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# MS *AHI1* genetic risk promotes IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells

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## ABSTRACT

**Objective:** To study the influence of the Abelson helper integration site 1 (*AHI1*) locus associated with MS susceptibility on CD4<sup>+</sup> T cell function.

**Methods:** We characterized the chromatin state of T cells in the MS-associated *AHI1* linkage disequilibrium (LD) block. The expression and the role of the *AHI1* variant were examined in T cells from genotyped healthy subjects who were recruited from the PhenoGenetic Project, and the function of *AHI1* was explored using T cells from *Ahi1* knockout mice.

**Results:** Chromatin state analysis reveals that the LD block containing rs4896153, which is robustly associated with MS susceptibility (odds ratio 1.15,  $p = 1.65 \times 10^{-13}$ ), overlaps with strong enhancer regions that are present in human naive and memory CD4<sup>+</sup> T cells. Relative to the rs4896153<sup>A</sup> protective allele, the rs4896153<sup>T</sup> susceptibility allele is associated with decreased *AHI1* mRNA expression, specifically in naive CD4<sup>+</sup> T cells ( $p = 1.73 \times 10^{-74}$ ,  $n = 213$ ), and we replicate this effect in an independent set of subjects ( $p = 2.5 \times 10^{-9}$ ,  $n = 32$ ). Functional studies then showed that the rs4896153<sup>T</sup> risk variant and the subsequent decreased *AHI1* expression were associated with reduced CD4<sup>+</sup> T cell proliferation and a specific differentiation into interferon gamma (IFN $\gamma$ )-positive T cells when compared with the protective rs4896153<sup>A</sup> allele. This T cell phenotype was also observed in murine CD4<sup>+</sup> T cells with genetic deletion of *Ahi1*.

**Conclusions:** Our findings suggest that the effect of the *AHI1* genetic risk for MS is mediated, in part, by enhancing the development of proinflammatory IFN $\gamma$ <sup>+</sup> T cells that have previously been implicated in MS and its mouse models. *Neurol Neuroimmunol Neuroinflamm* 2018;5:e414; doi: 10.1212/NXI.0000000000000414

## GLOSSARY

**AHI1** = Abelson helper integration site 1; **EAE** = experimental autoimmune encephalomyelitis; **eQTL** = expression quantitative trait locus; **GWAS** = genome-wide association study; **IFN $\gamma$**  = interferon gamma; **IL** = interleukin; **LD** = linkage disequilibrium; **MFI** = mean fluorescence intensity; **OR** = odds ratio; **PBMC** = peripheral blood mononuclear cell; **PGP** = PhenoGenetic Project; **SNP** = single nucleotide polymorphism; **TCR** = T cell receptor; **TF** = transcription factor; **Th** = T helper; **TSS** = transcription start site; **WT** = wild type.

MS is a chronic inflammatory disease of the CNS characterized by episodes of neuronal demyelination in genetically susceptible individuals on exposure to environmental triggers. Genome-wide association studies (GWASs) have identified and validated over 110 non-major histocompatibility complex genetic variants associated with MS, and many of these are implicated in the function of CD4<sup>+</sup> and CD8<sup>+</sup> T cells that are present in MS lesions.<sup>1,2</sup> Considerable work over the years in studies of patients with MS and in animal models of MS has implicated proinflammatory CD4<sup>+</sup> T helper (Th)1 cells (which produce interferon gamma [IFN $\gamma$ ]) and Th17 cells (interleukin [IL]-17-producers) as important mediators of new relapsing disease

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activity.<sup>3,4</sup> These 2 types of pathogenic cells are implicated in MS and could account for some of the immunologic and clinical heterogeneity of the disease.<sup>5</sup>

Recent analysis of Th17 cell development and pathogenicity revealed a critical role of IFN $\gamma$  for the severity of the disease in mouse models of MS. Indeed, in a reporter mouse model, it has been found that Th17 cells could play a pathogenic role in MS by converting their phenotype into a proinflammatory Th1 profile in which committed Th17 cells give rise to a progeny that shifts toward enhanced IFN $\gamma$  expression.<sup>6</sup> Similarly, in humans, there is evidence indicating that, in the presence of IL-12, Th17 cells also produce IFN $\gamma$ . These cells, which produce both IL-17 and IFN $\gamma$ , are called Th1/17 cells,<sup>7</sup> and, together with “non-classical Th1 cells,” they might contribute to disease pathogenesis through properties shared by both the Th1 and Th17 subsets.<sup>8</sup> Moreover, Th17 cells producing IFN- $\gamma$  are enriched in myelin oligodendrocyte glycoprotein-specific T cells.<sup>9</sup> IFN $\gamma$  produced by these cells could strongly activate macrophages whose infiltration in the CNS correlates with experimental autoimmune encephalomyelitis (EAE) severity.<sup>10</sup>

It is necessary to understand the functional consequences of disease-associated genetic variations as we strive to unravel the causal chain of events linking genetic risk factors to clinical syndromes. Separate from GWASs, expression quantitative trait locus (eQTL) studies have been used to map a genetic variants contribution to variation in gene expression.<sup>11</sup> However, moving from a list of eQTL effects to phenotypic changes, regulation of disease activity remains challenging and is often unfruitful.

In this study, we integrated results from large-scale, genome-wide disease gene discovery studies with eQTL studies to identify the MS susceptibility variant with a transcriptional effect that influences T cell phenotypes. We examined such a variant in the MS susceptibility gene Abelson helper integration site 1 (*AH1*) by first characterizing and validating its *cis*-eQTL effect, and we go on to demonstrate that the *AH1* genetic variant is involved in the regulation of human CD4<sup>+</sup> T cell proliferation and IFN $\gamma$  production. Furthermore,

we leveraged a mouse model with genetic deletion of *Abi1* to analyze the effects of *Abi1* deficiency on murine CD4<sup>+</sup> T cell function. Consistent with the human results, the mouse results suggest that the mechanism of the *AH1* genetic risk involves the differentiation of naive CD4<sup>+</sup> T cells into proinflammatory IFN $\gamma$ <sup>+</sup> T cells, which could contribute to MS onset.

**METHODS PhenoGenetic Project.** The PhenoGenetic Project (PGP) is a biobank of healthy individuals, older than 18 years, who are free from chronic inflammatory infections and metabolic diseases.<sup>11</sup> Genotyped individuals ( $n = 32$ ) of European descent bearing risk (TT), protective (AA), or heterozygous (AT) genotypes for rs4896153 were selected from this cohort and used for this study.

**PBMC isolation and CD4<sup>+</sup> T cell culture.** Peripheral venous blood was obtained from healthy control volunteers in compliance with protocols approved by the Institutional Review Board of Partners Healthcare as part of the PGP. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) gradient centrifugation. PBMCs were frozen at a concentration of  $1-3 \times 10^7$  cells/mL in 10% DMSO and stored at  $-80^\circ\text{C}$ . CD4<sup>+</sup>CD45RO<sup>-</sup>CD45RA<sup>+</sup> naive T cells were isolated from cryopreserved PBMCs by negative selection (Miltenyi Biotec, Auburn, CA). Naive CD4<sup>+</sup> T cells were cultured in 96-well round bottom plates (Costar, Cambridge, MA) at  $2 \times 10^4$  cells/well in serum-free X-Vivo 15 medium (BioWhittaker, Walkersville, MD) and stimulated with plate-bound anti-CD3 OKT3 (BioXCell, 10  $\mu\text{g}/\text{mL}$ ) and anti-CD28 (BioXCell, 1  $\mu\text{g}/\text{mL}$ ) antibodies for 6–8 days.

**Mouse CD4<sup>+</sup> T cell isolation and activation.** *Abi1*<sup>-/-</sup> mice were generated as described previously<sup>12</sup> and backcrossed >10 generations onto a Friend Virus B NIH Jackson background. CD4<sup>+</sup> T cells were isolated from the spleens of *Abi1*<sup>-/-</sup> and littermate control mice by negative selection (Miltenyi Biotec, Auburn, CA) and cultured in 96-well round bottom plates (Costar, Cambridge, MA) at  $1 \times 10^5$  cells/well in serum-free X-Vivo 15 medium (BioWhittaker, Walkersville, MD) supplemented with 1:1000 BME and stimulated with plate-bound anti-CD3 (BioXCell, 0.25–2  $\mu\text{g}/\text{mL}$ ) and anti-CD28 (BioXCell, 0.25–2  $\mu\text{g}/\text{mL}$ ) antibodies for 4 days. For Th1 cell polarization, recombinant IL-12 was used at 10 ng/mL. For Th17 cell polarization, recombinant IL-6 (10 ng/mL) and transforming growth factor- $\beta$ 1 (3 ng/mL) were used. All recombinant proteins were from R&D Systems (Minneapolis, MN).

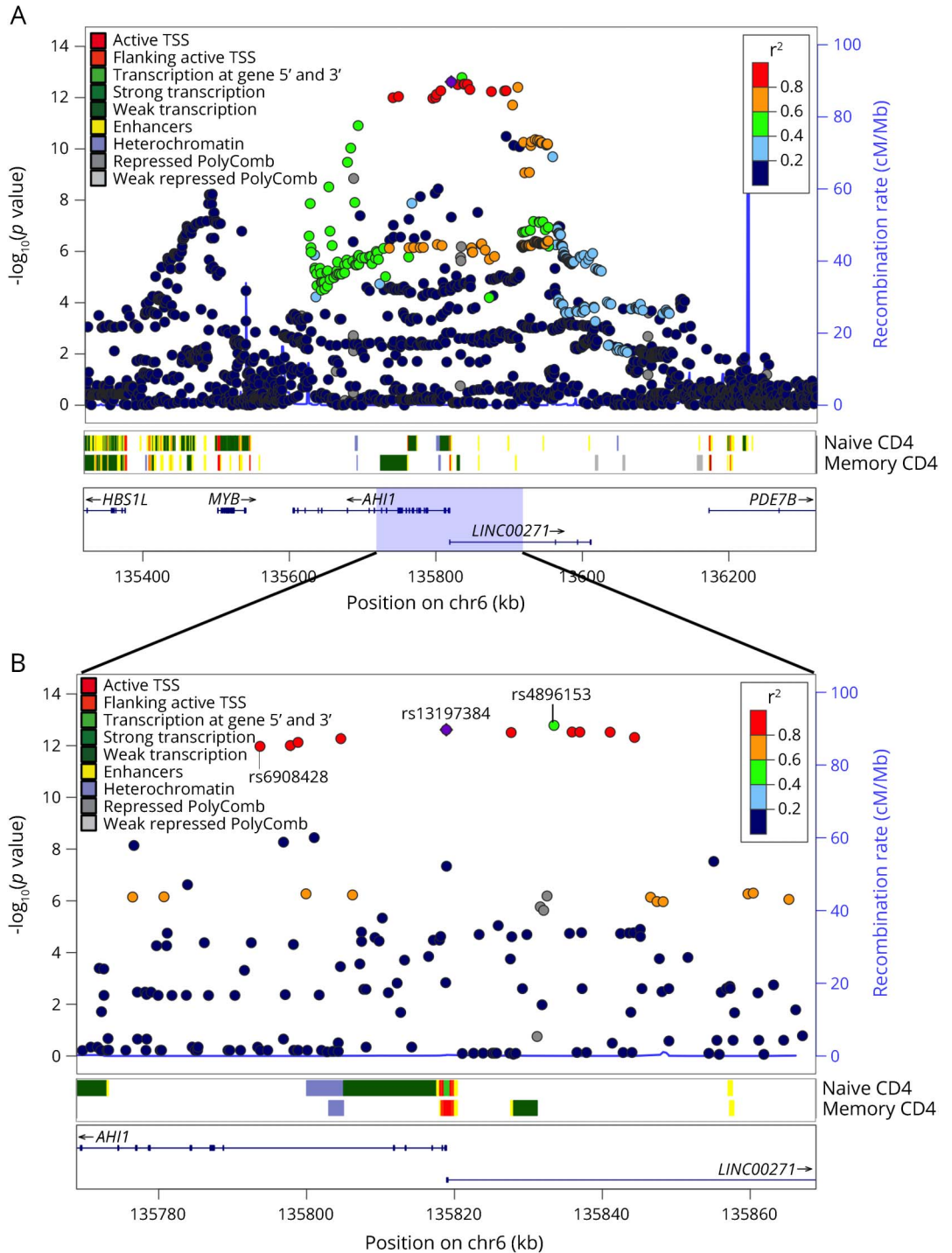
**Cytokine measurement by flow cytometry.** CD4<sup>+</sup> T cells were stimulated for 4 hours with PMA (50 ng/mL) and Ionomycin (250 ng/mL, both from Sigma-Aldrich, St. Louis, MO) in the presence of GolgiStop (BD Biosciences), then washed and fixed/permeabilized with Cytotfix/Cytoperm (BD Biosciences, San Jose, CA) according to manufacturer’s instructions. Cells were stained with 7-AAD (BD Biosciences), or Aqua LIVE/DEAD Fixable stain (Life Technologies, Carlsbad, CA) and FITC-IFN $\gamma$  (clone 25723; R&D Systems). Data were acquired on a FACSCalibur or LSRII (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

**RNA isolation and qPCR.** RNA was isolated using the Qiagen Plus Micro Kit (Qiagen, Venlo, the Netherlands) and converted to

cDNA via reverse transcriptase by random hexamers and *MuLV* transcriptase (Applied Biosystems, Foster City, CA). Samples were subjected to real-time PCR analysis on PRISM 7000

Sequencer Detection System (Applied Biosystems) under standard conditions. The primers used for the human *AH11* were purchased from Applied Biosystems: isoform A forward

**Figure 1** SNAP plot of the chromatin state overlapping the SNPs in LD with the *AH11* cis-eQTL and MS susceptibility variant, rs4896153 in CD4<sup>+</sup> T cells



(A) Naive (CD4<sup>+</sup>CD25<sup>-</sup>CDRA<sup>+</sup>) and memory (CD4<sup>+</sup>CD25<sup>-</sup>CDRA<sup>-</sup>) CD4<sup>+</sup> T cell-specific chromatin state mapping was generated using the chromHMM algorithm in 1-Mb and 100-kb views surrounding the *AH11* locus. ChIP-seq data generated by the ENCODE/ROADMAP project reveal various chromatin marks including enhancers, heterochromatin, and polycomb, and various transcription marks are color coded. All the discovered SNPs from dbGAP137 are listed including their *p* value of association to MS, the recombination rate. The LD structure is represented in the *r*-square value calculated from the Broad SNAP server and is assigned in red. The strongest MS-associated variant rs4896153 (green) and the strongest eQTL variant rs6908428 are labeled. (B) Zoom in of the region overlapping the rs13197384 showing the chromatin state in naive and memory T cells. eQTL = expression quantitative trait locus; LD = linkage disequilibrium; TSS = transcription start site.

primer 5'-CCAGCTAATCATGTGGCTAGTGAAACTG-3'; reverse primer 5'-CCTCAGGGCTTAAAGGAGGGGATGC-3'; isoform B forward primer 5'-TCAGACCGACAGTCACTTTGGTGAA-3'; and reverse primer 5'-TGGTGGGATCCCA GGTCGGCTCAGT-3'. Values are represented as the difference in Ct values normalized to  $\beta 2$ -microglobulin for each sample as per the following formula: relative RNA expression =  $(2^{-\Delta C_t}) \times 10^3$ . Murine *Abi1* was measured using commercially available assays (Applied Biosystems). Relative mRNA abundance was normalized against *Gapdh*.

**Proliferation assay.** Cells were cultured for 48 hours before the addition of 1  $\mu$ Ci of [ $^3$ H]-thymidine for 16 hours. The cells were harvested and quantified using an automated sample harvester (Perkin-Elmer, Waltham, MA). [ $^3$ H]-thymidine uptake is expressed in radiation counts per minute.

**Statistical analysis.** Statistical analysis was performed using Prism version 7.0 and R version 3.2.1. Cytokine and gene expression data were analyzed using unpaired *t* tests. Results from figure 1A are from Raj et al.<sup>11</sup> and represent meta-analysis of *cis*-eQTLs at false discovery rate 0.05 in CD4<sup>+</sup> T cells from 407 individuals.

**RESULTS *AHII* locus is associated with MS susceptibility and has a *cis*-eQTL effect.** The *AHII* locus has long been associated with MS. In 2011, a GWAS identified rs11154801<sup>A</sup> with an odds ratio (OR) of 1.13 ( $p = 1 \times 10^{-13}$ ) in the *AHII* locus, and the association was initially attributed to the *MYB* gene based on its proximity to the most associated

variant.<sup>13</sup> In a subsequent study using the Immuno-Chip array, the association with rs11154801<sup>A</sup> was replicated (OR 1.11;  $p = 2.3 \times 10^{-9}$ ) and assigned to the *AHII* gene.<sup>2</sup> Neither of these studies densely genotyped this region; thus, we leveraged results of a large-scale imputation based meta-analysis of the IMSCG to identify the most strongly associated single nucleotide polymorphisms (SNPs) within this locus. In this analysis,<sup>2</sup> the original lead SNP, rs11154801<sup>A</sup> was genome-wide significant ( $p = 1.0 \times 10^{-12}$ ). However, another SNP in the region had a more statistically significant *p* value: rs4896153<sup>T</sup> (OR 1.15,  $p = 1.65 \times 10^{-13}$ ). These 2 SNPs are in moderate linkage disequilibrium (LD), with an  $r^2$  of 0.55 and *D'* of 0.96 in the European panel data of the 1000 Genome Project. Of note, rs4896153 was not present in either of the previously published studies that reported rs11154801<sup>A</sup> as the most associated SNP. We therefore used rs4896153 as the tag SNP for this MS susceptibility locus.

Next, we tested whether rs4896153 exhibited a *cis*-eQTL effect in any of the nearby genes using a large-scale data set of naive CD4<sup>+</sup> T cells from healthy genotyped individuals. We found that the rs4896153<sup>T</sup> risk allele is associated with lower RNA expression of *AHII* ( $p = 2.14 \times 10^{-24}$ ); however, an SNP nearby, rs6908428, had a stronger effect ( $p = 8.77 \times 10^{-31}$ ) after correction for batch, age, and/or gender effects (table 1 and figure e-1, <http://links.lww.com/NXI/A5>). These 2 SNPs are in moderate LD, with an  $r^2$  of 0.58 and *D'* of 0.98. Of interest, rs6908428 and rs4896153 did not alter the expression of the neighboring genes in naive T cells, suggesting that *AHII* is the main target of these 2 SNPs (table 1). When rs6908428 and rs4896153 are modeled jointly with the expression of *AHII* as the outcome in CD4<sup>+</sup> T cells, the *p* values are 0.026 and 0.537, respectively. This result indicates that rs6908428 has a marginally independent association with the expression of *AHII*, but that rs4896153 does not, and that rs6908428 may be more likely to be the functional variant. However, 1 technical explanation for the superior eQTL effect of rs6908428 (MAF 0.3173, 1000 Genomes) is that it has a better imputation INFO score of 0.96 vs 0.55 for rs4896153 (MAF 0.3007, 1000 Genomes) in our sample of 211 subjects. For instance, the MAF of rs6908428 is much closer to the 1000 Genomes MAF of 0.31, than the MAF of rs4896153 (0.467). The lower imputation metric of rs4896153 implies a less accurate dosage measurement, thereby reducing power and likely artificially diminishing the level of statistical significance. In addition, the rs6908428 SNP is also a *cis*-eQTL for *AHII* in PBMCs of MS subjects ( $p = 2 \times 10^{-22}$ ).<sup>14</sup>

To visualize the MS susceptibility LD region associated with a *cis*-eQTL effect on *AHII*, an SNAP plot

**Table 1** rs4896153 and rs6908428 SNPs influence specifically *AHII* expression but not other neighboring genes in naive CD4<sup>+</sup> T cells

SNP_label	Gene_label	<i>p</i> Value	−log <sub>10</sub> ( <i>p</i> value)	False discovery rate
rs4896153	<i>AHII</i>	2.14684E-24	23.6682	1.71747E-23
rs6908428	<i>AHII</i>	8.77405E-31	30.0568	1.40385E-29
rs4896153	<i>FAM54A</i>	9.28325E-05	4.0323	0.000495107
rs6908428	<i>FAM54A</i>	0.002694635	2.5695	0.010778541
rs4896153	<i>BCLAF1</i>	0.301578238	0.5206	0.765292219
rs6908428	<i>BCLAF1</i>	0.515228645	0.288	0.765292219
rs4896153	<i>MYB</i>	0.396825891	0.4014	0.765292219
rs6908428	<i>MYB</i>	0.957634977	0.0188	0.957634977
rs4896153	<i>MAP7</i>	0.461636352	0.3357	0.765292219
rs6908428	<i>MAP7</i>	0.420436101	0.3763	0.765292219
rs4896153	<i>ALDH8A1</i>	0.214980961	0.6676	0.687939075
rs6908428	<i>ALDH8A1</i>	0.5261384	0.2789	0.765292219
rs4896153	<i>PDE7B</i>	0.751795982	0.1239	0.91966302
rs6908428	<i>PDE7B</i>	0.718455709	0.1436	0.91966302
rs4896153	<i>HBS1L</i>	0.808351037	0.0924	0.91966302
rs6908428	<i>HBS1L</i>	0.862184081	0.0644	0.91966302

Abbreviation: SNP = single nucleotide polymorphism.

Expression quantitative trait locus (eQTL) data were analyzed in naive CD4<sup>+</sup>CD62L<sup>+</sup> T cells isolated from the peripheral blood mononuclear cells of healthy European-American subjects carrying the susceptibility or protective alleles of rs4896153 or rs6908428 ( $n = 213$ ). Statistical analyses of log<sub>10</sub>-transformed values were performed by Spearman rank correlations.

was generated (figure 1, A and B). Eleven SNPs (red and purple) are in an LD block with rs4896153 (green) as the lead MS SNP. Ten SNPs are in strong LD with this lead SNP and are considered to be the “critical set” of SNPs that are likely to include the causal variant. To prioritize variants among this set of ten SNPs, we first looked at reference epigenomic data from CD4<sup>+</sup> T cells. The transcription start site (TSS) of *AHII* is flanked by enhancer marks in both naive and memory CD4<sup>+</sup> T cells, consistent with this gene’s expression in this cell type (figure 1, A and B). The SNP rs13197384 (purple) is 4 bp downstream of the +1 TSS of *AHII* and overlaps with a region of transcription at the 5’ or 3’, specifically in naive CD4<sup>+</sup> T cells (figure 1, A and B). This SNP is therefore highlighted as being in an area of differential transcriptional activity between naive and memory T cells by existing reference epigenomic data.<sup>15</sup> Next, we investigated the functional potential of these SNPs using RegulomeDB and HaploReg V4.2: rs13197384 (TSS), rs7750586 (upstream), and rs9399148 (upstream); all have evidence of functionality based on these SNPs altering known target sequences for DNA-binding motifs and being present within DNase hypersensitivity sites that mark chromosomal segments bound to a DNA-binding protein in naive CD4<sup>+</sup> T cells (supplementary material, table e-1, <http://links.lww.com/NXI/A6>). These data prioritize these 3 SNPs for follow-up functional assessment. In an attempt to understand the influence of the SNP position within the enhancer region in CD4<sup>+</sup> T cells, we used the R package motifbreakR version 1.4.0<sup>16</sup> to identify putative transcription factor (TF) binding sites that were likely to be disrupted by the studied polymorphism. TF motifs were obtained from the JASPAR 2014 database (177 motifs for homo sapiens),<sup>17</sup> and the motifbreakR algorithm was applied with default settings: the weighted sum method (weighted by relative entropy) was used to obtain scores, and putative bindings sites were reported if the scaled score (between 0 and 1) was greater than or equal to 0.8 for the reference or alternative allele. Binding sites whose unscaled and unweighted scores for the reference and alternative allele did not differ by at least 0.4 were considered as unaffected by the SNP and not reported (supplementary material, table e-2, <http://links.lww.com/NXI/A7>). We identified candidate TFs whose binding could be affected by the presence of 1 of these 3 SNPs. For instance, the transcription repressor CTCF also known as 11-zinc finger protein or CCCTC-binding factor that is involved in the induction of IFN $\gamma$  signaling<sup>18</sup> overlaps the rs13197384 variant (relative matching score = 0.8). These findings suggest that the rs13197384 variant, which is in LD with the lead MS SNP, may be

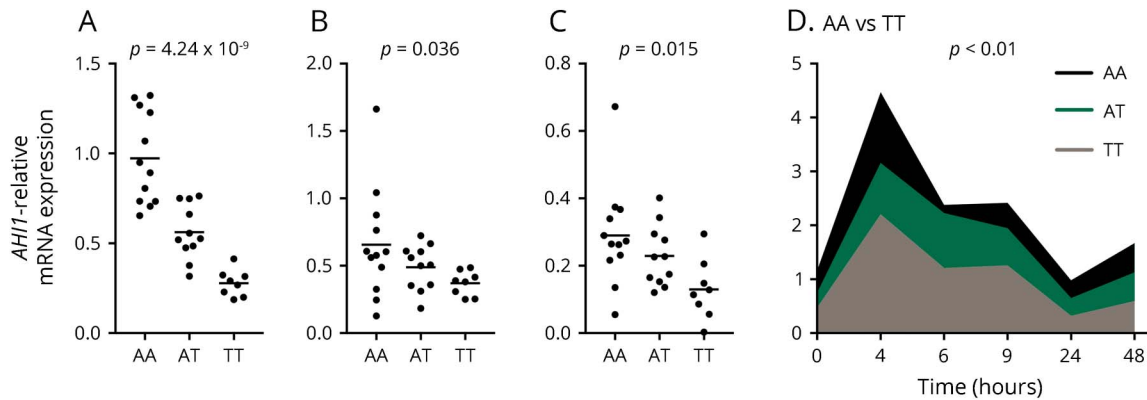
involved in the regulation of *AHII* transcription in CD4<sup>+</sup> T cells.

***AHII* eQTL replication in T cell receptor stimulated T cells.** To study the functional consequences of rs4896153 and its set of linked SNPs, we investigated the correlation between the rs4896153<sup>T</sup> risk allele and the adaptive immune system of healthy subjects. We used venous blood from healthy subjects, which allows for the examination of the immune system without any confounding effects of systemic inflammation or immune-modulating therapies in patients with autoimmune diseases.<sup>19</sup> Healthy genotyped subjects free of autoimmune and chronic infectious diseases were selected from the PGP who were homozygous for either rs4896153<sup>T</sup> (risk allele) or rs4896153<sup>A</sup> (protective allele). Using microbeads, naive CD4<sup>+</sup> T cells were isolated from PBMCs by negative selection. We first replicated the prior observation that rs4896153 has a *cis*-eQTL effect in naive CD4<sup>+</sup> T cells (figure 2A). Specifically, the rs4896153<sup>T</sup> risk allele showed significantly lower expression of *AHII* than the rs4896153<sup>A</sup> protective allele ( $\rho$  -0.837,  $p = 4.29 \times 10^{-9}$ ,  $n = 32$ ), as measured by quantitative PCR. This *cis*-eQTL effect was present for both of the 2 major *AHII* isoforms: isoform A (figure 2B, mRNA = NM\_017651.4, NM\_001134830.1, NM\_001134831.1; protein = NP\_001128303.1) and isoform B (figure 2C, mRNA = NM\_001134832.1; protein = NP\_001128304.1). *AHII* isoform B lacks the SRC Homology 3 (SH3) domain that is found in isoform A; SH3 is a signaling domain involved in mediating the interaction of proteins involved in signaling pathways.<sup>20</sup>

To investigate the temporal expression of *AHII* in CD4<sup>+</sup> cells, we measured *AHII* in genotyped samples over time in naive CD4<sup>+</sup> T cells after T cell receptor (TCR) stimulation with anti-CD3 and anti-CD28 monoclonal antibodies. We found that *AHII* mRNA expression is rapidly induced within 4 hours of stimulation then decreases over 24 hours, increasing again by 48 hours with the *cis*-eQTL effect remaining significant during TCR stimulation ( $p < 0.01$  for all time points) (figure 2D).

***AHII* risk variant promotes a CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> T cell phenotype.** Previous reports described *AHII* as an oncogene that enhances cancer cell proliferation.<sup>21–26</sup> To investigate the effects of the rs4896153 genotype on CD4<sup>+</sup> T cell function beyond RNA expression, we measured (1) T cell proliferation by [<sup>3</sup>H]-thymidine incorporation assays and (2) T cell cytokine profile. Naive CD4<sup>+</sup> T cells isolated from PBMCs of healthy genotyped subjects homozygous for either the *AHII* risk ( $n = 8$ ) or protective ( $n = 13$ ) alleles were exposed to TCR stimulation followed by analysis of T cell proliferation (day 3) and cytokine expression

**Figure 2** The MS risk allele rs4896153\* is associated with decreased *AHI1* expression in naive CD4<sup>+</sup> T cells and T cell receptor-stimulated CD4<sup>+</sup> T cells



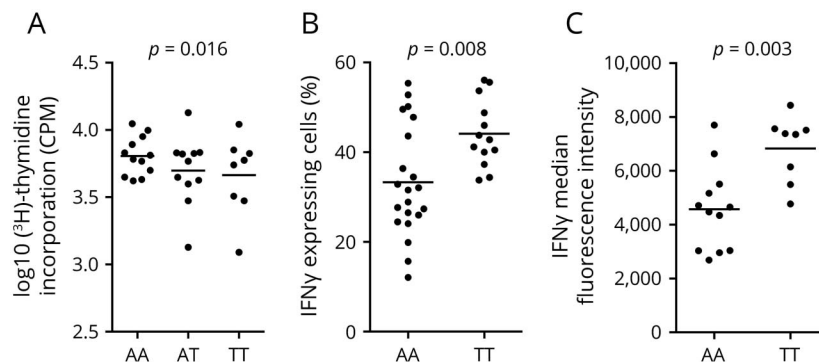
(A) Naive CD4<sup>+</sup> T cells were isolated from the peripheral blood of healthy subjects bearing risk (TT), protective (AA), or heterozygous (AT) genotypes for rs4896153, and *AHI1* expression was measured by Taqman qPCR. The 2 major isoforms of *AHI1* (B) isoform A (mRNA = NM\_017651.4, NM\_001134830.1, NM\_001134831.1; protein = NP\_001128303.1) and (C) isoform B (mRNA = NM\_001134832.1; protein = NP\_001128304.1) were measured in genotype individuals from (1A) by SYBR green qPCR. (D) Naive CD4<sup>+</sup> T cells were stimulated with anti-CD3/CD28 for 0–48 hours, and *AHI1* expression was measured by Taqman qPCR. Comparing the AA and TT genotypes, all data points are  $p < 0.01$ . Each dot represents an individual. Data are represented as mean  $\pm$  SEM.

(day 7). No significant difference was seen in proliferative capacity between the genotype categories (figure 3A). Of interest, T cells bearing the rs4896153<sup>TT</sup> genotype yielded significantly greater frequency of IFN $\gamma$ <sup>+</sup> T cells than the rs4896153<sup>AA</sup> genotype in TCR stimulated cells ( $p = 0.008$ ) (figure 3B). Within the IFN $\gamma$ -positive cells, more IFN $\gamma$  was expressed in cells from subjects with the risk genotype as measured by mean fluorescence intensity (MFI) (figure 3C), providing a link between an MS genetic susceptibility and a proinflammatory T cell phenotype. The effect of the *AHI1* risk allele on T cell phenotype may be specific to IFN $\gamma$  because we did not find any significant

alteration of IL-17 expression when naive CD4<sup>+</sup> T cells carrying the *AHI1* risk or protective alleles (data not shown).

**Increased IFN $\gamma$  expression in *Abi1* knockout CD4<sup>+</sup> T cells.** To further explore the role of *AHI1* in the regulation of IFN $\gamma$  production by CD4<sup>+</sup> T cells, we first measured *Abi1* expression in murine CD4<sup>+</sup> T cells. Naive CD4<sup>+</sup> T cells were isolated from spleens of wild-type (WT) mice, and cells were TCR stimulated for several time points. RNA lysates were prepared at each time point, and murine *Abi1*

**Figure 3** Decreased *AHI1* in human CD4<sup>+</sup> T cells is associated with decreased proliferation and increased IFN $\gamma$  production



(A) Naive CD4<sup>+</sup> T cells were isolated from the peripheral blood of healthy subjects bearing risk (TT), protective (AA), or heterozygous (AT) alleles for rs4896153, and cells were activated with anti-CD3/CD28 followed by the analysis of cell proliferation by [<sup>3</sup>H]-thymidine incorporation 60 hours after stimulation. (B, C) A subset of these cells was further stimulated for 7 days, and IFN $\gamma$  cytokine expression was measured by flow cytometry. (B) Frequency of CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> T cells bearing risk (TT) or protective (AA) alleles for the rs4896153 was analyzed by intracellular staining using flow cytometry. (C) Mean fluorescent intensity (MFI) of IFN $\gamma$  expression from the activated T cells is shown.  $p$  Values were generated using Student's  $t$  test. Each dot represents an individual. Data are represented as mean  $\pm$  SEM. IFN $\gamma$  = interferon gamma.

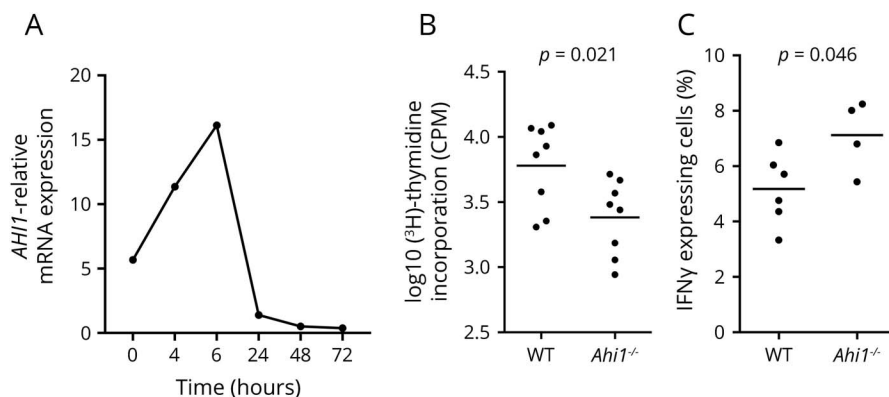
mRNA expression was assessed by quantitative PCR. In agreement with *AH11* expression in activated human CD4<sup>+</sup> T cells, we found that the mRNA levels of murine *Abi1* are moderately induced on TCR activation (4–6 hours) but rapidly decreased later as shown at 24–72 hours, suggesting a regulatory role of *Abi1* in the differentiation, phenotype, or function of CD4<sup>+</sup> T cells (figure 4A and figure e-2A, <http://links.lww.com/NXI/A5>). Similarly, the rapid but reversible increase in *Abi1* expression was also observed in CD4<sup>+</sup> T cells polarized under Th1 cells, a T cell subset that is known to express IFN $\gamma$ <sup>+</sup> T cells.

Next, we took advantage of total *Abi1* knockout (*Abi1*<sup>-/-</sup>) mice<sup>27</sup> to measure the role of *Abi1* in T cells using *Abi1* genetic deletion. Splens were dissected from *Abi1*<sup>-/-</sup> and WT littermates, and naive CD4<sup>+</sup> T cells were sorted and activated with mouse specific anti-CD3/CD28 antibodies. We found that, using a more physiologic stimulation that does not bypass costimulatory pathways (low anti-CD3/CD28 concentrations, each mAb at 0.25  $\mu$ g/mL), CD4<sup>+</sup> T cells from *Abi1*<sup>-/-</sup> mice exhibited impaired cell proliferation compared with their WT counterparts (figure 4B). Strikingly, we detected increased frequency of IFN $\gamma$ -producing T cells in *Abi1*<sup>-/-</sup> T cells (figure 4C), an effect that is in agreement with the human data. To study whether *Abi1* regulates T cell cytokine production under polarizing conditions, we analyzed IFN $\gamma$  and IL-17A expressions in CD4<sup>+</sup> T cells polarized toward a proinflammatory Th1 or Th17 fate. There was a trend implicating a modest increase in IFN $\gamma$ <sup>+</sup> frequency among Th1 cells (figure e-3A, <http://links.lww.com/NXI/A5>). Moreover, analysis of IL-17A in *Abi1*<sup>-/-</sup>

T cells polarized under Th17 condition did not reveal any significant alteration of IL-17A expression in the absence of *Abi1* (figure e-3B, <http://links.lww.com/NXI/A5>). Altogether, these findings suggest that *Abi1* regulates specifically IFN $\gamma$ -producing CD4<sup>+</sup> T cells in the absence of exogenous polarizing molecules.

**DISCUSSION** Rare mutations in *AH11/Abi1* have been reported to cause Joubert syndrome, a ciliopathic disorder, characterized by malformations of the cerebellar vermis and brainstem as well as clinical manifestations that include, breathing irregularities, hypotonia, developmental delays, and ocular motor apraxia.<sup>28</sup> The AH11 protein contains an N-terminal coiled-coil domain, 7 WD40 domains, and an SH3 domain; it is the only protein known to contain both a WD40 domain and an SH3 domain, suggesting a novel role in cell signaling for this protein.<sup>29</sup> In chronic myeloid leukemia cells, *AH11* knockdown showed reduced TNF $\alpha$ , IL-4, and IL-2 cytokine production.<sup>30</sup> On the other hand, overexpression of *AH11* conferred a growth advantage,<sup>25</sup> which is consistent with our mouse data in which reduced *Abi1* expression in CD4<sup>+</sup> T cells was associated with reduced proliferative capacity. However, the decrease in *AH11* expression did lead to increased cytokine production at least for IFN $\gamma$ , suggesting that the role of *AH11* in primary human T cells may involve distinct signaling pathways in comparison with tumor cells. In MS, proinflammatory cytokines such as IFN $\gamma$ , IL-17, IL-22, and GM-CSF are present in elevated amounts in the CSF and CNS lesions of MS patients compared with healthy controls, especially during the active phase of the disease.<sup>31–34</sup> The

**Figure 4** *Abi1* genetic deficiency decreases T cell proliferation and upregulates IFN $\gamma$  production



(A) *Abi1* mRNA expression in activated T cells. Wild-type (WT) naive CD4<sup>+</sup> T cells were isolated from the spleens of naive WT mice, and cells were activated with anti-CD3/CD28 for up to 72 hours (h). *Abi1* mRNA expression was measured by Taqman and calculated relatively to the house keeping gene *Gapdh*. (B, C) Effects of *Abi1* on T cell proliferation and IFN $\gamma$  expression. Naive CD4<sup>+</sup> T cells were isolated from the spleens of *Abi1*<sup>-/-</sup> or WT littermates, and cells were activated with anti-CD3/CD28 (0.25  $\mu$ g/mL) for (B) 60 hours to measure T cell proliferation by [<sup>3</sup>H]-thymidine incorporation or for (C) 4 days to measure IFN $\gamma$  production by intracellular staining using flow cytometry. Data represent the average of 3 independent experiments. Each dot represents a mouse. Data are represented as mean  $\pm$  SEM. IFN $\gamma$  = interferon gamma.



frequency of IFN $\gamma$  vs IL-17 responses in EAE can influence the localization of inflammation in the CNS.<sup>35</sup> Robust Th1 responses producing elevated levels of IFN $\gamma$  induced CD4<sup>+</sup> T cell infiltration in the spinal cord. By contrast, high IL-17 levels caused CD4<sup>+</sup> T cell infiltration preferentially into the brain parenchyma and induced atypical EAE.<sup>5</sup> In our study, although the *AHII* risk variant was associated with an increase in IFN $\gamma$  production by CD4<sup>+</sup> T cells, we did not detect a significant alteration in IL-17 levels. It should be noted that recent studies in several immunologic diseases including sarcoidosis and Crohn disease identified a subset of pathogenic IFN $\gamma$ -producing Th17 cells called Th17.1 cells expressing chemokine receptors CCR6 and CXCR3.<sup>36,37</sup> These cells are believed to be derived from classically polarized Th17 cells and differentiate into a Th1-like phenotype in which they produce significant IFN $\gamma$  but little amounts of IL-17.<sup>38</sup> Of interest, in patients with MS, circulating Th17 cells that respond to myelin peptides express high levels of IFN $\gamma$ , suggesting that these autoreactive T cells are plastic and express distinct cytokine profiles from that identified *in vitro*.<sup>39</sup> A more extensive cytokine characterization of CD4<sup>+</sup> T cells expressing the *AHII* risk variant may address this issue further.

In this study, we confirm that rs4896153, an MS susceptibility variant, has a strong *cis*-eQTL effect on overall *AHII* mRNA expression, affecting both *AHII* isoforms, and that this effect is maintained for at least 48 hours after TCR stimulation. To assess the functional outcome of this SNP on CD4<sup>+</sup> T cells, we measured proliferation and cytokine production following TCR stimulation. While we did not see a significant allelic difference in proliferation of human T cells, we consistently saw visibly less cell clonal expansion in the rs4896153<sup>TT</sup> cultures, an effect noticeable after only 24 hours of differentiation (data not shown). We postulate a role for *AHII* early on in TCR activation, given its peak mRNA expression 4–6 hours after anti-CD3/CD28 stimulation of both mouse and human CD4<sup>+</sup> T cells. *AHII* is localized to the mother centriole, and for TCR signaling, the reorientation and movement of the centriole and associated Golgi apparatus toward the contact area between a T cell and an antigen-presenting cell is required after an initial contact.<sup>40</sup> It is therefore possible that *AHII*, being localized to the mother centriole and involved in actin organization, may play a role in the formation or stabilization of the TCR synapse, which could be a mechanism for its association with MS.

Identification of the causal variant (the SNP/s that affect the trait), and determining their exact mechanism of action, is one of the main challenges and provides the next step in GWAS interpretation.<sup>41</sup> Our analysis of the GWAS identified MS variants near

the *AHII* gene and prioritized an LD block with several SNPs that are associated with decreased *AHII* expression in CD4<sup>+</sup> T cells carrying the MS risk allele. We identified 3 SNPs, rs13197384, rs7750586, and rs9399148, with suggestive functionality based on (1) being present within DNase hypersensitivity sites and (2) the potential alteration of TF binding to their DNA motifs in CD4<sup>+</sup> T cells. We identified candidate TFs whose binding could be affected by the presence of 1 of these 3 SNPs including the transcription repressor CTCF that is involved in the induction of IFN $\gamma$  signaling<sup>18</sup> in which the CTCF consensus binding site overlaps with rs13197384. Further functional studies, such as minigene reporter assays, are warranted to identify causal variants that influence a disease trait in MS.

We report that the rs4896153<sup>T</sup> allele reduces *AHII* RNA expression and is associated with a greater percentage of IFN $\gamma$ -producing CD4<sup>+</sup> T cells. While additional functional studies are required to further delineate the role of *AHII* in MS susceptibility, our results expand the repertoire of dysfunctional cytokine responses that are genetically implicated in MS susceptibility and may help establish a scaffold on which to assemble other susceptibility variants.

#### AUTHOR CONTRIBUTIONS

B.J.K. designed the study, performed experiments, analyzed data, and drafted the manuscript. T.S.B. performed experiments. C.W. performed statistical analyses. H.-U.K. and N.P. analyzed and interpreted data. J.R.B. and R.J.F. provided *Ahii*<sup>-/-</sup> mice and splenocytes. E.M.B. designed the study and interpreted data. P.L.D.J. and W.E. designed the study, interpreted data, supervised the study, and wrote the manuscript.

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