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A candidate regulatory variant at the *TREM* gene cluster associates with decreased Alzheimer's disease risk, and increased *TREML1* and *TREM2* brain gene expression

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Abstract

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Conflict of Interest Statement

Dr. Petersen has been a consultant to Genentech, Inc. Merck, Inc. and Roche, Inc. and has served on a data safety monitoring committee for Pfizer and Janssen Alzheimer Immunotherapy. Dr. Graff-Radford has multicenter treatment study grants from Lilly, TauRx and consulted for Cytox. Dr. Ertekin-Taner consulted for Cytox.

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INTRODUCTION—We hypothesized that common Alzheimer's disease (AD)-associated variants within the triggering receptor expressed on myeloid (*TREM*) gene cluster influence disease through gene expression.

METHODS—Expression microarrays on temporal cortex and cerebellum from ~400 neuropathologically diagnosed subjects, and two independent RNAseq replication cohorts were used for expression quantitative trait locus (eQTL) analysis.

RESULTS—A variant within a DNase hypersensitive site 5′ of *TREM2*, rs9357347-C, associates with reduced AD-risk and increased *TREML1* and *TREM2* levels (uncorrected-p=6.3×10-3 and 4.6×10-2, respectively). Meta-analysis on eQTL results from three independent datasets (n=1,006) confirmed these associations (uncorrected-p=3.4×10^{-2} and 3.5×10^{-3}, Bonferroni-corrected p=6.7×10^{-2} and 7.1×10^{-3}, respectively).

DISCUSSION—Our findings point to rs9357347 as a functional regulatory variant that contributes to a protective effect observed at the *TREM* locus in the International Genomics of Alzheimer's Project (IGAP) GWAS meta-analysis, and suggest concomitant increase of *TREML1* and *TREM2* brain levels as a potential mechanism for protection from AD.

Keywords

Alzheimer's disease; eQTL; *TREM2*; *TREML1*; regulatory variant

1. Introduction

Whole genome and exome sequencing are used as complementary approaches to uncover novel loci that can be missed by GWAS, and enabled the discovery of rare, missense alleles within *TREM2* that have a relatively large effect size on AD-risk [1, 2]. *TREM2* is a member of the triggering receptor expressed on myeloid (TREM) family, known to play a key role in modulating inflammation in the innate immune response [3]. This finding provided strong supportive evidence for the importance of inflammation in the etiology of AD, but the specific role played by *TREM2* in AD pathophysiology remains unclear [4].

Since the first two reports [1, 2], the risk effect of the most significant *TREM2* rare missense variant p.R47H (a.k.a. rs75932628) has been replicated in multiple Caucasian series [5–9], including a large meta-analysis of 24,086 AD cases and 148,993 controls [10]. *TREM2* resides within the *TREM* gene cluster on chromosome 6p21.1 (Fig. 1), which also includes the protein coding genes *TREM1*, *TREML1*, *TREML2*, *TREML4* that could be additional plausible AD-risk genes.

A missense variant in *TREML2*, p.S144G (a.k.a. rs3747742), that is not in linkage disequilibrium (LD) with *TREM2* p.R47H, was reported to associate with reduced AD-risk [11]. *TREML2* p.S144G is in tight LD with the intergenic variant, rs9381040, that demonstrated the most significant association at the *TREM* locus in the IGAP AD-risk GWAS meta-analysis (rs9381040 p=7.4×10^{-3} after Bonferroni correction for 11,632 variants tested in the combined IGAP stage 1 and 2 data sets; uncorrected p=6.3×10^{-7}) [12]. The authors concluded that *TREML2* p.S144G is the functional variant that accounted for the IGAP *TREM* locus signal, even though the significance of the AD-risk association with the

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intergenic rs9381040 is greater than that observed with p.S144G. Further, *TREML2* p.S144G does not have a predicted functional consequence (PolyPhen2 score=benign) or demonstrated functional outcome, suggesting that the IGAP signal at the *TREM* locus may be due to other functional variants.

Some variants at the *TREM* locus have been reported to show association with AD endophenotypes [11, 13, 14]. Cerebrospinal fluid (CSF) levels of AD biomarkers, tau and ptau, associate with three variants at the *TREM* locus that are not in LD with each other: *TREM2* p.R47H (rs75932628), rs6916710 located in intron 2 of *TREML2*, and rs6922617 located downstream from *NCR2* and outside the *TREM* cluster. Of these variants, only *TREM2* p.R47H was associated with AD-risk [13]. More recently, a variant upstream of *TREM2* (rs7759295) and a variant in intron 3 of *TREM1* (rs6910730) were reported to be independently associated with increased AD pathology burden and increased rate of cognitive decline [14]. However, neither of these two variants shows association with AD-risk in the IGAP meta-analysis (uncorrected $p>0.05$) [15]. Thus, other than *TREM2* p.R47H, none of the *TREM* locus variants previously reported to associate with AD endophenotypes show association with AD-risk. Functional AD-risk variants that influence AD endophenotypes are expected to show association both with these endophenotypes and risk of AD. Therefore, it is possible that these latter variants are not the functional variants *per se*, but merely markers of other un-tested functional variants.

Collectively, these prior findings suggest that besides the *TREM2* rare missense variants, there may be common variants at the *TREM* locus that influence AD-risk and/or its endophenotypes. We hypothesized that some of the common AD-risk variants at the *TREM* locus confer disease risk via regulation of transcript levels of coding genes at the *TREM* gene cluster. In this study, we characterized the brain expression levels of the *TREM* family genes using microarray expression data; validated expression levels by RNA sequencing (RNAseq); performed genetic associations with *TREM* locus genes reliably detected in cerebellum and temporal cortex with single nucleotide polymorphisms (SNP) that were also tested in the IGAP AD-risk GWAS meta-analysis; and annotated these variants for their effects on *TREM* gene expression levels and regulatory potential. Further, we obtained results for the top putative regulatory SNP from two other, independent cohorts with brain RNAseq data and performed meta-analysis of all three cohorts.

2. Materials and Methods

2.1 Variant selection

We restricted our analysis to variants located within 100kb of any coding *TREM* family gene at the chromosome 6p21.1 *TREM* gene cluster (Fig. 1). Variants were further selected based on the statistical significance of their AD-risk association in the IGAP stage 1 meta-analysis [12] (Supplementary Methods), where only those variants with uncorrected p-values 0.0015 were kept. This p-value cut-off was arbitrarily chosen to select those variants that existed in both the IGAP stage 1 AD GWAS and our discovery eQTL cohort, Mayo Clinic Whole Genome-DASL dataset, and that could be genotyped, if needed, in the replication eQTL cohorts, using cost-effective medium-throughput assays. Variants were further prioritized by their Regulome score. Regulome scores were obtained from the Regulome

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database, which annotates variants with regulatory information from 962 different datasets and a variety of sources, including ENCODE [16]. Regulome scores are on a scale from 1 to 6, and these numerical categories are sub-classified with letters based on the number of lines of evidence of functional consequence. A value of 1a is assigned to the variant with the most evidence of regulatory potential, while a score of 6 has the least [16].

2.2 Mayo Clinic Whole Genome-DASL dataset (Discovery eQTL cohort)

We utilized Illumina (Whole Genome-DASL=WG-DASL, Illumina, San Diego, CA) microarray gene expression data from our published human brain expression genome-wide association study (Mayo Clinic eGWAS) [17] conducted on brain tissue from autopsied AD patients (197 cerebellum, 202 temporal cortex) and non-AD subjects (177 cerebellum, 197 temporal cortex) (Table 1). All AD subjects had neuropathologic diagnosis of definite AD [2]. The non-AD subjects did not fulfill neuropathologic criteria for definite AD, but many had other unrelated pathologies. Expression measures were generated as described previously [17]. A description of this cohort and generation of expression measures is provided in the Supplemetary Methods.

2.3 RNAseq datasets (Replication eQTL cohorts)

Temporal cortex RNAseq data from two RNAseq cohorts: “Mayo Clinic RNASeq” and “ROS/MAP RNAseq” were employed for replication of the associations that were detected with the WG-DASL gene expression measurements. The Mayo Clinic RNASeq dataset is comprised of 84 LOAD and 48 non-AD brains from the Mayo Clinic Brain Bank that were not part of the Mayo Clinic WG-DASL cohort but whose neuropathological diagnosis followed the same criteria. The ROS/MAP RNAseq dataset is comprised of RNAseq data from 288 AD and 206 non-AD samples that are part of the ROS/MAP cohort (Table 1) previously described [18, 19]. Methodological details for the RNAseq data generation are provided in the Supplementary Methods.

2.4. Statistical Analysis

Normalized transcript expression levels, on a log2 scale, were tested for associations with *TREM* locus genotypes in each of the three datasets (Mayo WG-DASL, Mayo Clinic RNAseq and ROS/MAP RNAseq) via multivariable linear regression analyses implemented in PLINK [20]. An additive model was applied adjusting for age-at-death, sex, diagnosis, RNA Integrity Number (RIN) and adjusted RIN squared $(\text{RIN-RINmean})^2$ in all expression analyses, and *APOE* ε4 dosage and PCR plate in Mayo WG-DASL only, and flowcell in the Mayo Clinic RNAseq dataset only. The eQTL analysis in the discovery, WG-DASL dataset, included *APOE* ε4 dose as a covariate given the strong effect of this allele on AD. However, since a significant association was not detected with this covariate in the rs9357347 eQTL analyses in the discovery set, *APOE* ε4 dose was not included in the eQTL analyses implemented on the replication cohorts. For comparison, we have performed the eQTL analyses in all three datasets with and without adjustment for *APOE* ε4 dose and do not observe a substantial difference in the association results between these two models. Given the tight LD between variants at this locus, and the potential for co-regulation of *TREM* genes at this cluster across tissues, a correction for multiple testing was not applied, except

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for the final meta-analysis results, for which both the uncorrected and Bonferroni corrected p-value (adjusted for the two genes tested) are provided.

Meta-analyses were performed on eQTL results from the three independent datasets. For these analyses, METAL [21] was implemented using weighted average of z-scores from the individual study p-values, weighted according their sample size.

To assess if diagnosis is associated with *TREML1* and/or *TREM2* gene expression levels, linear regression analyses were performed in R in each of the three datasets, adjusting for all other covariates included in the eQTL analyses, as well as rs9357347 minor allele dose.

3. Results

In the WG-DASL gene expression data from the temporal cortex (n=399) and cerebellum (n=374) of neuropathologically diagnosed AD and non-AD subjects (Table 1), we observed that of the 5 *TREM* locus coding genes, only *TREML1* and *TREM2* were reliably detected (Table S1 and Fig. 2). *TREML1* was detected in both the temporal cortex and cerebellum, while *TREM2* was reliably detected only in the temporal cortex. We validated *TREML1* and *TREM2* WG-DASL temporal cortex gene expression measurements, using RNAseq data generated from a subset of 93 autopsied AD subjects who also had microarray data. There was highly significant correlation between WG-DASL and RNAseq measurements for both *TREML1* (r_s=0.65, $p<10^{-40}$) and *TREM2* (r_s=0.80, $p<10^{-40}$) (Fig. S1).

Variants located within 100kb of the 5′ or 3′end of any *TREM* coding gene that demonstrated association with AD-risk in the IGAP stage 1 meta-analysis (17,800 AD vs. 37,154 controls, p 0.0015), were evaluated for their association with *TREML1* expression in the temporal cortex and cerebellum, and with *TREM2* expression in the temporal cortex. Of the 1,002 variants tested at this locus in the IGAP stage 1 meta-analysis, 28 had p-values 0.0015, and 16 of these have been genotyped in the autopsied samples in the Mayo Clinic brain expression genome-wide association study (Mayo eGWAS). We also assessed 5 other variants at this locus previously reported to be associated with either reduced AD-risk (rs3747742) [11], increased AD pathology burden and cognitive decline (rs6910730, rs7759295) [14], or decreased CSF tau levels (rs6916710, rs6922617) [13]. Table 2 shows the association of *TREML1* and *TREM2* gene expression with these 21 variants. In 399 combined AD and non-AD temporal cortex samples tested for the 16 IGAP variants, 5 SNPs showed association (uncorrected p<0.05) with increased levels of both *TREML1* and *TREM2* (rs9381040, rs2093395, rs9357347, rs9394778, rs9296359), and a sixth variant (rs9394767) was significantly associated with increased *TREML1* levels only. As shown in Fig. 3, four of the six variants that associate with increased levels of *TREML1* and *TREM2* are in a single LD block (block 2: rs9357347, rs9381040, rs2093395 and rs9394767) and in tight linkage disequilibrium with each other (r^2 0.90). Of these variants, rs9381040 has the most significant AD-risk association in the IGAP stage 1 meta-analysis (Table 2). This IGAP "hit" is located 5.5kb downstream from *TREML2* and 23.7kb upstream from *TREM2* and is associated with *TREML1* and *TREM2* expression (uncorrected p=0.0083, beta=0.086 and uncorrected p=0.048, beta=0.091, respectively). Given that the expression measures were on a log2 scale, these changes in expression are equivalent to *TREML1* and *TREM2*

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fold-changes of 1.06 and 1.07, for each copy of the minor allele, respectively. Notably, the minor allele of the IGAP "hit" rs9381040 is associated with both decreased AD-risk and increased *TREML1* and *TREM2* levels. However, based on data from the Roadmap Epigenomics Consortium [22], rs9381040 lacks evidence of regulatory potential in brain regions relevant to AD.

The variant with the most significant association with brain *TREML1* expression, which also associates with *TREM2* levels, is rs9357347 in block 2 (Fig. 3). This SNP is located 6.9kb downstream from *TREML2* and 19.6kb upstream from *TREM2* and is in tight LD with the IGAP "hit" rs9381040 (D'=0.99, r^2=0.96). As expected, the minor allele of rs9357347 is associated with reduced AD-risk (OR=0.95, 95% CI=0.91–0.98, uncorrected p=0.001) in the IGAP meta-analysis [12] and with increased *TREML1* and *TREM2* expression in the temporal cortex in the Mayo Clinic WG-DASL eQTL analysis (uncorrected p=0.0063, beta=0.088 and uncorrected p=0.046, beta=0.090, respectively) (Table 2 **and** Fig. S2). These beta coefficients can be interpreted as an estimated 1.06-fold change of both *TREML1* and *TREM2,* per rs9357347 minor allele, in this temporal cortex dataset. Given the tight LD between variants at this locus, and the potential for co-regulation of *TREM* genes at this cluster across tissues, correcting for the 21 variants and two genes tested is unwarranted. It should be noted however, that although the association of rs9357347 with gene expression would not survive a strict Bonferroni correction for all 21 variants evaluated, its association with *TREML1* gene expression in temporal cortex remains significant (Bonferroni corrected p=0.019) when applying a Bonferroni correction for the tissues and genes tested (*TREML1* in cerebellum, *TREML1* in temporal cortex and *TREM2* in temporal cortex). Unlike the IGAP "hit" (rs9381040), rs9357347 lies within sequence subject to histone modifications and within a DNAse hypersensitive site detected by the Roadmap Epigenomics Consortium [22] in brain regions relevant to AD pathology such as the hippocampus. Furthermore, this variant is predicted to affect transcription factor binding (SP1 and PPAR) as catalogued in HaploReg (http://www.broadinstitute.org/mammals/haploreg/haploreg.php) [23]. Consequently, it has a compelling Regulome score of 2b (http://www.regulomedb.org/) due to the evidence of its regulatory potential [16] (Table 2). Indeed, of all the variants with an AD-risk p-value<0.0015 in the IGAP meta-analysis, and p-values<0.05 in our WG-DASL eQTL analysis of temporal cortex *TREML1* and *TREM2* gene expression levels, rs9357347 had the greatest regulatory potential as determined by their Regulome scores (Fig. S3 and Fig. S4).

The other two variants with gene expression associations in the temporal cortex are in a different LD block (block 4: rs9394778 and rs9296359) and in tight LD with each other (r^2 = 0.67). These SNPs are more significantly associated with *TREM2* than with *TREML1* expression; however, neither has compelling evidence of regulatory potential as both have Regulome scores of 6 (Table 2). In the 374 AD and non-AD subjects with cerebellum expression measures, none of the 16 IGAP AD-risk associated variants that were tested, associate with *TREML1* gene expression (p>0.05).

We determined the extent of linkage disequilibrium (LD) between the likely regulatory variant rs9357347, the IGAP "hit" rs9381040 and the significant *TREM2* rare missense AD-risk variants p.D87N (rs142232675) and p.R47H (rs75932628) [1]. As shown in Fig. 3,

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these two *TREM2* rare missense AD-risk variants are not in LD with either rs9357347 or rs9381040. This suggests that the protective effect of the regulatory rs9357347 and the IGAP "hit" are independent of the rare, missense *TREM2* variants.

We next evaluated LD amongst variants tested at this locus, including common *TREM* locus variants previously reported to have associations with AD-risk (rs3747742) [11], increased AD pathology burden and cognitive decline (rs7759295 and rs6910730) [14], or with lower CSF ptau (rs6922617 and rs6916710) [13]. The missense *TREML2* variant rs3747742 (p.S144G) is in LD with the regulatory variant implicated in our study, rs9357347. As reported, rs3747742 is also in LD with rs9381040 (IGAP hit); and as expected associates with reduced AD-risk (uncorrected p=0.009), however with slightly lesser significance than the AD-risk association of the regulatory rs9357347 (uncorrected p=0.001) or the IGAP "hit" rs9381040 (uncorrected p=0.0006). Further, the association of rs3747742 with *TREML1* expression is not as significant as that of rs9357347. In addition, rs3747742 has no association with brain *TREM2* levels, and has a weak Regulome score of 6 (Table 2).

Of the four common *TREM* locus variants that associate with AD endophenotypes, only rs6916710 is in tight LD with the regulatory rs9357347 ($D'=0.91$, $r^2=0.62$). However, rs6916710, does not show significant association with AD-risk in the IGAP meta-analysis (uncorrected p=0.103) nor with *TREML1* or *TREM2* gene expression levels (Table 2).

None of the other three common *TREM* locus variants with reported AD-endophenotype associations are in tight LD with the regulatory rs9357347 or any of the other *TREM* locus variants that are associated with AD-risk. Only rs7759295 showed association with *TREML1* gene expression (uncorrected p=0.04), but neither this nor any of the other AD-endophenotype-associated SNPs have evidence of AD-risk association or Regulome scores that are indicative of likely regulatory function (Fig. 3 and Table 2).

Utilizing publicly available RNAseq data from two independent cohorts (Table 1) that do not overlap with the samples included in the WG-DASL eQTL analysis, we sought replication of the rs9357347 association with *TREML1* and *TREM2*. Although in the ROS/MAP RNAseq dataset a significant association was only detected with the levels of *TREM2;* and the Mayo Clinic RNAseq dataset showed no evidence of association with either *TREM2* or *TREML1* (Table 3), meta-analysis from the three independent study p-values (Mayo WG-DASL, Mayo RNAseq and ROS/MAP RNAseq) yielded significant results (*TREML1* uncorrected $p=3.4\times10^{-2}$; *TREM2* uncorrected $p=3.5\times10^{-3}$), confirming the association of the rs9357347 minor allele with increased *TREML1* and *TREM2* gene expression. The evidence of association with *TREM2* expression was greater upon meta-analysis compared to the association observed in our discovery dataset; whereas the evidence of association with *TREML1* expression was slightly greater in our discovery dataset compared to the meta-analysis.

The association of *TREML1* and/or *TREM2* gene expression with diagnosis was also tested. The box plots in Fig. S5 show the difference in expression between AD and nonAD subjects, and indicate the significance of the association for each comparison (uncorrected p-values). We observe a consistent trend of higher *TREML1* and *TREM2* expression in AD versus

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nonADs, although some of these associations do not reach statistical significance (Mayo WG-DASL: *TREML1* p=1.9×10^{-6}, *TREM2* p=1.1×10^{-1}; Mayo Clinic RNAseq: *TREML1* p=1.4×10^{-2}, *TREM2* p=8.5×10^{-7}; ROS/MAP RNAseq: *TREML1* p=6.6×10^{-1}, *TREM2* p=5.2×10^{-2}).

4. Discussion

In this study, we first sought to characterize the brain expression of *TREM* locus genes based on the premise that those *TREM* cluster genes that are expressed in the brain are likely to be candidate AD-risk genes. We determined that besides *TREM2*, only *TREML1* has reliable expression in the brain regions we studied. Whereas *TREML1* is expressed in both cerebellum and temporal cortex of all subjects, *TREM2* is expressed in 98% of temporal cortex and 41% of cerebellum samples. This suggests that cerebellar levels of *TREM2* are lower than those for temporal cortex, consistent with previous reports showing higher gene levels in the temporal cortex than cerebellum [24] and higher protein levels correlating with AD neuropathology [25]. In contrast, *TREM1, TREML2* and *TREML4* are expressed in only 0%–17% of the subjects. While lack of reliable brain expression of these genes does not definitively rule them out as plausible AD-risk genes, our findings provide the strongest evidence for *TREML1*, besides *TREM2*, as most likely *TREM* locus genes for further studies in AD.

Consequently, we focused our studies on *TREML1* and *TREM2*, and utilized their brain expression levels as endophenotypes to identify putative regulatory variants that modify risk for AD. Focusing on brain *TREML1* and *TREM2* expression associations with variants at the *TREM* locus that also show evidence of AD-risk association in the publicly available IGAP meta-analysis, we identified a putative regulatory variant, rs9357347, located between *TREM2* and *TREML2.* The minor allele of this variant is associated with both decreased AD-risk and with increased *TREML1* and *TREM2* brain expression in the temporal cortex, although not reaching genome-wide or transcriptome-wide significance criteria. The direction of effect of this variant on AD-risk and brain expression levels of these genes appears to be biologically congruent based on the known functions of these genes.

TREML1, which is also known as TREM-like transcript 1 (TLT-1), is a myeloid receptor expressed exclusively in the α-granules of platelets and megakaryocytes [26]. Identification of higher levels of soluble TREML1 (sTLT-1) in septic patients vs. controls and development of hemorrhage in mice lacking *Treml1* when exposed to inflammatory injury led to the conclusion that TREML1 functions to maintain vascular integrity during inflammation [27]. Further, TREML1 was shown to dampen leukocyte activation during sepsis, and inhibited pro-inflammatory activation of TREM1 by competing with its ligand [28]. These studies strongly support a role for TREML1 in promoting vascular homeostasis and limiting inflammation.

Functional, *in-vitro* studies of *TREM2* rare, missense mutations revealed reduced TREM2 function as a consequence of decreased maturation and ectodomain shedding, also supported by findings of decreased soluble TREM2 levels in the cerebrospinal (CSF) levels of patients with these mutations [13, 29]. TREM2 deficiency also led to increased amyloid pathology

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and neuronal loss in the 5XFAD mouse model of AD [30]. Interestingly, TREM2 deficiency in an ischemic mouse model resulted in reduced phagocytosis and resorption of infarcted brain tissue, and worse neurological recovery [31]. Collectively, these findings support a neuroprotective role for TREM2 in various neuronal injury models. There are, however, studies with contradictory results for TREM2. In a different mouse model of AD (APP/PS1), knock-out of *Trem2*, resulted in reduction of macrophages infiltrating from the periphery, along with less brain inflammation and reduced amyloid and tau pathology [32]. These opposite findings of *Trem2* knock-out could be due to differences in the mouse models of Alzheimer's disease tested, different *Trem2* knockout mouse lines, and analyses performed at different time points (early stages versus later stages of Alzheimer's disease).

Given these collective data, a regulatory variant that enhances levels of *TREML1* in platelets, and levels of *TREM2* in brain resident microglia could conceivably promote vascular homeostasis and limit inflammatory damage to neurons in AD and potentially other nervous system diseases. Indeed, rs9357347 has compelling evidence of regulatory potential as it is located in a known DNase hypersensitive site and affects histone modification in the hippocampus and transcription factor binding, according to the evidence compiled in the Regulome database and HaploReg [16, 23]. Interestingly, rs9357347 is predicted to affect transcription factor binding (SP1 and PPAR) as catalogued in HaploReg. These two transcription factors are known be important in regulating key players in the inflammatory response and lipid metabolism [33, 34]. Further, rs9357347 shows the most significant association with *TREML1* gene expression amongst variants at the *TREM* locus with IGAP meta-analysis AD-risk p-values 0.0015, in addition to its association with brain *TREM2* levels.

The regulatory rs9357347 SNP is in the same haplotype block as the variant with the most significant AD-risk association at the *TREM* locus in the IGAP meta-analysis, rs9381040, which is an intergenic variant downstream of *TREML2*. Though this IGAP *TREM* locus "hit" SNP has greater evidence of AD-risk association than rs9357347, there is no evidence of regulatory potential for rs9381040 in brain regions relevant to AD.

While the fold change estimates in gene expression associated with rs9357347-C are modest at 6–7%, the biological impact of the increase attributed to each copy of the minor allele, can be significant and may provide sufficient protection from disease in some individuals, particularly when considered over a lifetime. Furthermore, these estimates are based on RNA isolated from tissue samples and not microglial cells where both *TREM2* and *TREML1* are predominantly expressed [35], and where expression levels of these genes may be impacted to a greater extent by regulatory variants. Indeed, it is worth noting that we observed a trend toward higher *TREML1* and *TREM2* expression in AD subjects, which could be a reflection of microglial activation and/or proliferation known to occur in AD brains. Future gene expression studies from isolated microglia of AD subjects and controls would provide additional valuable insights as to the impact of these expression changes on the biology of microglial cell function.

The *TREML2* p.S144G variant [11], which associates with reduced AD-risk, is also in LD with both rs9357347 and rs9381040. Though proposed to be the functional variant that

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accounts for the IGAP signal at this locus, *TREML2* p.S144G is not predicted to have a functional consequence based on PolyPhen2 nor does it have evidence of regulatory potential. Further, *TREML2* expression is too low to be reliably measured in brain tissue (TCX and CER). This raises the possibility that the association with *TREML2* p.S144G is due to its LD with a functional variant(s) that influences the function or level of a nearby *TREM* gene(s), such as *TREML1* or *TREM2.* Alternatively, the protective effect of p.S144G could be mediated directly through the function of TREML2 in a cell with abundant expression, such as macrophages, in which TREML2 is known to be upregulated in response to inflammation, [36]. It is also possible that significant rs9357347 eQTL associations would be detected with *TREML2* or other *TREM* locus transcripts in tissues were these genes are more abundantly expressed.

Our findings therefore challenge the conclusion that p.S144G is the only functional variant accounting for the protective effect detected in the IGAP meta-analysis at this locus, and propose rs9357347 as an alternative functional variant with regulatory effects. In reality, both variants could have functional consequences and contribute to the IGAP signal. It should be emphasized that, as demonstrated in our LD analysis, *TREM2* p.R47H is not in LD with these two variants, and thus affects AD-risk independently. Both rs9357347 and p.S144G should be tested for their functional potential and influence on outcomes of inflammation and neuroprotection. It remains possible that rs9357347 is in LD with an untested true functional variant with effects on transcription and AD-risk. It is likewise possible that while rs9357347 is associated with both AD-risk and gene expression levels, these joint effects are coincidental due to LD, rather than being related. These possibilities need to be explored through sequencing of the entire *TREM* locus, or via targeted sequencing of LD block 2 where rs9357347 resides. Thus, our findings provide a testable hypothesis for a strong candidate functional variant, specific transcription factors and their effects on *TREML1* and *TREM2* levels.

Furthermore, our investigation of variants previously shown to associate with AD-related endophenotypes [13–15] suggests that these are unlikely to be functional AD-risk variants *per se*, though it remains possible that they are markers of functional variants at the *TREM* locus.

In summary, we characterized expression of *TREM* genes in cerebellum and temporal cortex and determined *TREML1* and *TREM2* to be the only reliably expressed *TREM* genes in these brain regions. We identified rs9357347 as a putative regulatory variant that is associated with protection from AD and with increased *TREML1* and *TREM2* brain levels, and nominate rs9357347 as one of the functional variants that accounts for the IGAP AD-risk signal. Additional studies are needed to validate the function of this variant, and to explore the possibility of the presence of other variants at this locus that could contribute to associations observed with rs9357347. Importantly, these findings suggest a potential link between *TREML1* and *TREM2*, as well as vascular homeostasis and neuroinflammation as related mediators of neuronal protection and injury in AD and possibly other central nervous system diseases.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Research in Context

Systematic review

We performed a comprehensive review of existing literature investigating the role of the *TREM* locus in AD. Although the involvement of *TREM* genes in AD pathophysiology and the underlying variants modifying AD-risk remain unclear, there have been several studies demonstrating association with AD risk and its endophenotypes.

Interpretation

We hypothesized that some variants at the *TREM* locus may modify AD-risk via regulation of *TREM* gene expression. We found a variant in a regulatory region (rs9357347-C) at the *TREM* locus that associates with reduced AD risk and higher *TREML1* and *TREM2* brain gene expression.

Future directions

Our findings nominate regulation of brain *TREML1* and *TREM2* as a potential mechanism for AD risk modification by *TREM* locus variants. In-depth sequencing of the *TREM* locus is needed to fully characterize regulatory variants at this locus that may modify AD-risk.

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Highlights

- rs9357347-C, 5′ *TREM2*, is associated with reduced AD-risk ($p_{uncorrected}=1\times10^{-03}$).
- *TREM2* and *TREML1* are the only *TREM* cluster genes with reliable brain expression.
- Higher brain levels of *TREM2* and *TREML1* associate with rs9357347-C.
- rs9357347 is predicted to affect transcription factor binding (SP1 and PPAR).
- Increased gene expression of *TREML1* and *TREM2* may reduce AD-risk.

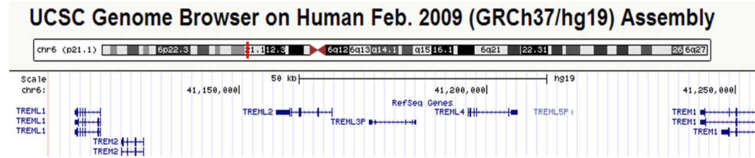

Fig. 1. *TREM* gene cluster on Chr 6p21.1

The chromosomal positions are based on the human genome assembly from February 2009 (GRCh37/hg19). There are seven RefSeq genes at the *TREM* locus (*TREM1*, *TREML1*, *TREM2*, *TREML2*, *TREML3P*, *TREML4* and *TREML5P*); however, *TREML3P* and *TREML5P* are non-coding pseudogenes. The transcript figures are taken from the UCSC Genome Browser.

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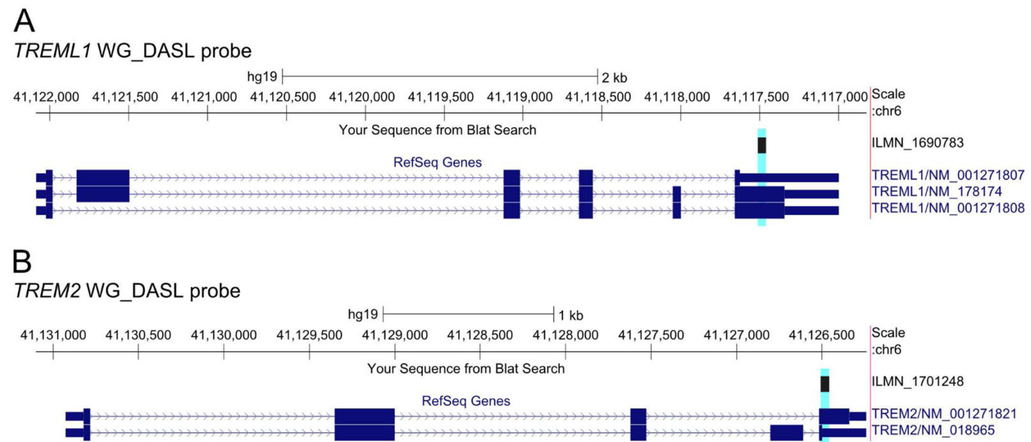

Fig. 2. Location of *TREML1* and *TREM2* WG-DASL probes

The location of the (A) *TREML1* and (B) *TREM2* WG-DASL probes (highlighted in light blue) are shown relative to their Refseq transcripts. The chromosomal positions are based on the human genome assembly from February 2009 (GRCh37/hg19). As shown, both of these probes are complementary to all RefSeq transcripts for the respective gene. The transcript figures are taken from the UCSC Genome Browser.

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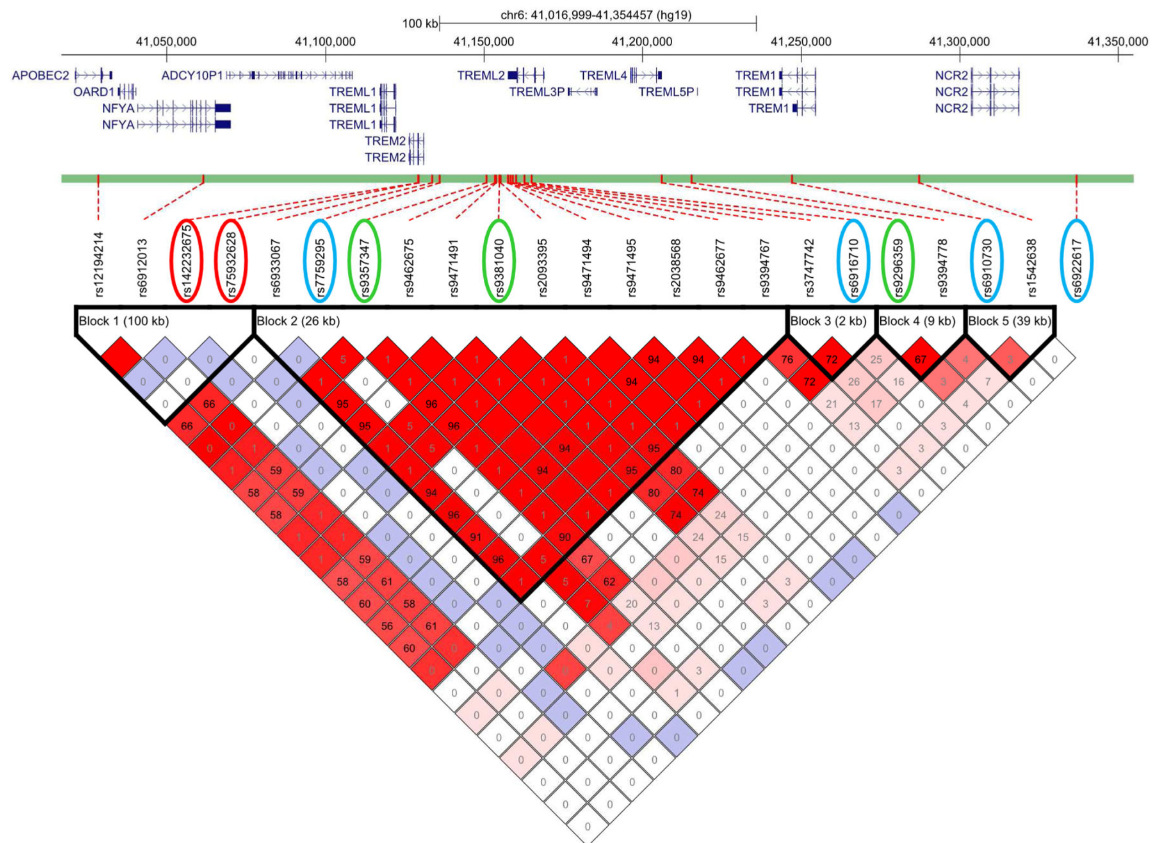

Fig. 3. LD Plot of *TREM* locus variants

LD plot of *TREM* locus variants where haplotype blocks were determined with the solid spine definition; square colors correspond to D′ (tight LD=warmer colors, weak LD=cooler colors) and r^2 values are shown within the squares (Supplementary Methods). Red circles: The rare *TREM2* AD-risk missense variants rs142232675 (p.D87N) and rs75932628 (p.R47H) [1]. Blue circles: Variants that associate with increased AD pathology burden and cognitive decline (rs7759295 and rs6910730) [14], or with lower CSF ptau (rs6922617 and rs6916710) [13]. Green circles: The variant with the most significant AD-risk association in the IGAP meta-analysis (rs9381040); rs9357347, which has the most significant *TREML1* gene expression association, also shows association with *TREM2* gene expression, IGAP AD-risk association and the best Regulome score within all tested SNPs; and rs9296359 which has the most significant association with *TREM2* expression. RefSeq gene transcripts are shown above the LD plot relative to the variant position according to the February 2009 human genome assembly (GRCh37hg19) across the targeted genomic region (*TREM* gene +/−100 kb: chr6:41016999–41354457).

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Table 1

Description of samples included in the discovery and replication cohorts utilized for eQTL analysis.

	Mayo Clinic WG-DASL				Mayo Clinic RNAseq		ROS/MAP RNAseq	
	CER		TCX		TCX		PFCX	
	AD	Non-AD	AD	Non-AD	AD	non-AD	AD	non-AD
N	197	177	202	197	84	48	288	206
Mean age +/− SD	73.6 ± 5.6	71.7 ± 5.5	73.6 ± 5.5	71.6 ± 5.6	83.2 ± 8.7	85.7 ± 8.3	89.8 ± 5.8	86.5 ± 7.2
Female, N (%)	101 (51%)	63 (36%)	108 (53%)	78 (40%)	48 (57%)	26 (54%)	186 (65%)	121 (59%)
% *APOE* ε4+	64%	25%	61%	25%	51%	17%	34%	12%

Samples included in the Mayo Clinic eGWAS (discovery cohort), with cerebellar (CER) and temporal cortex (TCX) gene expression measurements from Illumina WG-DASL arrays have been previously described [17]. Samples in the Mayo Clinic RNAseq cohort (replication cohort #1) had temporal cortex gene expression measurements, and did not overlap with the Mayo eGWAS (WG-DASL) cohort. The ROS/MAP RNAseq cohort (replication cohort #2) had dorsolateral prefrontal cortex (PFCX) gene expression measurements, and did not overlap with the Mayo eGWAS (WG-DASL), or with the Mayo Clinic RNAseq cohort. The RNAseq data for these two cohorts is available at the Sage Synapse, AMP AD Knowledge Portal (https://www.synapse.org/#!Synapse:syn2580853/wiki/66722), under synapse IDs syn3388564 (ROS/MAP RNAseq) and syn3163039 (Mayo RNAseq).

Table 2

with AD-risk and *TREM* WG-DASL brain gene expression levels.

D-Risk (IGAP Stage1 Meta-analysis)			Brain eQTL (Mayo Clinic eGWAS)						Regulome Score	HapMap CEU MAF
Non Effect Allele	OR (95% CI)	P-value	TREML1 CER BETA	TREML1 CER P	TREML1 TCX BETA	TREML1 TCX P	TREM2 TCX BETA	TREM2 TCX P		
C	**0.94 (0.91 – 0.98)**	**5.97E-04**	**0.021**	**2.30E-01**	**0.086**	***8.30E-03***	**0.091**	***4.80E-02***	**NA**	**26.70%**
G	0.94 (0.91 – 0.98)	6.40E-04	0.021	2.30E-01	0.086	*8.30E-03*	0.091	*4.80E-02*	6	27.90%
G	1.14 (1.05 – 1.23)	7.93E-04	0.018	7.80E-01	–0.08	3.90E-01	–0.186	1.60E-01	5	8.30%
A	1.16 (1.06 – 1.26)	8.36E-04	–0.081	9.40E-02	–0.104	2.70E-01	–0.129	3.20E-01	6	4.20%
G	1.15 (1.06 – 1.25)	9.54E-04	–0.015	7.50E-01	–0.114	1.60E-01	–0.207	6.50E-02	5	3.60%
T	1.15 (1.06 – 1.25)	1.07E-03	–0.013	7.60E-01	–0.098	2.10E-01	–0.134	2.10E-01	7	3.50%
A	**0.95 (0.91 – 0.98)**	**1.10E-03**	**0.013**	**4.60E-01**	**0.088**	***6.30E-03***	**0.09**	***4.60E-02***	**2b**	**28.10%**
A	0.95 (0.91 – 0.98)	1.14E-03	0.011	5.70E-01	0.096	*6.50E-03*	0.083	1.00E-01	5	28.80%
A	1.06 (1.02 – 1.09)	1.14E-03	–0.022	2.20E-01	–0.035	2.90E-01	–0.064	1.60E-01	4	28.30%
C	1.15 (1.05 – 1.26)	1.31E-03	–0.015	7.50E-01	–0.114	1.60E-01	–0.207	6.50E-02	7	3.50%
C	1.15 (1.05 – 1.25)	1.40E-03	0.014	8.30E-01	–0.099	2.90E-01	–0.235	7.20E-02	7	3.50%
T	1.15 (1.05 – 1.25)	1.41E-03	0.016	8.10E-01	–0.099	2.90E-01	–0.235	7.30E-02	7	4.30%
G	0.95 (0.92 – 0.98)	1.44E-03	0.015	3.30E-01	0.065	*2.70E-02*	0.099	*1.50E-02*	6	39.80%
C	1.15 (1.05 – 1.25)	1.46E-03	0.01	8.70E-01	–0.102	2.60E-01	–0.221	8.20E-02	6	4.50%
T	1.15 (1.05 – 1.25)	1.48E-03	–0.076	1.20E-01	–0.104	2.70E-01	–0.124	3.40E-01	5	2.70%
G	0.95 (0.92 – 0.98)	1.48E-03	0.017	2.80E-01	0.066	*2.40E-02*	0.116	*4.60E-03*	6	27.40%
T	0.96 (0.92 – 0.99)	8.56E-03	0.018	2.90E-01	0.072	*2.30E-02*	0.064	1.50E-01	6	28.30%

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D-Risk (IGAP Stage1 Meta-analysis)			Brain eQTL (Mayo Clinic eGWAS)						Regulome Score	HapMap CEU MAF
Non Effect Allele	OR (95% CI)	P-value	TREML1 CER BETA	TREML1 CER P	TREML1 TCX BETA	TREML1 TCX P	TREM2 TCX BETA	TREM2 TCX P		
C	0.97 (0.94 – 1.01)	1.03E-01	0.013	4.30E-01	0.054	7.70E-02	0.072	9.20E-02	7	38.40%
C	0.98 (0.93 – 1.03)	3.66E-01	−0.023	3.50E-01	0.094	*4.00E-02*	−0.008	9.00E-01	6	13.30%
A	0.99 (0.94 – 1.04)	6.86E-01	−0.046	8.50E-02	−0.079	1.20E-01	−0.032	6.50E-01	4	8.40%
G	0.99 (0.93 – 1.05)	6.98E-01	−0.033	2.60E-01	−0.098	7.40E-02	0.011	8.90E-01	7	8.50%

that had an AD-risk p 0.0015 in the IGAP stage 1 meta-analysis (top 16 rows), as well as 5 common *TREM* locus variants with previous reports
SNP marked with an *). AD-risk association results are from the publicly available IGAP meta-analysis stage 1. Brain gene expression associations
(CER) and temporal cortex (TCX) gene expression measurements with Illumina WG-DASL arrays with *TREML1* probe ILMN_1690783 and
s table are uncorrected, given that many of the variants tested at this locus are correlated due to LD, and the genes tested are likely co-regulated.
rected p<0.05) are underlined and in italic font. The variant with the most significant AD-risk association in the IGAP meta-analysis (**rs9381040**),
sociation and best Regulome score (**rs9357347**) are in bold font. OR (95% CI): odds ratio and 95% confidence interval. Given that the eGWAS
the Mayo eGWAS beta coefficients = 2^{beta}.

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Table 3

Meta-analysis of rs9357347 eQTL results from three independent datasets.

Dataset	Sample size	MAF	*TREML1*			*TREM2*		
			beta	**SE**	**P**	**beta**	**SE**	**P***
Mayo WG-DASL	380	0.307	0.088	0.032	6.28E-03	0.090	0.045	4.61E-02
Mayo Clinic RNAseq	132	0.311	−0.030	0.108	7.82E-01	0.084	0.128	5.13E-01
ROS/MAP RNAseq	494	0.281	0.089	0.114	4.36E-01	0.124	0.060	3.77E-02
Meta-analysis	1006		+−+		**3.36E-02 (6.72E-02)** *	+++		**3.54E-03 (7.08-03)** *

Meta-analysis of rs9357347 eQTL results from temporal cortex (Mayo WG-DASL and Mayo Clinic RNAseq) and dorsolateral prefrontal cortex samples (ROS/MAP). MAF = minor allele frequency. SE = standard error. Since in all three datasets the expression measures analyzed were on a log2 scale, fold-change for the beta coefficients = 2^{beta}. The meta-analysis was performed using METAL, with weighted average of z-scores from the individual study p-values, weighted according their sample size.

* All p-values shown on this table are uncorrected, except for a strict Bonferroni correction applied to the p-value shown in parenthesis, which accounts for the two different genes that were tested.