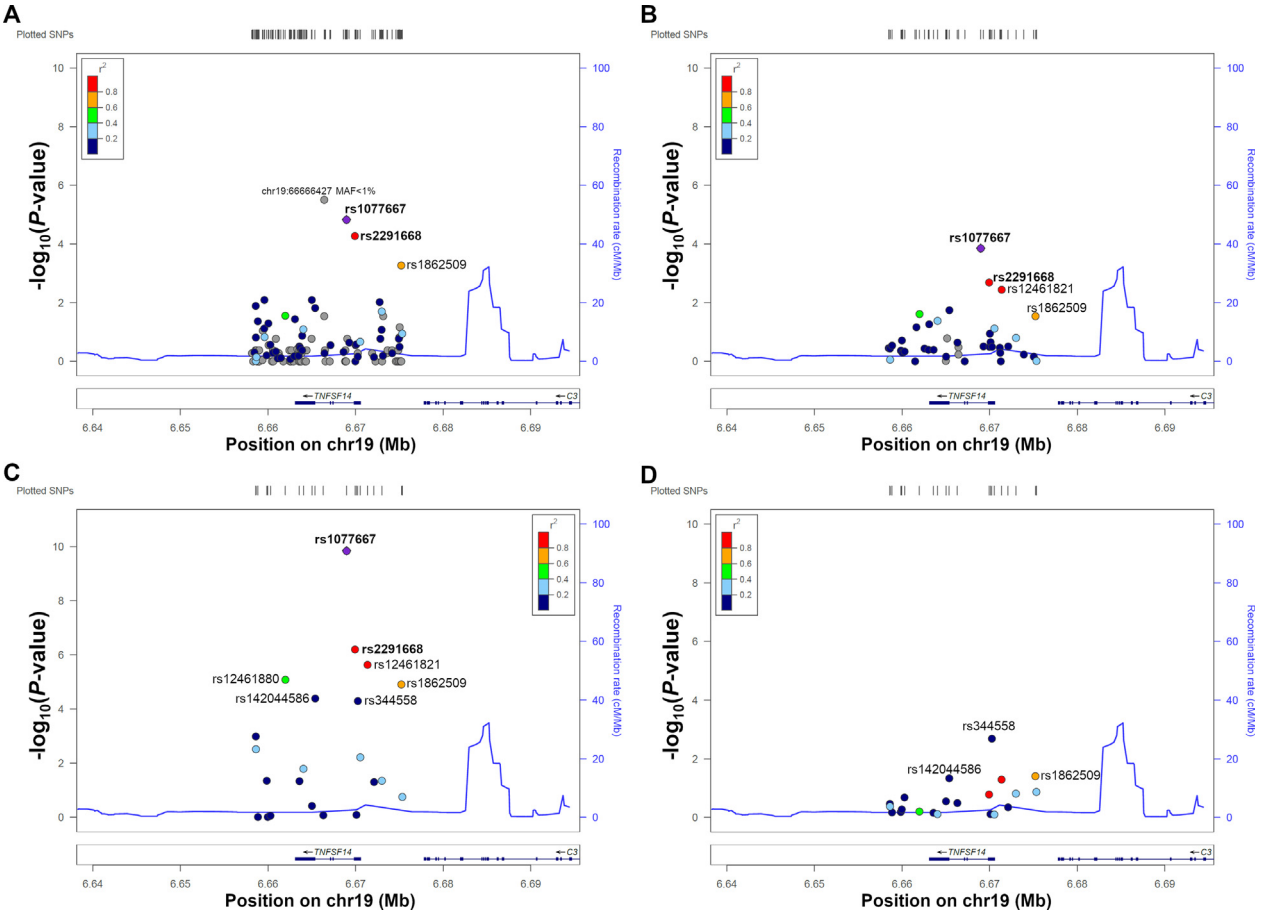

Fig. 2. Regional association plots on *TNFSF14* gene. **A**: Sequencing dataset 1. **B**: Analysis of targeted genotyping platform. **C**: Meta-analysis of targeted genotyping platforms, GWAS dataset 1 and GWAS dataset 2. **D**: Meta-analysis conditioning on rs1077667. All SNPs with at least one nominal *P* value are indicated. The intronic variant rs1077667 (circled blue dot) and exonic variant rs2291668 (red dot) are indicated in bold letters. In panel **A**, the strongest association signal is that of the rare intronic variant chr19: 66666427 (MAF < 1% in MS patients), not previously reported in public databases, showing no statistically significant association in target genotyping platform analysis ($P = 0.68$; Table S2).

observed in the pools. The intronic rs1077667 variant showed the strongest association ($P = 3.2 \times 10^{-5}$). The other five variants displayed various LD values (r^2 range: 0.76–0.16) with the intronic rs1077667 variant and did not maintain a statistically significant association after conditioning on rs1077667 (Table S2).

To increase the statistical power of our fine-mapping, we enriched the previously mentioned dataset with two additional sample sets, one containing 734 patients with MS and 1,250 HCs (GWAS dataset 1) and the other comprising 1,236 MS and 370 HCs (GWAS dataset 2), both imputed with the 1000 Genomes dataset, for a total of 2,837 patients with MS and 2,498 HCs. After meta-analysis of these three sample sets, with 22 SNPs in common, we observed a significant association for 14 SNPs ($P < 0.05$), comprising the 6 SNPs significantly associated in the previous analysis. The intronic rs1077667 was still the most significantly associated signal also in the meta-analyzed dataset ($P = 1.363 \times 10^{-10}$), followed by the exonic variant rs2291668 ($P = 6.199 \times 10^{-7}$) (Fig. 2C). After conditioning on rs1077667, the association of this exonic variant was no longer statistically significant ($P = 0.1623$), whereas three other SNPs, with various LD values with rs1077667, maintained only a nominally statistically significant association, namely, rs142044586 ($P = 0.04637$, $r^2 = 0.16$), rs1862509 ($P = 0.03893$, $r^2 = 0.76$), and rs344558 ($P = 0.0023$, $r^2 = 0.028$) (Fig. 2D). After conditioning on each of these four variants, rs1077667 maintained the strongest statistical significance ($P = 6.202 \times 10^{-5}$, 1.23×10^{-6}, 3.19×10^{-6}, and 4.4×10^{-9}, Table S3).

Altogether, these results indicate that rs1077667 displays the strongest association signal independently of any other tested common variants (MAF > 1%) in the *TNFSF14* region, including those with a residual nominal independent association. Regarding the aggregate contribution of rare variants, we carried out a burden test for rare *TNFSF14* coding variants (eight with MAF < 0.01, including two nonsynonymous variants) identified through NGS sequencing from 1,092 patients with MS and 912 HCs (sequencing dataset 1 and sequencing dataset 2; Table S1), which did not detect any statistically significant association (Supplementary Methods; Tables S4 and S5). Thus, rs1077667 appears to be the main genetic variant associated with MS in the *TNFSF14* region.

eQTL analysis from public databases

We next investigated expression quantitative trait locus (eQTL) data available in the following public databases: Geuvadis consortium (Lappalainen et al., 2013), Blood eQTL browser (Westra et al., 2013), Bioportal (Holm et al., 2010), Gtex portal (Melé et al., 2015), and Brain eQTL Almanac (Ramasamy et al., 2014) (Table S6). The MS risk allele (C) of the rs1077667 variant was consistently associated with significantly lower *TNFSF14* expression levels in Epstein Barr Virus (EBV)-transformed lymphoblastoid cell lines, peripheral whole blood cells, and peripheral blood mononuclear cells (PBMCs). No significant eQTL association was found in brain cells apart from the hippocampus (Table S6). These data were consistent with the allelic imbalance of the exonic variant rs2291668 (in high LD with rs1077667, $r^2 = 0.808$), based on RNAseq data from EBV cell lines derived from 97 heterozygous individuals (Geuvadis). Indeed, allelic expression of rs2291668 was significantly unbalanced to the detriment of the allele in phase with the risk allele rs1077667-C with lower eQTL expression. More precisely, 68% of individuals showed fewer RNAseq reads carrying the C allele compared with those harboring the T allele (C<T, Wilcoxon paired-samples test: $P < 0.0001$). The same trend was observed in Gtex EBV cell lines (33 samples, C<T: 60.7%) and PBMCs (129 samples, C<T: 53.2%), albeit these differences were not statistically significant. Meta-analysis of EBV cell lines from both Geuvadis and Gtex datasets confirmed the allelic imbalance ($P = 0.001$). Conversely, a statistically significant opposite trend was observed in other Gtex tissues (i.e., lung, 23 samples, C<T: 21.7%, $P = 0.0264$; liver, 37 samples, C<T: 16.2%, $P < 0.0001$; adipose visceral tissue, 56 samples, C<T: 17.9%, $P < 0.0001$; and esophagus muscularis, 20 samples, C<T: 20%, $P = 0.0020$).

TNFSF14 gene expression in patients with MS and HCs

To confirm the previously mentioned eQTL results, we evaluated *TNFSF14* expression levels by SYBR green quantitative polymerase chain reaction (qPCR) in two different sample sets: (1) PBMCs from 45 patients with MS and 64 HCs; and (2) whole blood from 39 patients with MS and 16 HCs. In both sample sets, patients with MS were treatment naïve.

As aforementioned, there exist two alternatively spliced transcript variants encoding different isoforms of *TNFSF14*: a full-length transcript of 1,491 bp (ENST00000599359.1) and a shorter transcript (ΔTM isoform) of 1,169 bp (ENST00000245912.7) (Granger et al., 2001). This shorter transcript is generated by joining a cryptic splice donor in exon 1 to the canonical splice acceptor of intron 1, resulting in the removal of nucleotides comprising the exonic SNP rs2291668 encoding the transmembrane domain. Thus, based on this information, we decided to test the association of rs1077667 genotypes with RNA expression of both *TNFSF14* isoforms in both cohorts (Figs. S1-S4). To this end, we performed linear regression analysis (Table 1) to test the association of rs1077667 genotypes with *TNFSF14* gene expression in the two aforementioned sample sets, accounting for sex and disease status effect as possible confounders. In the presence of the rs1077667 MS risk allele (C), both full-length and ΔTM isoforms in either sample set were expressed at lower levels (beta value < 0), and we recorded a statistically significant value only for the full-length isoform in sample set 1 ($P = 0.0021$). However, when the two sample sets were subjected to meta-analysis, we obtained statistical significance for both isoforms (fixed effects model, full length: $P = 0.0029$; ΔTM: $P = 0.011$), in good agreement with what previously observed in the eQTL analysis.

Interestingly, expression levels of both the full-length (Mann-Whitney *U* test; sample set 1: $P = 0.0072$; Cohort 2: $P = 0.0010$; Fig. 3A and 3B) and ΔTM (sample set 2: $P = 0.0001$; Fig. 3D) *TNFSF14* isoforms were lower in patients with MS compared with HCs. However, when we covaried for sex and genotype, this trend was statistically significant only in sample set 2 (full-length isoform: $P = 0.00023$; ΔTM: $P = 3.0 \times 10^{-6}$).

Taken together, these results indicate that *TNFSF14* gene expression in blood cells from patients with MS tends to be lower than that observed in HCs, which is consistent with the lower expression levels observed in carriers of the risk allele of the most significantly MS-associated variant rs1077667. Finally, the superimposable expression patterns of the two *TNFSF14* isoforms (Figs. S1-S5) suggest that the MS risk allele modulates the expression of these splice variants in a similar fashion.

LIGHT protein expression in blood cells according to rs1077667 genotype

To evaluate the association of rs1077667C/T variant with LIGHT surface expression, we performed flow cytometry analyses on peripheral blood of 20 HCs with different rs1077667 genotypes (10 CC, 8 CT, and 2 TT), testing the percentage of LIGHT$^+$ cells in CD8$^+$ T cells, CD4$^+$ T cells, DCs (CD11c$^+$), monocytes (CD14$^+$), NK cells (CD56^{dim}/CD16^{bright}, CD56$^-$/CD16^{bright}, CD56^{dim}/CD16$^-$), and B cells (CD19$^+$, Fig. 4A). The mean percentage of LIGHT$^+$ cells among the analyzed populations ranged between 17.4% (CD4$^+$ T cells) and 0.3% (B cells, the only cell population with less than 1% of LIGHT$^+$ cells). Among the cell populations with a percentage of LIGHT$^+$ cells higher than 1%, we observed a significant association with rs1077667 genotype in

Table 1
Association between *TNFSF14* expression levels and genotypes of the primarily MS-associated variant (rs1077667 intronic variant).

rs1077667 allele	*TNFSF14* isoform	Cohort 1[a]		Cohort 2[b]		Meta-analysis[c]		
		Effect (beta)[d]	*P* value	Effect (beta)[d]	*P* value	Effect (beta)[d]	*P* value	I^{2}[e]
C	Full length	−0.0012	**0.0021**	−1.2 × 10-4	0.90	−0.0010	**0.0029**	8.10
C	ΔTM	-4.1×10^{-5}	0.071	-5.2×10^{-5}	0.077	-4.5×10^{-5}	**0.011**	0.00

A linear regression analysis correlating the number (0, 1, or 2) of the risk alleles (C) and qPCR $2^{-\Delta CT}$ values, covariate for sex and disease status, was performed in both cohorts. The increase in the number of risk alleles is associated with the decrease in $2^{-\Delta CT}$ values, which means that the risk allele is associated with lower *TNFSF14* expression levels. Bold values represent statistically significant *P* values ($P < 0.05$).

[a] PBMC, peripheral blood mononuclear cells.
[b] Whole blood.
[c] Fixed effects model.
[d] Beta value of linear regression, referred to qPCR $2^{-\Delta CT}$ values.
[e] Heterogeneity index.

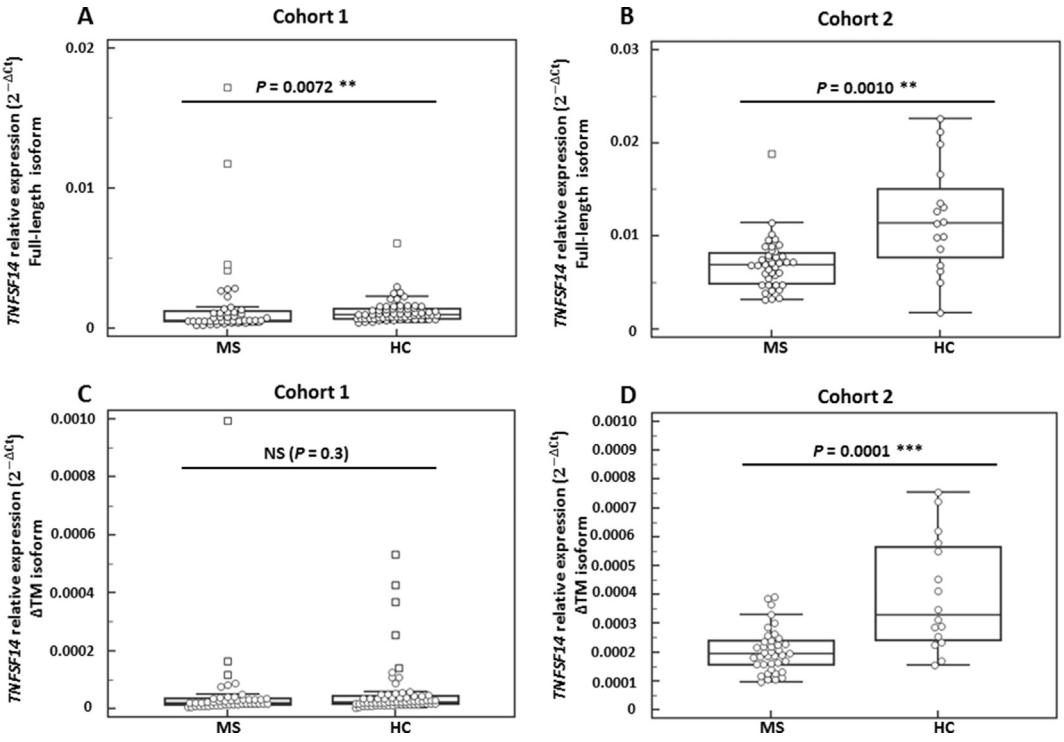

Fig. 3. *TNFSF14* expression levels. **A** and **B**: Box plots displaying the expression levels of full-length *TNFSF14* isoform in HCs and MS patients (MS) of Cohort 1 (**A**) and Cohort 2 (**B**). **C** and **D**: Box plots displaying the expression levels of ΔTM *TNFSF14* isoform in HCs and MS patients (MS) of Cohort 1 (**C**) and Cohort 2 (**D**). 2. $2^{-\Delta CT}$ values (*Y*-axis) are displayed, and each dot represents an individual. *P* values were derived the from Mann-Whitney test. **, $P < 0.01$; ***, $P < 0.001$.

myeloid DCs ($CD11c^{+}$). Indeed, the mean percentage of $LIGHT^{+}$ cells in homozygous individuals for the MS risk allele (CC) was significantly higher compared with individuals with other genotypes (CT + TT) (Student's *t*-test, $P = 0.02$; Fig. 4A). The association was maintained when we extended the flow cytometry analysis of this cell population ($CD11c^{+}$) to additional controls, reaching a total of 37 HCs (18 CC, 15 CT, and 4 TT, Student's t-test, $P = 0.035$; Fig. 4B).

To choose the best *in vitro* model of DCs with which we could replicate our *ex vivo* data from blood samples, we measured LIGHT expression in five different types of monocyte-derived DCs (MDDCs), obtained by growing cultures of monocytes from 5 HCs for 5 days in the presence of GM-CSF + IL-4, GM-CSF + IFNβ, IL-3 + IFNβ, IL-3, or GM-CSF + IL-15. Surface expression of LIGHT was then analyzed by flow cytometry in these MDDCs cultured for 2 days in the presence (activated/mature MDDCs) or absence (resting/immature MDDCs) of lipopolysaccharide (LPS). The results showed that LIGHT expression was relatively weak in all types of immature MDDCs and tended to be upregulated in mature MDDCs, particularly in MDDCs obtained with GM-CSF + IFNβ (Student's *t*-test, $P = 0.037$) or GM-CSF + IL-15 (Student's *t*-test, $P = 0.04$), where the upregulation was statistically significant (Fig. S6).

Next, we selected the MDDC population showing the highest percentage of $LIGHT^{+}$ cells, which corresponded to that cultured in the presence of GM-CSF + IL-15 ($MDDC^{IL15}$), and we increased the sample set to a total of 22 HCs with different genotypes of the rs1077667 C/T variant (12 CC, 9 CT, and 1 TT). This analysis showed that individuals carrying the MS risk genotype (CC) had a higher percentage of $LIGHT^{+}$ cells than that of the other groups (TT + CT), with a statistically significant difference in mature $MDDCs^{IL15}$ (Mann-Whitney *U* test, $P = 0.04$, Fig. 5).

Because LIGHT can be produced as a soluble form, we also measured the levels of secreted LIGHT in the culture supernatants of immature and mature $MDDCs^{IL15}$ from the same set of 22 HCs and compared it with LIGHT surface expression. The results confirmed that LIGHT surface expression was significantly higher in mature *vs.* immature $MDDCs^{IL15}$ (paired Student's *t*-test, $P = 0.0002$) (Fig. S7A).

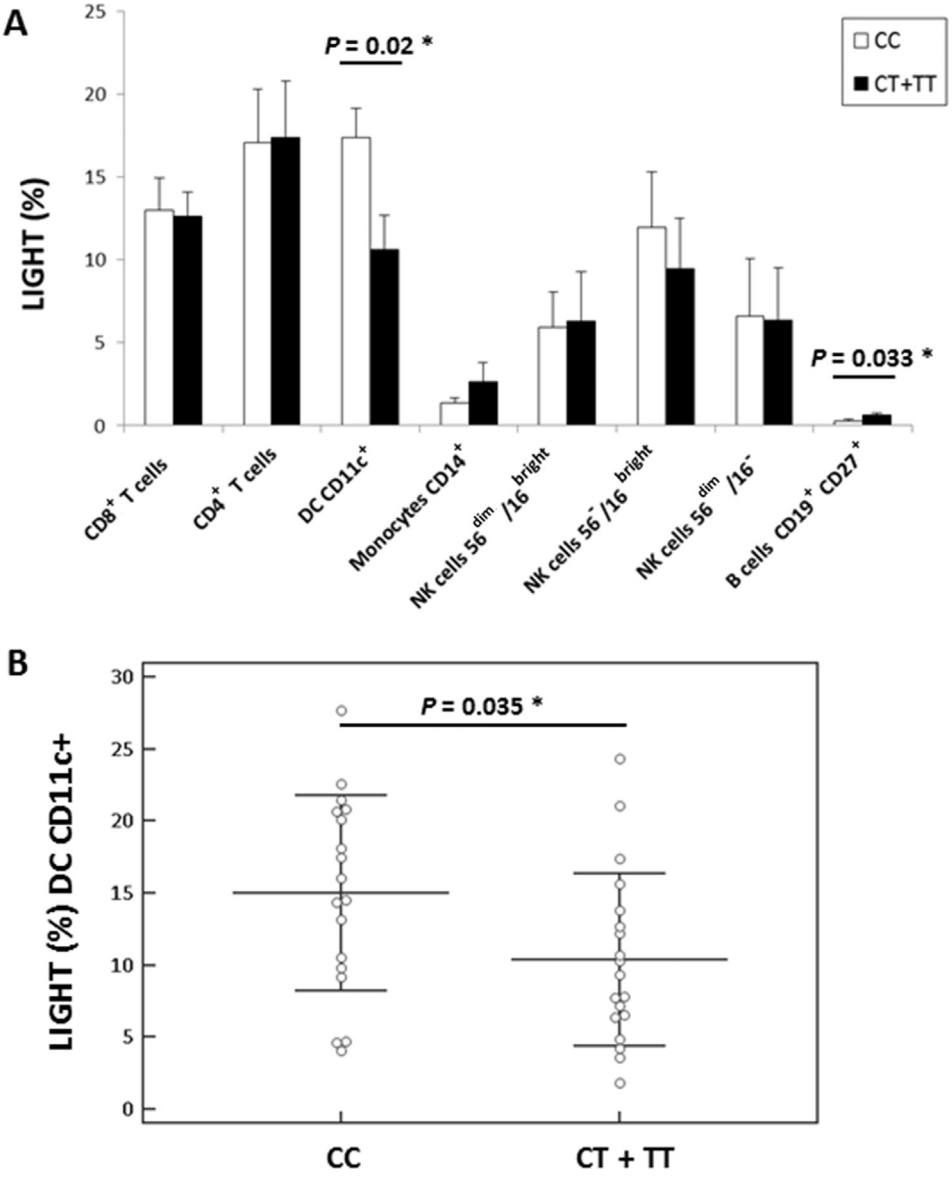

Fig. 4. Association of the rs1077667C/T variant with LIGHT surface expression in blood cells from healthy donors. **A**: Mean percentage (± standard error of the mean, SEM) of LIGHT-positive cells detected by flow cytometry in $CD8^+$ T cells, $CD4^+$ T cells, $CD11c^+$ myeloid DCs, monocytes ($CD14^+$), NK cells ($56^{dim}/16^{bright}$, $56^-/16^{bright}$, $56^{dim}/16^-$), and B cells ($CD19^+$) in blood from 20 HCs stratified according to the rs1077667 genotype (CC *vs.* CT + CT). **B**: Percentage of LIGHT-positive myeloid DCs ($CD11c^+$) in blood from 37 individuals stratified according to the rs1077667 genotype (CC *vs.* CT + CT). Each dot represents an individual; the lines in the graph represent the mean and standard deviation. *P* values were derived from Student's *t*-test. *, $P < 0.05$.

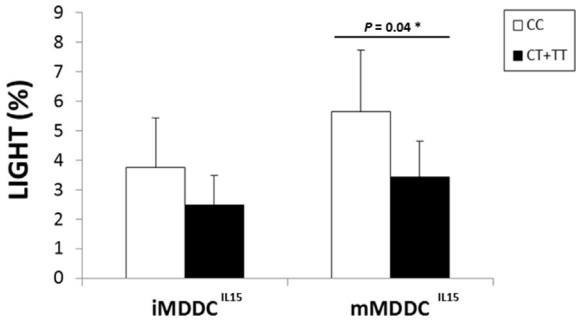

Fig. 5. Association of the rs1077667C/T variant with LIGHT surface expression in monocyte-derived DCs from healthy donors. Mean percentage (± standard error of the mean, SEM) of LIGHT-positive immature (iMDDC) and mature (mMDDC, LPS-activated), monocyte-derived DCs (MDDCs) obtained by culturing monocytes with GM-CSF + IL-15 from 22 healthy donors (12 CC, 9 CT, and 1 TT), (detected by flow cytometry) stratified according to the rs1077667 genotype. *P* values were derived from Mann-Whitney test. *, $P < 0.05$.

By contrast, soluble LIGHT levels displayed an opposite behavior as enhanced levels were detected in the supernatants of immature $MDDCs^{IL15}$ and were downregulated in their mature counterparts (paired Student's *t*-test, $P = 0.007$) (Fig. S7B). Stratification analysis according to rs1077667 genotype revealed similar expression changes in both CC and CT + TT strata for both the surface and soluble LIGHT isoforms, indicating that the modulation of LIGHT isoform expression triggered by $MDDC^{IL15}$ maturation is not influenced by the rs1077667 intronic variant (Fig. S8).

Discussion

The genetic association between the *TNFSF14* locus and MS was first reported in IMSGC GWAS in 2011 (Sawcer et al., 2011) and subsequently confirmed by other international IMSGC studies (Beecham et al., 2013; International Multiple Sclerosis Genetics Consortium, 2019). Here, we have identified the SNP rs1077667 located in intron 1 of the *TNFSF14* gene as the primarily MS-

associated variant within the *TNFSF14* locus in a large Italian cohort of patients with MS and HCs analyzed using a dense list of markers derived by public databases and sequencing of Italian patients with MS and HCs. Among the 201 non-MHC MS-associated loci, this is one of the few loci dissected with this high degree of resolution.

According to computationally integrated ChIP-seq data using a Hidden Markov Model (Ernst and Kellis 2010) available on UCSC genome browser, the rs1077667 SNP is located in a 2,600 bp region showing an enrichment of the H3K4me3 histone mark and identified as an active promoter (Fig. S9), suggesting a regulatory role of the SNP in *TNFSF1*4 mRNA expression. The hypothetical function of this variant is further supported by cis-eQTL data from different databases, showing that this MS risk allele is significantly associated with a lower *TNFSF1*4 mRNA expression in EBV-transformed lymphoblastoid cell lines (Geuvadis, Bioportal, Gtex), PBMCs (Gtex), and whole blood (Blood eQTL browser). These data are consistent with the imbalance of the risk allele observed in heterozygous individuals in EBV-transformed lymphoblastoid cell lines (Geuvadis, Gtex) from RNAseq analyses. These results are supported by our RT-PCR expression analysis on blood cells from 84 Italian MS and 80 HCs, measuring the expression of the two *TNFSF14* isoforms (full length and ΔTM). Interestingly, we show that the MS risk allele is associated with lower expression levels of both *TNFSF14* isoforms. This is in agreement with previous findings showing downregulation of LIGHT protein expression in the serum of patients with MS homozygous for the risk allele (Malmeström et al., 2013). In addition, we show that patients with MS tend to produce less LIGHT mRNA in comparison with HCs independently of the *TNFSF14* genotype. A similar trend was previously observed in smaller sample sets without taking into account the *TNFSF14* genotype (Jernås et al., 2013; Romme Christensen et al., 2013).

In silico predictions by TRANSFAC (Matys et al., 2006) and MatInspector (Cartharius et al., 2005) suggest that the rs1077667 variant can modify the binding of the AhR transcription factor (Fig. S9). Specifically, the existence of an AhR consensus binding site is only predicted in the presence of the MS risk allele. AhR was initially discovered and characterized as a transcription factor responsible for the activation of genes encoding different enzymes involved in xenobiotic metabolism (Vogel et al., 2014). Further studies showed that AhR activation is also involved in the regulation of the immune system (Singh et al., 2007) and differentiation of regulatory T cells and T-helper 17 cells (Th17) (Kimura et al., 2008). The involvement of AhR in Th17 cell differentiation is particularly interesting, as the expansion of these cells in the peripheral blood is associated with the active phase of MS (Durelli et al., 2009).

Inflammation is one of the key pathogenic mechanisms in MS, at least in the early stages of disease (Frischer et al., 2009), and nuclear factor kappa B (NF-κB) activation has been proposed as one of the key events mediating inflammatory processes. Here, we show that LIGHT, which plays a role in NF-κB activation, is downregulated in MS PBMCs. In this regard, it is interesting to observe that other genes involved in NF-κB activation are downmodulated in PBMCs of patients with MS (Navone et al., 2014).

LIGHT modulates T cell activation by interacting with two cellular receptors, the lymphotoxin-β receptor (LTβR) and the herpesvirus entry mediator (HVEM). Through LTβR-mediated TRAF-3 recruitment and caspase activation, LIGHT, in particular contexts, can trigger cell death (Granger and Rickert, 2003), whereas binding to HVEM has a costimulatory effect on T cell activation (Shui et al., 2011). Thus, it is likely that an imbalance in the modulation of LIGHT production can predispose individuals to inflammatory and neurologic conditions typical of MS.

Evidence supporting a role of LIGHT in MS pathogenesis comes from the observation that LIGHT-deficient mice develop severe experimental autoimmune encephalomyelitis (EAE), leading to an atypically high mortality rate (Maña et al., 2013). In these mice, LIGHT expression was also crucially involved in controlling activated macrophages/microglia during autoimmune CNS inflammation (Maña et al., 2013). In addition, LIGHT has been shown to be essential for DC maturation by cooperating with CD154 (CD40 ligand) (Morel et al., 2001) and interacting with licensed natural killer cells (Holmes et al., 2014). Interestingly, we show that LIGHT is expressed in peripheral blood myeloid DCs ($CD11c^+$) and that MS risk allele of rs1077667 is associated with an increased percentage of LIGHT-positive cells. These *ex-vivo* results were then confirmed in MDDCs differentiated *in vitro* in the presence of different cytokine milieus. Indeed, we found that LIGHT expression is weak in immature MDDCs, whereas it is upregulated in mature MDDCs, particularly in $MDDCs^{IL15}$. Moreover, an expanded analysis of $MDDCs^{IL15}$ revealed that the MS risk allele is associated with an increased percentage of LIGHT-positive cells in mature $MDDCs^{IL15}$.

Our findings differ from a previous study by Tamada et al. (2000), showing that immature MDDCs differentiated with GM-CSF + IL-4 express high levels of LIGHT, which are downmodulated on LPS treatment. This discrepancy might be ascribed to technical differences between the two studies, probably because of the fact that Tamada et al. (2000) measured LIGHT expression by indirect immunofluorescence using a polyclonal rabbit antibody raised against a small synthetic peptide derived from LIGHT conjugated to KLH. By contrast, we used an anti-LIGHT mAb detected by direct immunofluorescence, which should increase analysis specificity.

The association between the MS risk allele and increased percentage of LIGHT surface-positive DCs seems to be in disagreement with our observation that the MS risk allele is associated with decreased LIGHT mRNA levels in peripheral blood cells (whole blood or PBMCs) and EBV-derived B cell lines. This apparent contradiction may be explained by the fact that in peripheral blood cells, LIGHT mRNA levels can be substantially influenced by changes in the distribution of white cell types, which basally express different amounts of LIGHT. For instance, T cells express high levels of LIGHT, whereas B cells and monocytes are low expressors. Thus, an unbalance between these cell types is very likely to have a profound impact on overall LIGHT expression in the blood. By contrast, changes in LIGHT expression in myeloid DCs, which represent a small fraction of white blood cells (1%–3%), would only have a minimal impact on blood LIGHT expression. However, this explanation may not apply to EBV-derived B cell lines, where the risk allele might actually influence LIGHT mRNA expression. In this scenario, it is intriguing that we detected a minimal percentage of peripheral blood B cells expressing LIGHT, while this percentage was significantly lower in subjects carrying the risk allele compared with the other subjects ($0.3 \pm 0.3\%$ *vs.* $0.6 \pm 0.4\%$, $P = 0.033$), which is in line with the EBV-derived B cell lines RNA data. Notably, our analyses of eQTL databases reinforce the hypothesis that the eQTL effect of rs1077667 on *TNFSF14* gene expression is tissue dependent because in some tissues, we detected an opposite effect to that observed in PBMCs. However, no data specifically focused on DCs were available in these eQTL databases.

Given that LIGHT can be produced in a soluble form, we also analyzed LIGHT protein released in the culture supernatants of $MDDCs^{IL15}$ and found an inverse relationship between the soluble and surface form. Specifically, we observed that immature $MDDCs^{IL15}$ express high levels of soluble LIGHT and low levels of surface LIGHT. Conversely, mature MDCs tend to express high levels of cell surface LIGHT and low levels of soluble LIGHT. However, no significant association was found between the production of soluble LIGHT and the risk allele. As these data refer to the production of soluble LIGHT in DCs, they are not comparable with the significant association between rs107667 genotypes and serum levels of LIGHT, as reported by Malmeström et al. (2013), which may reflect

the activity of multiple cell and tissue types, with only a minor contribution of DCs.

The involvement of DCs in MS has been well established in humans and mice. In particular, Serafini et al., (2006) detected both immature and mature DCs in the meninges and parenchymal lesions of patients with primary and secondary progressive MS. MDDCs from the cerebrospinal fluid of patients with MS display a mature phenotype because they express high levels of HLA-DR, CD80, CD86, and CD40 and produce high levels of the pro-inflammatory cytokine IL-6 (Pashenkov et al., 2001). As aforementioned, LIGHT plays a crucial role in the maturation of DCs, and, when expressed on DCs, increases IFNγ production by T cells (Tamada et al., 2000), which is relevant to MS pathogenesis given the central role played by IFNγ in T cell-mediated autoimmune response in MS. This would also fit with our data showing that the MS-associated *TNFSF14* variant is associated with increased percentage of LIGHT^{+} in MDDCs^{IL15} cells, which have been reported to be particularly efficient in supporting cytotoxic T lymphocytes (CTLs), thereby playing a key role during T cell-mediated autoimmunity in MS (Banchereau and Palucka, 2005).

In conclusion, our work based on a fine-mapping approach identifies the intronic SNP rs1077667 as the primarily MS-associated variant in the *TNFSF14* region and suggests that altered *TNFSF14* expression in immune cells driven by this variant may contribute to MS pathogenesis. In particular, the MS risk allele is associated with lower *TNFSF14* mRNA expression, and according to in silico predictions, this may be mediated by differential binding of the AhR transcription factor to the two intronic SNP alleles. Furthermore, patients with MS produce lower mRNA levels of LIGHT compared with HCs, suggesting that genetically mediated downregulation of LIGHT may be involved in MS pathogenesis. This hypothesis is further supported by the observation that LIGHT-deficient mice develop severe EAE. Finally, our data indicate cell-specific *TNFSF14* SNP-mediated regulation of LIGHT expression. Indeed, the MS-associated variant seems to be associated with low *TNFSF14* RNA expression in a mixed population of PBMCs and with increased percentage of LIGHT^{+} cells in DCs. Because LIGHT is required for DC maturation and DCs play a functional role in MS pathogenesis, it is tempting to speculate that an imbalance between DC functional subsets triggered by LIGHT may contribute to MS autoimmunity. The results of this study pave the way to design further experiments to explain how a modulation of LIGHT expression can influence MS pathogenesis.

Materials and methods

Samples

A total of 8,101 individuals of continental Italian ancestry were recruited across several Italian MS centers after approval by the ethics committee of the local hospitals and after obtaining written informed consent for genetic analysis for research purposes from all study participants. In our study, we included 4,198 patients with MS (2.0:1 female/male ratio) and 3,903 HC (1:1.8 female/male) ratio, 31.67 (±10) mean age of disease onset, mean EDSS 3.16 (± 2.25), 7% with primary progressive MS (Table S1).

Patients with MS were diagnosed according to McDonald criteria, whereas HCs did not have family history of autoimmune diseases and have geographic provenience similar to that of the patients. A peripheral blood sample with Ethylenediaminetetraacetic acid (EDTA) was obtained from each study participant.

Nucleic acids were extracted from whole blood according to standard protocols (salting out or QIAamp DNA Blood Mini kit and RNeasy Plus Mini Kit provided by QIAGEN). For Cohort 1 consisting of 64 HCs and 45 patients with MS, PBMCs were isolated by density gradient centrifugation using Lympholyte-H (Cedar Lane, Burlington, NC, USA) and stored at −80°C with RNAlater (QIAGEN, GmbH, Hilden, Germany) for RNA preservation. RNA was extracted by RNeasy Plus Mini Kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions. For Cohort 2, consisting of 16 HCs and 39 patients with MS, RNA was isolated from whole blood using the ABI Prism 6100 Nucleic Acid Prep Station (Life Technologies, Monza, Italy) following the manufacturer's instructions.

Sequencing of the *TNFSF14* region

The *TNFSF14* gene was sequenced in 1,092 patients with MS and 912 HC, pooled in groups of 12 individuals each, in two different sequencing experiments. The libraries were prepared with the "SureSelectXT Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library" (Agilent Technologies). The DNA quantity was properly balanced in each pool to equally represent each genome. In the first experiment (sequencing dataset 1), the whole region (17,500 bp including exons, introns, UTRs, and 5 kb upstream and downstream of the gene) was sequenced in 588 patients with MS and 408 HCs grouped in 83 pools. Paired-end (PE) multiplexed sequencing was performed on the Illumina GaIIx platform (Illumina, San Diego, CA), combining 6 pools tagged with different index sequences in each lane and producing 2 × 85 bp read lengths.

In the second experiment (sequencing dataset 2), only the coding regions of *TNFSF14* gene were sequenced in 504 patients with MS and 504 HC grouped in 84 pools. PE multiplexed sequencing was performed on the Illumina NextSeq 500 (Illumina San Diego) platform, producing 2 × 150 bp read length.

The two datasets were analyzed with the same bioinformatics pipeline. Briefly, the raw-reads were first checked for quality using FastQC software (Andrews, 2015). The QC-checked PE reads of each pool were mapped to NCBI human reference genome (build GRCh37) using Burrows-Wheeler Algorithm (BWA v 0.7.5) (Li and Durbin, 2009), and the duplicate reads because of PCR amplification during library preparation were removed using SAMTools (Li et al., 2009a). A variant caller, specifically designed for pooled samples (Bansal, 2010), was used to call the variants. Genomic and functional annotation of the variations was performed by ANNOVAR (Wang et al., 2010).

Allelic frequencies (AF) in patients and controls were estimated using an ad-hoc custom pipeline, which was developed to guarantee accurate AF estimation with pooled NGS data (Anand et al., 2016). Specifically, a threshold was applied to single pool alternative AF to remove spurious reads. The thresholds (2.6% for the first experiment and 2.4% for the second one) were empirically determined as described in Anand et al. (2016). To remove false-positive variants, those variants with sequencing call quality >100 were chosen.

The 588 patients with MS in the first dataset have been previously individually genotyped either with the Illumina 660Q chip or with the Immunochip platform (Cortes and Brown, 2011; Beecham et al., 2013; Parkes et al., 2013), and AF comparison with these platforms was used to demonstrate a high correlation with AF in the pools ($R^2 = 0.987$). Similarly, a high correlation between pooled AF and frequencies reported in public databases was observed (1000 Genomes_EUR $R^2 = 0.980$, ExAC $R^2 = 0.970$).

Replication and fine-mapping

Sixty-two *TNFSF14* variants were genotyped on an independent, individually typed cohort of 1812 samples using a

TaqMan OpenArray Genotyping System (Applied Biosystems, Foster City, CA, USA). DNA samples were loaded at a concentration of 50 ng/mL and amplified according to the manufacturer's instructions. The auto-calling method, implemented in the TaqMan Genotyper software version 1.3, was used to assign genotypes. Seven SNPs were removed from the analysis because of failure in the design of the probes, and 13 variants were removed after QC because of bad clustering. All remaining SNPs showed a call rate > 90%. Individuals showing a call rate < 80% were removed during QC, yielding in a final dataset of 867 MS and 878 HC. Association effects sizes from this cohort were meta-analyzed with those of two other sample sets:

a) 734 MS and 1,250 HC (GWAS dataset 1) genotyped with Human610-Quad platform (Sawcer et al., 2011) and imputed with Mach software (Li et al., 2009b; Li et al., 2010) on the 1000 genomes dataset (Abecasis et al., 2012). Pre-imputation QC was performed as described elsewhere (Sawcer et al., 2011). After imputation, we retained SNPs showing imputation quality index $R^2 > 0.3$ and MAF > 0.01.
b) 1,236 MS and 370 HC (GWAS dataset 2) genotyped with Illumina HumanOmniExpress-12 BeadChip and HumanOmni-2.5 BeadChip (~550 K markers in overlap) and imputed on 1000 Genomes phase 3 ALL reference panel (Abecasis et al., 2012) using SHAPEIT (Delaneau et al., 2011) for prephasing step and Minimac for genotype imputation (Howie et al., 2012; Fuchsberger et al., 2015). After imputation, we retained SNPs showing $R^2 > 0.8$ and MAF > 0.01.

Gene expression analysis

The expression of the two splicing isoforms of *TNFSF14* on two different cohorts of patients and controls (Cohort 1 consisting of frozen PBMC from 64 HC and 45 MS; Cohort 2 consisting of whole blood from 16 HC and 39 MS) was determined by quantitative RT-PCR with SYBR Green method using the GoTaq 2-step RT-qPCR system (Promega). Its components allow the synthesis of cDNA using GoScript Reverse Transcription System and the subsequent quantification by GoTaq qPCR Master Mix. The qRT-PCR reaction was performed by means of a C1000 Thermal Cycler CFX96 Real-Time System (Bio-Rad). Each sample was tested in triplicate for the two *TNFSF14* splicing isoforms and for *β-actin* (housekeeping gene). The analysis of gene expression data was performed with CFX Manager Software Bio-Rad. We analyzed expression levels with the $2^{-\Delta CT}$ method (Livak method) (Livak and Schmittgen, 2001).

qRT-PCR primers: *TNFSF14* full-length isoform forward: GGTGGGTCTGGGTCTCTT; *TNFSF14* full-length isoform reverse: AGACCTTCGCTCTTGTATCAGC; *TNFSF14* ΔTM isoform forward: AGTGTGGCCCGGGACGGA; *TNFSF14* ΔTM isoform reverse: GCTGGAGTTGGCCCCTGTGA; *β-actin* forward: CGCCGCCAGCTCACCATG; *β-actin* reverse: CACGATGGAGGGGAAGACGG.

eQTL analysis and allelic imbalance

eQTL data from five public resources were examined: (1) Geuvadis project (Lappalainen et al., 2013), which performed mRNA sequencing on 465 lymphoblastoid cell line samples from five populations of the 1000 Genomes Project: the CEPH (CEU), Finns (FIN), British (GBR), Toscani (TSI), and Yoruba (YRI); (2) the Brain eQTL Almanac (BRAINeac) (Ramasamy et al., 2014), which is a Web-based resource to access the UK Brain Expression Consortium dataset; (3) the Gtex portal (Melé et al., 2015), which collects RNA sequencing data from 1,641 samples across 43 tissues from 175 individuals; (4) the eQTL blood browser (Westra et al., 2013); and (5) the SNPexp, a web tool for calculating and visualizing correlation between HapMap genotypes and gene expression levels in lymphoblastoid cell lines (Holm et al., 2010). In addition, we used Wilcoxon paired-samples test to compare the number of individuals with a difference in reads supporting risk (C) *vs.* protective allele (T) (allelic imbalance analysis) on RNAseq data of 97 heterozygous EBV cell lines individuals from Geuvadis and Gtex (version phs000424.v6.p1) in EBV cell lines (33 samples), in PBMCs (129 samples), lung (23 samples), liver (37 samples), adipose visceral (56 samples), and esophagus muscularis (20 samples). The results from the various EBV cell line datasets were meta-analyzed with CMA software (Borenstein and Higgins, 2013) under a fixed effect model.

Cell analysis

Flow cytometry experiments (BD FACSCalibur 2 Laser, Marshall Scientific) were performed on blood samples from healthy donors to detect the LIGHT/TNFSF14 surface expression (Human Allophycocyanin Mab, Clone 115520, R&D System) in several immune cell types: $CD8^+$ T cells, $CD4^+$ T cells, B cells ($CD19^+$), myeloid DCs ($CD11c^+$), NK cells ($CD56^{dim}/CD16^{bright}$, $CD56^-/CD16^{bright}$, and $CD56^{dim}/CD16^-$), and monocytes ($CD14^+$).

MDDCs were prepared from $CD14^+$ monocytes isolated with the EasySep Human CD14 Negative Selection Kit (STEMCELL Technologies, Vancouver, BC, USA). We compared the expression of LIGHT in MDDC obtained by culturing monocytes for 5 days with GM-CSF + IL-4 or GM-CSF + IFNβ or IL-3 alone or IL-3 + IFNβ or GM-CSF + IL-15, which are different MDDC types described in the literature (Banchereau and Palucka, 2005). Finally, we stimulated them with LPS for 2 days to obtain mature (activated) MDDCs. The soluble LIGHT was detected in the cell supernatant of MDDCs by ELISA (Human LIGHT/TNFSF14 Quantikine ELISA Kit, R&D System). Further details are present in the supplementary methods.

Statistical analysis

Genotype association analysis was conducted with PLINK software (Purcell) (Purcell et al., 2007). Conditional analysis was performed fitting a logistic regression model, incorporating sex as covariate and conditioning on one SNP at a time. For RNA expression data, we used Mann-Whitney *U* test to compare expression levels in patients with MS *vs.* HCs and between homozygous for the risk allele of the rs1077667 variant and other genotypes (Figs.S1-S4). Furthermore, we performed linear regression analysis testing the association between expression levels and genotypes, assuming an additive model and using sex and individual status (case or control) as covariates. Meta-analysis was conducted with PLINK software under a fixed effect model.

Regarding the cell analysis, in each group, the normal distribution of values of LIGHT expression and cytokines production was verified by Kolmogorov-Smirnov test, and the differences in mean expression levels were tested by Student's *t*-test or by Mann-Whitney test, as appropriate, with MedCalc Statistical Software version 15.5 (MedCalc Software bvba, 2015).

Data availability

Nucleotide sequence data reported are available in European Variation Archive (EVA). Accession number: Project, PRJEB32114; Analyses, ERZ858083.

CRediT authorship contribution statement

Miriam Zuccalà: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Nadia Barizzone**:

Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision. **Elena Boggio**: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Luca Gigliotti**: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Melissa Sorosina**: Methodology, Validation, Investigation, Data Curation, Writing - Review & Editing. **Chiara Basagni**: Methodology, Validation, Formal analysis, Investigation, Writing - Review & Editing. **Roberta Bordoni**: Methodology, Validation, Investigation, Writing - Review & Editing. **Ferdinando Clarelli**: Methodology, Software, Validation, Data Curation, Formal analysis, Writing - Review & Editing. **Santosh Anand**: Methodology, Software, Validation, Formal analysis, Writing - Review & Editing. **Eleonora Mangano**: Methodology, Software, Validation, Formal analysis, Writing - Review & Editing. **Domizia Vecchio**: Writing - Review & Editing, Data Curation, Resources. **Elena Corsetti**: Methodology, Investigation, Writing - Review & Editing. **Serena Martire**: Conceptualization, Data Curation, Writing - Review & Editing, Resources. **Simona Perga**: Conceptualization, Data Curation, Writing - Review & Editing, Resources. **Daniela Ferrante**: Formal analysis, Writing - Review & Editing. **Alberto Gajofatto**: Writing - Review & Editing, Data Curation, Resources. **Andrei Ivashynka**: Writing - Review & Editing, Data Curation, Resources. **Claudio Solaro**: Writing - Review & Editing, Data Curation, Resources. **Roberto Cantello**: Writing - Review & Editing, Data Curation, Resources. **Vittorio Martinelli**: Writing - Review & Editing, Data Curation, Resources. **Giancarlo Comi**: Writing - Review & Editing, Data Curation, Resources. **Massimo Filippi**: Writing - Review & Editing, Data Curation, Resources. **Federica Esposito**: Conceptualization, Writing - Review & Editing, Data Curation, Resources. **Maurizio Leone**: Conceptualization, Writing - Review & Editing, Data Curation, Resources. **Gianluca De Bellis**: Conceptualization, Writing - Review & Editing, Data Curation, Resources. **Umberto Dianzani**: Conceptualization, Supervision, Data Curation, Writing - Review & Editing. **Filippo Martinelli-Boneschi**: Conceptualization, Supervision, Resources, Data Curation, Writing - Review & Editing. **Sandra D'Alfonso**: Conceptualization, Supervision, Project administration, Funding acquisition, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing.

Conflict of interest

For the following authors this is the conflict of interest statement. No conflict of interest for all the other authors. Prof. Filippi is Editor-in-Chief of the *Journal of Neurology*, Associate Editor of *Human Brain Mapping*, Associate Editor of *Radiology*, and Associate Editor of *Neurological Sciences*; received compensation for consulting services and/or speaking activities from Alexion, Almirall, Bayer, Biogen, Celgene, Eli Lilly, Genzyme, Merck-Serono, Novartis, Roche, Sanofi, Takeda, and Teva Pharmaceutical Industries; and receives research support from Biogen Idec, Merck-Serono, Novartis, Roche, Teva Pharmaceutical Industries, Italian Ministry of Health, Fondazione Italiana Sclerosi Multipla, and ARiSLA (Fondazione Italiana di Ricerca per la SLA).

Dr. Gajofatto received fees from Biogen and Merck to participate in advisory boards. Dr. Vittorio Martinelli received compensation for speaking and/or for consultancy and support for travel expenses and participation in Congresses from Biogen, Merck-Serono, Novartis, Roche, Genzyme and Teva Pharmaceutical Industries. Prof. Filippo Martinelli Boneschi has received compensation for consulting services and/or speaking activities from Teva Pharmaceutical Industries, Sanofi Genzyme, Merck-Serono, Biogen Idec, Roche, Medday, Excemed, and received research support from Merck, Teva Pharmaceutical Industries, Italian Ministry of Health, Fondazione Italiana Sclerosi Multipla and Fondazione Cariplo.

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Supplementary data

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