The transcription factor nuclear factor κB (NFκB) is a central regulator of inflammation, and genome-wide association studies in subjects with autoimmune disease have identified a number of variants within the NFκB signaling cascade. In addition, causal variant fine-mapping has demonstrated that autoimmune disease susceptibility variants for multiple sclerosis (MS) and ulcerative colitis are strongly enriched within binding sites for NFκB. We report that MS-associated variants proximal to NFκB1 and in an intron of TNFRSF1A (TNFR1) are associated with increased NFκB signaling after tumor necrosis factor–α (TNFα) stimulation. Both variants result in increased degradation of inhibitor of NFκB α (IκBα), a negative regulator of NFκB, and nuclear translocation of p65 NFκB. The variant proximal to NFκB1 controls signaling responses by altering the expression of NFκB itself, with the GG risk genotype expressing 20-fold more p50 NFκB and diminished expression of the negative regulators of the NFκB pathway: TNFα-induced protein 3 (TNFAIP3), B cell leukemia 3 (BCL3), and cellular inhibitor of apoptosis 1 (CIAP1). Finally, naïve CD4 T cells from patients with MS express enhanced activation of p65 NFκB. These results demonstrate that genetic variants associated with risk of developing MS alter NFκB signaling pathways, resulting in enhanced NFκB activation and greater responsiveness to inflammatory stimuli. As such, this suggests that rapid genetic screening for variants associated with NFκB signaling may identify individuals amenable to NFκB or cytokine blockade.

INTRODUCTION

Nuclear factor κB (NFκB) was one of the first transcription factors identified and is a central regulator of inflammation (1). The canonical p50/p65 NFκB signaling cascade is critical for activation of immune responses downstream of T and B cell receptors, toll-like receptors, and cytokines, including tumor necrosis factor–α (TNFα) and interleukin-1β (IL-1β). Moreover, alterations in NFκB have been associated with both autoimmune disease and malignancies (2, 3). Inflammatory autoimmune diseases, which reflect complex interactions between genetic variation and environment, are important systems for genetic investigation of human disease. These diseases share a substantial degree of immunopathology, with increased activity of autoreactive CD4+ T cells secreting inflammatory cytokines and loss of regulatory T cell function (4–7). Multiple sclerosis (MS) is one such autoimmune disease where there is chronic inflammation in the central nervous system (CNS), with infiltration of activated mononuclear cells into the CNS that damages both myelin and axons. This complex genetic disease is associated with environmental factors that appear to drive a predominantly T cell autoimmune response against CNS antigens (8, 9).

Genome-wide association studies (GWAS) and subsequent targeted genomic studies have identified 97 variants associated with MS susceptibility (10–12). Although each of these variants contributes only a small increase in the complex phenotype of disease risk, the biological function associated with individual allelic variants has been striking (13–17). Many of these variants fall within specific signaling cascades, suggesting that alterations in pathways, rather than individual genes, may be the key to understanding how individual variants with small odds ratios result in disease susceptibility (18–20). About 17% (17 of 97) of MS susceptibility variants identified by GWAS fall either within or proximal to NFκB signaling genes, including variants proximal to NFκB1 itself and within TNF receptor 1 (TNFR1) (10, 12, 21).

We recently integrated genetic and epigenetic fine-mapping to identify potentially causal variants in autoimmune disease–associated loci and explore their functions by generating cis-regulatory element maps for a spectrum of immune cell types. About 60% of likely causal variants map to enhancer-like elements, with preferential enrichment in stimulus-dependent CD4+ T cell enhancers. When overlapping causal single-nucleotide polymorphisms (SNPs) with 31 transcription factor binding maps generated by ENCODE, SNPs were strongly enriched within binding sites for immune-related transcription factors, and variants associated with different diseases correlate to different combinations of transcription factors that control immune cell identity and response to stimulation. In patients with MS, SNPs preferentially coincide with NFκB-, EBF1 (early B cell factor 1)–, and MEF2A (myocyte enhancer factor 2A)–bound regions (22).

Previous studies have shown that total peripheral blood mononuclear cells (PBMCs) from relapsing-remitting MS (RRMS) patients exhibit increased levels of active NFκB (23). We similarly observed that naïve CD4 T cells from RRMS patients exhibit increased activation of the canonical p65 NFκB pathway compared to healthy controls, suggesting that this difference is not due to the activation status of the cells. Thus, we hypothesized that the alterations in NFκB signaling seen in patients with MS are a result of SNPs in the NFκB signaling cascade associated with MS susceptibility. Because NFκB signaling is triggered by many environmental stimuli through toll-like receptors, this may represent a critical intersection between genetic and environmental factors resulting in MS development. However, the impact of autoimmunity-associated genetic variants on the biological function of this major transcription factor is unknown.
It has been shown that mice with a constitutively active form of p65 NFκB exhibit multiorgan inflammation and die within 3 weeks of birth. However, when these mice with constitutively active p65 NFκB were bred back to mice lacking TNFR1, they were protected from inflammation and exhibited only a mild Sjögren’s syndrome–like ocular keratitis, indicating a critical role of the NFκB/TNF pathway in regulating autoimmune disease (24). Variants within or proximal to both TNFRSF1A (TNFR1) and NFκB1 have been identified by GWAS as being associated with risk of developing MS. Therefore, we chose to focus our studies on these SNPs to determine whether they alter NFκB responses.

Here, we investigated the effect of MS risk haplotypes on the function of the NFκB signaling cascade. We demonstrate that both the haplotype with the variant proximal to NFκB1 (rs228614-G) and that with the variant within TNFR1 (rs1800693-C) result in enhanced NFκB responses to TNFα. The rs228614-G risk allele also enhances responses to phorbol 12-myristate 13-acetate (PMA), suggesting that this variant strongly controls NFκB signaling. Mechanistically, we found that rs228614-G is associated with a 20-fold increase in p50 NFκB expression and decreased expression of the negative regulators of NFκB signaling. These findings elucidate a genetic mechanism controlling NFκB responses that result in predisposition to developing MS.

RESULTS

Patients with MS have increased activation of the canonical NFκB cascade

Previous reports suggest that total PBMCs from patients with MS exhibit increased activity of NFκB, consistent with excessive immune activation (23, 25). However, it was not possible to determine whether this increased NFκB activation is due to up-regulation of NFκB or to the increased frequency of activated and memory CD4 T cells in patients with MS. To resolve this question, we isolated total PBMCs from patients with MS and healthy control subjects ex vivo and directly stained for phospho-p65 NFκB. We found that naïve CD4 cells from patients with MS exhibit significantly higher phospho-p65 NFκB than those from age-matched healthy control donors, and this increased activation of p65 NFκB was mitigated by treatment (Fig. 1; subject demographics are listed in table S1). This increased constitutive expression of phospho-p65 NFκB was repeated in a second cohort of MS patients and healthy controls (fig. S1). The presence of enhanced activation of NFκB in naïve CD4 cells demonstrates that this is not due to an increase in the number of activated or memory cells but is rather due to a hyperactivated state of CD4 cells.

The MS risk variant rs228614 near NFκB1 is associated with increased NFκB signaling

To determine whether the increased NFκB activation seen in patients with MS may be due to genetic variation in the NFκB signaling cascade associated with disease susceptibility, we next assessed whether the MS risk variant proximal to NFκB1 increases NFκB signaling. The SNP rs228614 on chromosome 4 is associated with MS susceptibility (odds ratio of 1.09 per G allele carried; \( P = 1 \times 10^{-8} \)) and lies near the NFκB1 gene (12). It is part of a haplotype of more than 90 variants in tight linkage disequilibrium (LD) that spans the region encoding both NFκB1 and MANBA (Fig. 2A). Although this strong LD precludes identifying this specific SNP as the causal variant for disease, we stratified donors into those carrying one or two copies of the G risk allele (rs228614-G) and correlated NFκB signaling to genotype in total PBMCs. In addition, we also investigated a second variant, rs7665090, in LD to rs228614 (\( r^2 > 0.8 \)) that was identified by fine-mapping as the most associated variant in the region (10). This allowed us to examine two variants within the same region to determine the impact on NFκB signaling and thus address the difficulty of identifying the causal variant in genetic mapping studies. We used healthy control subjects carrying the risk or protective variants to avoid the confounding factors of ongoing inflammation and therapeutic intervention seen in patients with MS.

We collected blood samples from healthy donors in our previously genotyped biorepository of recallable subjects (Yale Phenogenetic Project) (see demographics in tables S2 and S3). We found that the MS risk alleles rs228614-G and rs7665090-G are significantly associated with an increase in inhibitor of NFκB α (IκBα) degradation after TNFα stimulation (allelic logistic regression: rs228614, \( P = 0.0078 \), and rs7665090, \( P = 0.01 \); Fig. 2B). This difference is also significant when comparing homozygous risk and protective genotypes (Student’s t test between GG and AA carriers: rs228614, \( P = 0.0091 \); rs7665090, \( P = 0.0089 \); Fig. 2B). Risk allele homozygotes showed consistently increased phosphorylation of p65 NFκB (Student’s t test between GG and AA carriers: rs228614, \( P = 0.029 \); rs7665090, \( P = 0.039 \)); Fig. 2C, representative histogram and gating strategy in fig. S2). A trend of increased signaling from both rs228614 and rs7665090 was also observed at 15, 30, and 45 min (fig. S3). Thus, the region containing the rs228614 and rs7665090 SNPs modulates TNFα signaling through NFκB.

To determine whether this represents a global impact on NFκB signaling or a specific alteration in TNFα signaling, we stimulated total PBMCs from subjects with the different genotypes at rs228614 with PMA, an activator of NFκB independent of TNFα. The risk genotype (GG) resulted in greater degradation of IκBα and phosphorylation of p65 NFκB in naïve CD4 cells after stimulation with PMA, suggesting a modulation of global NFκB responses rather than to a specific stimulus (Fig. 2D; IκBα, \( P = 0.019 \) and pNFκB, \( P = 0.027 \)). To determine whether this region was also affecting NFκB signaling in MS patients, we stimulated total PBMCs from rs7665090 homozygous risk (GG) and protective (AA) subjects with TNFα and determined degradation of IκBα and phosphorylation...
of p65 NFκB by flow cytometry. Similar to the changes in NFκB signaling seen in healthy controls, the risk variant also resulted in increased IkBα degradation (Fig. 2E) and phosphorylation of p65 NFκB (Fig. 2F) in naïve CD4 cells in MS patients. We hypothesized that these divergent NFκB responses should result in differential rates of nuclear localization of p65 NFκB. We tested this by comparing healthy donors with AA (protective) and GG (risk) genotype at rs228614. We stimulated CD4 cells with TNFα for 15 or 30 min, stained for p65 NFκB and 4′,6-diamidino-2-phenylindole (DAPI), and determined nuclear localization of p65. We found that GG carriers demonstrate increased p65 nuclear localization after 30 min, confirming stronger signaling responses (Fig. 3, A and B). Together, these data demonstrate that the MS risk haplotype captured by rs228614 and rs7665090 strongly modulates NFκB responses, with the GG risk genotype resulting in enhanced NFκB signaling.

rs228614 is associated with changes in the expression of NFκB1 and the negative regulators of the NFκB pathway

We did not find differences in IkBα and phospho-p65 NFκB expression in resting CD4 cells, suggesting that the effect of the rs228614 risk variant is only evident after signaling induction (fig. S4). This suggests that the underlying causal variant perturbs regulatory elements controlling stimulus-dependent activation of the NFκB gene, consistent with the observation that most GWAS risk variants alter gene regulation rather than structure (26). The haplotype block containing rs228614 spans the coding regions of NFκB1 and MANBA and the intergenic space between them, which contains at least 62 transcription factor binding sites, 12 regions of DNA hypersensitivity, 3 insulator regions, and multiple putative enhancer regions active in both T cell and myeloid cell lineages (27–33), suggestive of important regulatory functions. We therefore looked for changes in expression of p50 NFκB in rs228614 GG and AA genotype carriers by Western blot. We found that subjects with the GG risk genotype express p50 NFκB at a 20-fold higher level than the AA genotype (Fig. 4A and fig. S5, uncut blot). We also found a
commensurate decrease in mRNA levels in total PBMCs of three key negative regulators of NFκB [TNFα-induced protein 3 (TNFAIP3), B cell leukemia 3 (BCL3), and cellular inhibitor of apoptosis 1 (CIAP1)] in the GG homozygotes (Fig. 4B). Together, these results suggest that an increase in total p50 NFκB and a loss of negative regulation of this pathway are responsible for enhanced signaling. Although IκBα degradation is upstream of NFκB, signaling through NFκB regulates the expression of NFκB1 itself, as well as the negative regulators of NFκB. Thus, we demonstrate alterations in the expression of both NFκB1 and the negative regulators of this pathway, suggesting that the MS risk allele causes a fundamental shift in the regulation of NFκB signaling.

NFκB responses are stable over time and are not associated with age or gender

If the strength of NFκB signaling is primarily mediated by genetic variability between individuals and not by changes in environmental or external stimuli, we would expect consistent strength of signal across multiple time points. We redrew 15 subjects on an average of 6 months after the first blood draw. We found no statistically significant difference between draws (fig. S6). Although there was significant variability between individuals in the strength of NFκB signaling, the stability in this variability across multiple draws was consistent with a genetically mediated threshold for NFκB signaling.

It has been shown that individuals 65 years or older have an increase in constitutive activation of p65 NFκB (34). The Phenogenetic Proj-

rs1800693 in TNFR1 is associated with enhanced NFκB responses to TNFα

Because both NFκB and TNFR1 are important in autoimmune inflammation and variants near both NFκB1 (rs226814) and TNFRSF1A (rs1800693) are associated with risk of developing MS (12), we investigated the impact of the TNFRSF1A variant on NFκB signaling. Unlike the NFκB1 locus, where tight LD precludes identifying the likely causal SNP, rs1800693 alone best explains the association signal in the TNFRSF1A region and is thus likely causal (10). The variant falls 10 base pairs upstream of TNFRSF1A exon 6 in a splice acceptor site (Fig. 5A). We and others have previously shown that homozygous carriers of the rs1800693-C risk allele show loss of exon 6 and a premature stop codon in ~10% of TNFR1 mRNAs (35, 36). Moreover, this variant results in an exaggerated cytokine production response from monocytes after stimulation with TNFα (35), suggesting that this transcriptional change results in changes in overall signaling responses. We evaluated this hypothesis by stimulating PBMCs and purified monocytes with TNFα and assessing resultant IκBα degradation (see demographics in table S4). We found that carriers of the CC risk genotype show increased degradation of IκBα after stimulation with TNFα in both naïve CD4 cells (P = 0.0092; Fig. 5B) and monocytes (P = 0.01; Fig. 5C) compared to TT genotype carriers. Unlike the variant proximal to NFκB1, there were no differences in signaling after PMA stimulation between the CC and TT genotypes (Fig. 5B). These data suggest that rs1800693 is associated with changes in signaling only when TNFα binds to TNFR1.

TNFα can signal through two receptors, TNFR1 and TNFR2. Naïve CD4 cells primarily express TNFR1, with TNFR2 being up-regulated after activation (37). Our results suggest that altered TNFα-induced signaling is due to rs1800693-C exerting regulatory effects on TNFR1. To validate this model, we blocked TNFR1 and found that TNFα signaling in naïve CD4 cells occurs exclusively through TNFR1, with no contribution from TNFR2 (fig. S8). As such, the changes in signaling observed in naïve CD4 cells can be attributed directly to alterations in TNFR1.
To confirm these changes in NFκB signaling in CD4 cells, we purified total CD4 cells, stimulated them with TNFα for 15 or 30 min, and determined the amount of p65 NFκB nuclear localization. We observed an increase in nuclear localization 15 min after stimulation in carriers of the CC genotype compared to the TT genotype (Fig. 6, A and B). The CC genotype did not result in changes in the cell surface expression of TNFR1 on naïve CD4 cells, either as a percentage of cells expressing TNFR1 or the expression level of TNFR1 on the surface (fig. S9). This suggests that the change in signaling is not due to changes in overall TNFR1 levels on the cell surface. We and others have shown that expression of the prematurely terminated transcript missing exon 6 caused by the rs1800693-C allele results in altered intracellular accumulation of TNFR1 in human embryonic kidney (HEK) 293 cells or HeLa cells (35, 36). We investigated whether this is true in primary immune cells directly ex vivo and found that CD14+ monocytes from CC risk genotype carriers exhibit altered intracellular accumulation of TNFR1 into punctate structures within the cytoplasm (Fig. 7A, single-stained control, fig. S10). Together, these results demonstrate that the variant in TNFR1 results in increased signaling to TNFα in both naïve CD4 cells and monocytes and that this altered signaling may be due to altered localization of TNFR1 within the cell.

**Fig. 4.** rs228614 allelic variant results in increased NFκB1 expression and decreased expression of the negative regulators of NFκB. (A) Representative Western blot of p105 and p50 NFκB in total PBMCs and densitometry of total p50 NFκB by Western blot (GG, n = 7; AA, n = 7). (B) mRNA expression by quantitative polymerase chain reaction (qPCR) of BCL3, TNFAIP3, and CIAP1 in total PBMCs (GG, n = 6; AA, n = 7). P value shown for unpaired t test.

rs1800693 in TNFR1 is associated with increased plasma cytokines in healthy controls consistent with levels seen in MS patients

Because TNFα signaling drives the expression of inflammatory cytokines, we examined whether changes in TNFR1-mediated signaling associated with the rs1800693-C risk allele result in altered plasma cytokines levels. We measured plasma levels for 29 cytokines in healthy subjects and found that CC genotype carriers have increased plasma levels of IL-7, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-inducible protein 10 (IP10), and monocyte chemoattractant protein 1 (MCP1) (Fig. 7B and table S5). This is consistent with our previous findings that IP10 is overexpressed in monocytes after TNFα stimulation in CC genotype carriers (35). It has been reported that IL-7, GM-CSF, IP10, IL-8, and MCP1 are increased in serum of patients with MS (38–42). In addition, recent twin studies have shown that serum concentrations of all five of these cytokines are highly heritable (43). This demonstrates that the CC homozygous risk genotype alters inflammation levels in healthy control subjects, consistent with that seen in MS.

**DISCUSSION**

NFκB is a central regulator of inflammation, controlling the activation, proliferation, and cytokine production of immune responses. We observed that naïve CD4 T cells from patients with the autoimmune disease MS exhibit increased activation of p65 NFκB, prompting us to investigate the genetic control of NFκB signaling in the disease. We found that variants near genes in the NFκB signaling cascade have large effects on NFκB signaling. Specifically, the allelic variant proximal to NFκB1 controls signaling responses by altering the expression of NFκB itself, with the GG risk genotype expressing 20-fold more p50 NFκB. This genotype is associated with altered NFκB responses to both TNFα and PMA, suggesting a global control of NFκB signaling. Because causal SNPs are strongly enriched within binding sites and signaling molecules for NFκB, these results demonstrate a central role for NFκB in driving the pathophysiology of MS.

The region spanned by the MS risk haplotype near NFκB1 contains a large number of gene regulatory elements, as mapped by the ENCODE project (27–33). This suggested the hypothesis that the MS risk allele affects NFκB1 expression and thus alters NFκB signaling. To investigate this potential mechanism, we examined how allelic variation in this region influenced expression of p50 NFκB and found that MS risk variants are strongly associated with large increases in expression. Consistent with increased NFκB signaling, there was a decrease in expression of the negative regulators of NFκB, suggesting a global disruption in the NFκB cascade. The central role of NFκB in immune activation would suggest that this MS haplotype broadly increases inflammatory responses, thereby predisposing individuals to autoimmunity.

We recently investigated the overlap of potentially causative SNPs with 31 transcription factor binding maps generated by ENCODE and observed that they were strongly enriched within binding sites for immune-related transcription factors (22). Moreover, variants associated with different autoimmune diseases correlate with different combinations of transcription factors that control immune cell identity and response to stimulation. We found that MS SNPs preferentially coincide with NFκB-, EBF1-, and MEF2A-bound regions, whereas rheumatoid arthritis and celiac disease SNPs preferentially coincide with IRF4 (interferon regulatory factor 4) regions (22). Thus, both GWAS and epigenetic mapping strongly implicate NFκB as a critical pathway.
The TNFR1 variant rs1800693 results in increased TNFα responses. (A) Association with MS risk in the region surrounding TNFRSF1A. Y axis shows the GWAS \(-\log(P)\) value for the allelic test of association as reported in (10). We have highlighted rs1800693, the most associated variant in the region. With further replication data in independent samples, rs228614 meets the GWAS significance threshold of \(P < 5 \times 10^{-5}\). (B) Degradation of IκBα after 30 min of TNFα stimulation in CD4+CD45RA+CD45RO+ T cells (CC, \(n = 14\); TT, \(n = 20\)). (C) Degradation of IκBα after 30 min of TNFα stimulation in CD14+ monocytes (CC, \(n = 5\); TT, \(n = 12\)). P value shown for unpaired t test.

The haplotype block surrounding the intergenic region downstream of NFκB1 contains more than 90 variants in strong LD, and we were unable to identify a single most likely causal variant by genetic and epigenetic fine mapping. In the future, the use of probabilistic identification of causative SNPs in larger cohorts may allow further fine mapping of this region, which may better identify the most likely causative SNP (22). In spite of this lack of resolution in the discovery process, we are able to show that the underlying MS variant in this region tagged by the rs228614-G allele is strongly associated with NFκB responses to TNFα.

We demonstrated that significant interindividual variation exists in NFκB responses, and this variability is partially mediated by genetic and epigenetic fine-mapping. Because many autoimmune diseases and cancers have genetic variants associated with NFκB, it is likely that genetically mediated variability in this pathway is central to disease risk. In addition, because the variants associated with each disorder are different, variation in the NFκB pathway may result in changes in signaling that are specific to each disease. Finally, the increased NFκB signaling we observed is consistent with the enhanced activation of the canonical NFκB pathway observed in naïve CD4 cells from patients with MS. Hyperactivation of NFκB in mice results in rapid postnatal death from massive multiorgan inflammation not seen in human disease (24, 49). As such, it is likely that dysregulation of
NFκB represents a necessary factor in the development of autoimmunity but that other factors are also required to determine the location and nature of the inflammatory insult. Current GWAS have identified variants associated with disease susceptibility but do not determine how these variants are affecting disease severity or progression. Given that NFκB signaling is critical for both inflammation and neuronal degeneration (50), this pathway may represent a node that both predisposes to disease and contributes to disease progression.

In conclusion, naïve CD4 T cells from patients with MS exhibit increased p65 activation, consistent with changes in NFκB signaling. We observe equivalent changes in healthy controls carrying MS risk variants, which alter NFκB responses to TNFα, suggesting that disease risk is mediated by gene regulatory changes resulting in altered NFκB signaling. Because SNPs causal for MS are enriched in binding sites and signaling molecules for NFκB, these data demonstrate a central role for NFκB in driving the pathophysiology of MS. Identifying these critical nodes may suggest novel therapeutics aimed at specifically targeting the underlying causes of disease while leaving systemic immune responses primarily intact.

MATERIALS AND METHODS

Study design
Healthy subjects between the ages of 18 and 56 years at the time of initial assessment were enrolled in the Yale Phenogenetic Project and could be recalled up to six times a year for up to 4 years. Subjects with autoimmune disorders were excluded from the repository. In addition, any subjects exhibiting illness or fever at the time of enrollment were excluded. Investigation of the impact of rs228614 and rs7665090 on phospho-p65 NFκB and total IκBα was performed blinded, with the strength of signal determined before genotyping the samples. Samples were unblinded, and comparison of strength of signal to genotype was performed after unblinding. For blinded studies, power calculations determined that a singleton quantitative trait locus with a variance of 10% and an allele frequency of 20% could generate a significance of 0.05 for overall association with 50 samples and 0.01 with 90 samples. Because rs228614, rs7665090, and rs1800693 all have minor allele frequencies of >30%, these studies were sufficiently powered to generate significance. Nuclear localization, Western blotting, and qPCR studies were performed on recalled samples with specific genotypes. All TNFRSF1A studies were performed on samples of risk and protective genotypes recalled from the Brigham and Women’s Hospital (BWH) Phenogenetic Project. All studies were performed to at least three biological replicates.

Fig. 6. TNFR1 rs1800693 CC risk genotype results in increased NFκB nuclear localization. (A) Representative nuclear localization images from TT protective and CC risk genotypes in CD4+ T cells by Amnis Imagestream®. Green, p65 NFκB; purple, DAPI; colocalization, overlap of p65 and DAPI by Pearson coefficient. (B) Composite nuclear localization of p65 NFκB after TNFα stimulation in CD4+ T cells (CC, n = 9; TT, n = 9). Nuclear localization is shown normalized to unstimulated cells. P value shown for unpaired t test.

Fig. 7. The TNFR1 variant rs1800693 results in altered intracellular accumulation of TNFR1 and plasma cytokines. (A) Confocal microscopy of CD14+ monocytes from the TT (protective) or CC (risk) variant in the TNFR1 region. Blue, DAPI; green, TNFR1. Two of four subjects from each genotype are shown. (B) Concentrations of IL-7, IL-8, GM-CSF, MCP1, and IP10 in plasma samples from healthy control subjects with the CC (risk) or TT (protective) genotypes (CC, n = 25; TT, n = 40). P value shown for unpaired t test.
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Blood repositories
Samples were acquired from the Yale Phenogenetic Project or the BWH PhenoGenetic Project. These samples have been genotyped for autoimmunity-associated SNPs by Illumina Chip. Blood was drawn into heparin tubes. Samples from Yale were prepared immediately after blood draw. TNFR1 samples from BWH were shipped overnight with room temperature gel packs (SAF-T-PAK) and prepared the following morning. RMRs from age and gender-matched blood was drawn at the Yale MS Clinic and prepared the same as the healthy control samples. All samples were compared to samples from the same source (Yale or BHW).

PBMC preparation and cell purification
PBMCs were prepared from whole blood by Ficoll-Hypaque density gradient centrifugation. For some experiments, total CD4 cells or CD4⁺ monocytes (containing both CD16⁺ and CD16⁻) were purified by magnetic negative selection using EasySep magnetic separation kits (Stem Cell Technologies).

Cytokine stimulation
Total PBMCs were rested for 1 hour in RPMI medium without fetal bovine serum (FBS) after isolation. Monocytes were plated overnight in RPMI medium containing 5% FBS, 1-glutamine, nonessential amino acids, sodium pyruvate, and Hepes. Monocytes were washed two times with RPMI and rested for 1 hour with RPMI without FBS before stimulation. Stimulation was performed with TNFα (50 ng/ml; R&D Systems) or PMA (500 ng/ml; Sigma-Aldrich). Cells were fixed at 15, 30, or 45 min with BD fixation buffer (BD Biosciences), washed two times with phosphate-buffered saline (PBS), and permeabilized with ice-cold BD permeabilization buffer III (BD Biosciences). Cells were permeabilized overnight at −80 °C. After being washed, the cells were stained for CD4 phycoerythrin (PE), CD45RA AF700, CD45RO B42, and IκBα A488 (Cell Signaling Technologies). Flow cytometry was performed on a BD LSRII Fortessa (BD Biosciences). Analysis was performed using FlowJo software (TreeStar).

Nuclear localization
Total CD4 cells were stimulated with TNFα (50 ng/ml; R&D Systems) for 15 or 30 min. Cells were fixed with 3% formalin for 10 min, washed, and permeabilized with 0.1% Triton X-100 + 2% FBS in PBS (no Ca/Mg). Cells were blocked with Fc Block (eBioscience) and a cocktail of 1% FBS, 1% donkey serum, and 1% human serum. TNFR1 was stained with polyclonal TNFR1 antibody (R&D Systems) and labeled with FITC-labeled anti-rabbit IgG (Jackson ImmunoResearch). Images were acquired using a Leica microscope (Leica Biosystems) and analyzed using ImageJ software (NIH).

Quantitative PCR
RNA was extracted from whole PBMCs using Qiagen RNEasy Plus Micro Kit according to the manufacturer’s instructions. Sample concentration and purity were determined by spectrophotometer analysis on NanoDrop 2000. Complementary DNA was prepared from RNA through reverse transcription–PCR with reverse transcriptase kits from Life Technologies. qPCR was performed using primers for TNFAIP3 (hs00234713_m1), BCL3 (hs00184033_m1), CIAP1 (BIRC2, Hs01112284_m1), and the housekeeping genes HPRT (hs01003267_m1) and β2M (Hs00984230_m1) with TaqMan Fast Universal PCR Master Mix (No AmpErase Uracil N-Glycosylase) (Life Technologies). Samples were run on an ABI Prism quantitative PCR machine (Life Technologies).

Western blot
Total PBMCs were lysed with radioimmunoprecipitation assay buffer (Pierce) containing Halt protease inhibitors (Pierce). Total protein was determined by biocinchoninic acid assay (Pierce), and 10 μg of total protein was run per lane. Samples were prepared with loading buffer (Life Technologies) and heated for 10 min at 72 °C before running on Western blot. Samples were run on a 10% bis-tris gel and transferred onto nitrocellulose with the XCELL Western blot transfer apparatus (Life Technologies). Blots were blocked with 1x tris-buffered saline (TBS) and 0.1% Tween 20 with 5% (w/v) nonfat dry milk overnight. p105/p50 NFκB and actin (Cell Signaling Technology) were stained overnight, washed three times with 1x TBS and 0.1% Tween 20, and labeled with horseradish peroxidase–linked anti-mouse IgG (Cell Signaling Technologies). Blots were developed using ECL Prime (GE Healthcare). Densitometry was performed using ImageJ analysis software [National Institutes of Health (NIH)].

Confocal microscopy
Two hundred thousand monocytes were plated overnight in 24-well plates with poly-1-lysine–coated coverslips. Cells were incubated overnight, fixed with 3% formalin, and permeabilized with 0.1% Triton X-100 + 2% FBS in PBS (no Ca/Mg). Cells were blocked with Fc Block (eBioscience) and a cocktail of 1% FBS, 1% donkey serum, and 1% human serum. TNFR1 was stained with polyclonal TNFR1 antibody (R&D Systems) and labeled with FITC-labeled anti-rabbit IgG (Jackson ImmunoResearch). Images were captured on a Leica microscope and analyzed using ImageJ software (NIH).

Luminex
Plasma was prepared by centrifuging total blood at 5000 rpm for 10 min and removing the plasma fraction from the red cell fraction. Plasma was immediately frozen at −80 °C until run on a 30-plex Luminex (Millipore).

Statistics
Genotype-phenotype associations were calculated in two ways: an allelic test as implemented in PLINK (51) and comparing opposite homozygote groups by unpaired t test, as implemented in PRISM (GraphPad). In both cases, uncorrected P values were reported.

Study approval
The study was conducted in compliance with the Declaration of Helsinki. Before study initiation, approval was obtained from the ethics committee of Yale-New Haven Hospital. Informed consent was received from all subjects before inclusion in the study.

SUPPLEMENTARY MATERIALS
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Fig. S1. Confirmation of increased pNFκB in MS patients.
Fig. S2. Gating strategy and representative histogram for rs228614 GG (risk) and AA (protective) variants.
Fig. S3. n228614 and n7665090 proximal to NFκB1 result in increased IκBα degradation after TNFα treatment.

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REFERENCES AND NOTES


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In patients with autoimmune diseases, such as multiple sclerosis, immune cells attack the tissues that they are supposed to protect. However, it remains unclear why these self-targeted cells become activated in some individuals and not in others. Now, Housley et al. show that at least some multiple sclerosis patients have genetic variants that result in increased NFκB signaling after TNFα stimulation. These variants, in effect, lower the activation threshold of CD4 T cells, making them more responsive to inflammation and thus more likely to contribute to autoimmunity. Patients with these variants may be good candidates for therapies that block either NFκB signaling or inflammatory cytokines.