The role of the *CD58* locus in multiple sclerosis

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Multiple sclerosis (MS) is an inflammatory disease of the central nervous system associated with demyelination and axonal loss. A whole genome association scan suggested that allelic variants in the *CD58* gene region, encoding the costimulatory molecule LFA-3, are associated with risk of developing MS. We now report additional genetic evidence, as well as resequencing and fine mapping of the *CD58* locus in patients with MS and control subjects. These efforts identify a CD58 variant that provides further evidence of association with MS ($P = 1.1 \times 10^{-6}$, OR 0.82) and the single protective effect within the *CD58* locus is captured by the rs2300747^{G} allele. This protective rs2300747^{G} allele is associated with a dose-dependent increase in *CD58* mRNA expression in lymphoblastic cell lines ($P = 1.1 \times 10^{-10}$) and in peripheral blood mononuclear cells from MS subjects ($P = 0.0037$). This protective effect of enhanced CD58 expression on circulating mononuclear cells in patients with MS is supported by finding that CD58 mRNA expression is higher in MS subjects during clinical remission. Functional investigations suggest a potential mechanism whereby increases in CD58 expression, mediated by the protective allele, up-regulate the expression of transcription factor FoxP3 through engagement of the CD58 receptor, CD2, leading to the enhanced function of CD4^{+}CD25^{high} regulatory T cells that are defective in subjects with MS.

genetic | human | RNA | quantitative trait | inflammation

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system associated with demyelination, axonal loss, and brain atrophy; susceptibility to this disease is affected by both genetic variation and environmental risk factors (1, 2). The initial episode of neurologic dysfunction results in a clinical diagnosis of a clinically isolated demyelinating syndrome (CIS), and a second episode leads to a diagnosis of MS (1). Increasing evidence suggests that activated, autoreactive T cells play a central role in MS pathophysiology, as evidenced by the efficacy of treatments such as Natalizumab (anti-VLA-4 monoclonal antibody) that block lymphocyte egress from the vascular compartment into the CNS (3). Furthermore, the control of activated T cells by natural regulatory CD4^{+} T cells is impaired in subjects with MS (4). This population of regulatory CD4^{+} T cells expresses high levels of the IL-2 receptor (CD25) and FoxP3, an important transcription factor for regulatory T cells (4). We have now begun to integrate these immunologic observations with results of our genetic studies in patients with MS.

Two novel MS susceptibility loci have recently been identified using a genome-wide association scan approach, and these 2 loci, *IL2RA* and *IL7R*, have now been validated in independent subject collections (5–9). In the genome scan, several other loci, including the *CD58* locus, displayed suggestive evidence of association with MS susceptibility. Since CD58 (LFA-3) costimulates and enhances T cell receptor signaling by engaging CD2 (10), the *CD58* locus is an attractive target for understanding the role of genetic variation in immune system dysfunction associated with MS. Here, we first refine and then enhance the association between a polymorphism in *CD58* and MS susceptibility. Then, using data from Epstein-Barr virus (EBV) transformed B cells (lymphoblastic cell lines), we find the putative *CD58* protective allele to be significantly associated with higher CD58 RNA levels, and we validate this observation by measuring mRNA expression in circulating mononuclear cells isolated ex vivo from subjects with MS. Moreover, we present evidence that a higher level of *CD58* mRNA expression is seen during the clinically quiescent phase of MS and, finally, that higher CD58 expression may function in part by enhancing FoxP3 expression in regulatory T cells.

Results

Fine Mapping, Resequencing, and Validation of the *CD58* Susceptibility Locus in MS. We recently performed a whole genome association screen for MS susceptibility genes and identified a suggestive association at SNP rs12044852 both in the screening and replication phase ($P = 1.9 \times 10^{-5}$ in the combined analysis) (Fig. 1; ref. 5). We therefore initiated a fine mapping effort in the chromosomal region that contains rs12044852 to better characterize this association to MS. Using our collection of subjects with MS from the Brigham and Women's Hospital in Boston, MA [supporting information (SI) Table S1], we initially surveyed

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The authors declare no conflict of interest.

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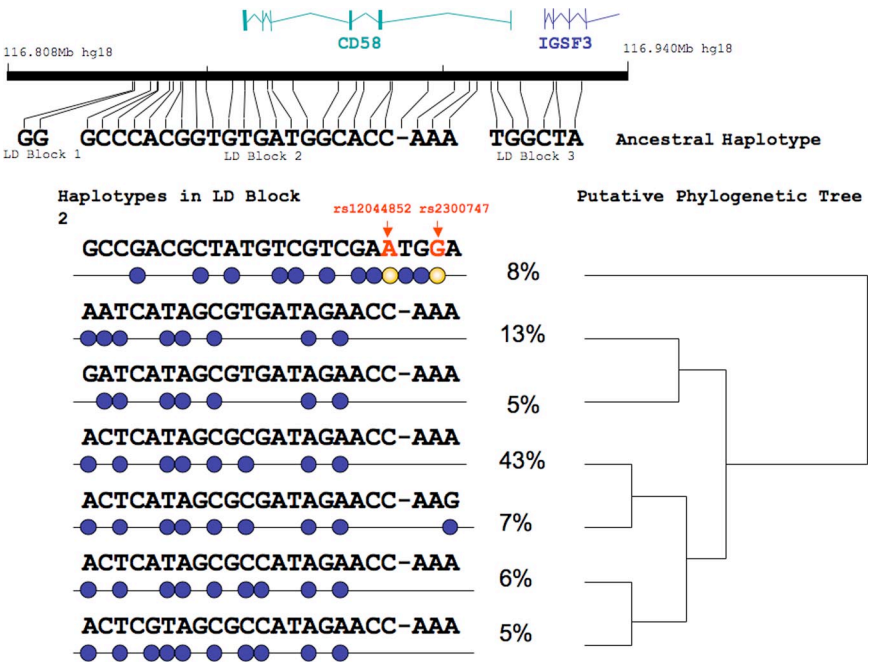

Fig. 1. The minor alleles of the 2 SNPs with the lowest association *P*-values are found on the same haplotype that spans most of the *CD58* gene. The segment of chromosomal DNA under study is shown in black, with its physical boundaries noted based on human genome assembly 18; the location of the *CD58* gene is shown in light blue. The flanking genes are *IGSF3* (centromeric, navy blue) and *ATP1A1* (telomeric, outside of the segment shown here). *Below*, we show the 31 SNPs and 1 insertion/deletion polymorphism (indel: −/T) found within this chromosomal segment that we have genotyped. These polymorphisms are divided into 3 distinct blocks of linkage disequilibrium based on genotyping data from both cases and healthy control individuals from the Brigham and Women's Hospital collection (see Fig. S1 for the detailed linkage disequilibrium structure and list of haplotypes found in this region). All haplotypes in the second block of linkage disequilibrium with a frequency >0.05 in the Brigham and Women's Hospital samples are shown with their unique sequences of alleles. The ancestral alleles of each polymorphisms, as recorded in the HapMap (27) or dbSNP resources (http://www.ncbi.nlm.nih.gov/projects/SNP/), are used to determine the ancestral haplotype, which serves as the reference haplotype, at the top of the figure. The derived (non-ancestral) allele of a SNP is highlighted by a navy blue circle on each haplotype. The minor alleles of the 2 SNPs (rs12044852 and rs2300747; Table S2) with a *P* value < 0.0005 in our primary association analysis to MS are highlighted in yellow; both minor alleles are on the same haplotype that is found at a frequency of 8% in the Brigham and Women's Hospital samples. Their location is highlighted in red. It is interesting to note that this 8% haplotype, which is underrepresented in subjects with MS, is the most divergent of the haplotypes with a frequency >5% in subjects of European ancestry studied here. The frequency of each haplotype is noted to the right, and the dendritic tree illustrates a possible evolutionary relationship of the different haplotypes.

24 SNPs that capture common variation within this region of the genome (Table S2). These SNP data allowed us to define groups of markers that are correlated and define chromosomal segments that tend to be inherited as a block (Fig. 1). Further analysis in the context of this linkage disequilibrium structure shows that the association to MS is located within the central chromosomal segment (Fig. S1). More specifically, one version of this chromosomal segment, a haplotype found at 8% frequency in our subjects, contains the minor "A" allele of rs12044852 (rs12044852^{A}) and is under-represented in subjects with MS (P = 0.0015). None of the haplotypes containing the more common "G" major allele at rs12044852 has significant evidence of association (Fig. S1). These results suggest that an allele protecting subjects from MS exists somewhere within a 76 kb segment of DNA that only contains the *CD58* gene (Fig. 1).

We then sequenced 16 selected individuals over this 76,048 bp DNA segment. Seventeen putative new SNPs were identified and underwent validation (Table S3). To identify a better marker for the *CD58* association to MS, we then genotyped those SNPs that had been validated and demonstrated some level of correlation with rs12044852. We also genotyped additional SNPs that provided information regarding genetic variation within the *CD58* gene region but which had not yet been assessed by the initial panel of 24 SNPs (Table S2). Using these data, we assessed the possibility of other independent associations to MS susceptibility within the *CD58* locus (allelic heterogeneity) by performing a conditional analysis in our fine mapping data to account for the effect of the most associated SNP (Table S4). Once the effect of the associated *CD58* protective allele is accounted for, as estimated by either of the 2 best markers (rs12044852^{A} or rs2300747^{G}), there is no residual evidence of association to MS susceptibility within the *CD58* locus. This result suggests that a single allele or a group of alleles that are strongly correlated explains the association of the *CD58* locus to MS susceptibility.

We then extended our mapping effort by genotyping the 15 most associated SNPs from our original fine mapping screen in an additional 1,278 trio families with MS (Table S5*a*). The 2 best SNPs from this trio analysis [rs2300747 and rs12044852, which are strongly correlated (r^2 = 0.929) in HapMap CEU samples; ref. 11] were then genotyped in an additional 3,341 MS cases and their controls. Once all data are pooled, rs2300747 is the most associated marker ($P = 1.1 \times 10^{-6}$, odds ratio 0.82, 95% confidence interval 0.75–0.89) (Fig. S2 and Table S5*b*). The minor allele rs2300747^{G} is found in the protective haplotype that contains the *CD58* gene (Fig. 1), and we therefore consider this allele to be a marker for a protective effect in MS susceptibility.

The *CD58* Protective Allele Affects RNA Expression. The rs2300747 polymorphism is found within the first intron of *CD58* and does not have a known functional consequence. Thus, we investigated the effect of the MS associated allele on expression of *CD58* RNA using data generated by the Sanger Institute from EBV-transformed lymphoblastic cell lines (LCL) used in the HapMap project (11, 12). Using the quantitative trait analysis module implemented in the PLINK toolkit (13), we find that the protective rs2300747^{G} allele is associated with increased expres-

sion of *CD58* RNA in 60 unrelated LCLs of European ancestry [Centre d'Etude du Polymorphisme Humain (CEPH), Utah residents with ancestry from northern and western Europe (CEU) LCLs] ($P = 0.038$) and in 89 unrelated LCLs of East Asian ancestry [Han Chinese in Beijing (CHB) and Japanese in Tokyo (JPT) LCLs] ($P = 1.1 \times 10^{-10}$). The higher frequency of rs2300747^{G} in the larger sample of East Asian LCLs (frequency = 0.66 vs. 0.13 in the CEU LCLs) explains in part the more extreme association of rs2300747^{G} with higher levels of *CD58* expression in the East Asian LCLs. This association of rs2300747^{G} with higher expression of *CD58* RNA is best illustrated by plotting the RNA expression values of individual LCLs and organizing them by genotype class (Fig. 2*A*): the rs2300747^{GG} homozygote class has a higher level of *CD58* RNA expression than does the rs2300747^{AA} homozygote class, and the rs2300747^{AG} heterozygote class has an intermediate level of expression. This effect on *CD58* RNA expression is also observed in an independent set of 400 independent LCLs from subjects of British ancestry for which similar data have been collected (14). In these samples, rs2300747 has not been genotyped, but the minor allele of rs6677309, a SNP which is strongly correlated with rs2300747 ($r^2 = 0.87$ in CEU HapMap samples; ref. 11), is seen to be associated with higher levels of CD58 RNA expression ($P = 2.1 \times 10^{-5}$). In addition, the correlation of rs2300747^{G} with RNA expression is specific to *CD58*: it is not seen with the 2 genes flanking *CD58*. Using the more informative East Asian HapMap LCLs, we repeated the quantitative trait analysis and found no evidence for association of rs2300747^{G} with RNA expression of the flanking *ATP1A1* gene ($P = 0.96$) on the telomeric side or of the *IGSF3* gene ($P = 0.50$) on the centromeric side. The LCLs from individuals of European ancestry showed similar results (data not shown). Thus, this putative "protective" *CD58* allele for MS may exert its effect on disease risk by specifically increasing the expression of *CD58* RNA in a dose-dependent manner.

We next validated this in vitro observation using ex vivo data: we examined a data set derived by extracting mRNA from circulating mononuclear cells from 239 subjects with remitting-relapsing MS (RR MS) or CIS for evidence of correlation between rs2300747 and the expression of *CD58* RNA. Since some of these subjects with MS were treated at the time of sampling, we first established that there was no significant difference in *CD58* RNA expression among subjects that are untreated ($n = 81$), treated with glatiramer acetate ($n = 64$), or treated with an IFN beta formulation ($n = 94$) (Fig. S3). We then pooled all of these subjects into a genotypic analysis. There is only one rs2300747^{GG} homozygote, so we compared the level of *CD58* RNA expression in rs2300747^{AA} homozygotes to the expression level observed in subjects bearing at least one rs2300747^{G} allele. This analysis validates our in vitro LCL results by demonstrating that the protective rs2300747^{G} allele is associated with higher expression of CD58 RNA ($P = 0.0037$) in mononuclear cells of RRMS and CIS subjects (Fig. 2*B*).

The *CD58* Locus and Clinical Manifestations of MS. To assess whether CD58 mRNA levels correlated with clinical disease activity, we analyzed RNA data captured from a different set of subjects with MS who were experiencing either a clinical relapse or remission. These data had been generated as part of an independent project that analyzed changes in RNA expression of 9,381 genes to discover relapse- and remission-specific patterns of gene expression in whole blood of untreated subjects with MS (15). Of the 38 putative MS susceptibility loci with evidence of replication in the recent whole genome association scan for MS (5), *CD58* is the only one whose RNA expression is enhanced in subjects in clinical remission (Fig. S4). The expression of *CD58* RNA in whole blood during a remission is, on average, 1.7-fold greater than baseline expression in healthy control subjects, and this remission-associated increased expression of *CD58* is significantly greater than the levels of *CD58* expression seen in subjects with MS that are sampled during a relapse ($P = 0.011$) (Fig. 3).

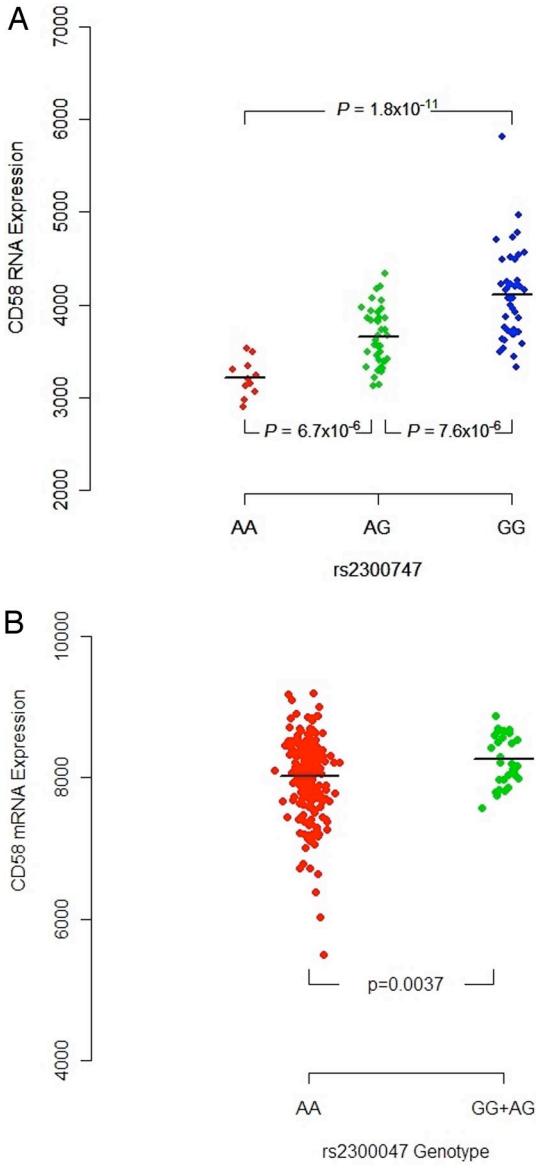

Fig. 2. The *CD58* protective allele increases *CD58* RNA expression. (*A*) To illustrate the association of the rs2300747^{G} marker to higher *CD58* expression, we have plotted the distribution of RNA expression values by genotype and show the results of pairwise comparisons between genotype categories. The location of the mean value for each category is denoted by a black line. We present the results of the genotypic comparisons among the 89 LCLs of East Asian ancestry that offer a much more robust estimate of the allele's effect given the higher frequency (0.66) of rs2300747^{G} in this population. The allele frequency of rs2300747^{G} is much lower (0.13) in the smaller collection of 60 LCLs of European ancestry; this precludes a robust pairwise assessment of genotype categories. The cell surface expression of CD58 in these cell lines shows the same pattern of association to genotype—higher surface expression in rs2300747^{GG} subjects—but does not reach statistical significance given the limited number of cell lines that are available (data not shown). (*B*) To validate the initial observation obtained from the LCL data, we present evidence that the correlation of higher *CD58* RNA expression with rs2300747^{G} is also seen ex vivo in RNA data obtained from mononuclear cells of subjects with remitting-relapsing MS or CIS. Since a single MS subject had the rare rs2300747^{GG} genotype, this subject was pooled with the 29 rs2300747^{AG} heterozygotes (green) and compared to the 209 rs2300747^{AA} homozygotes (red) with MS or CIS. The location of the mean value for each category is denoted by a black line.

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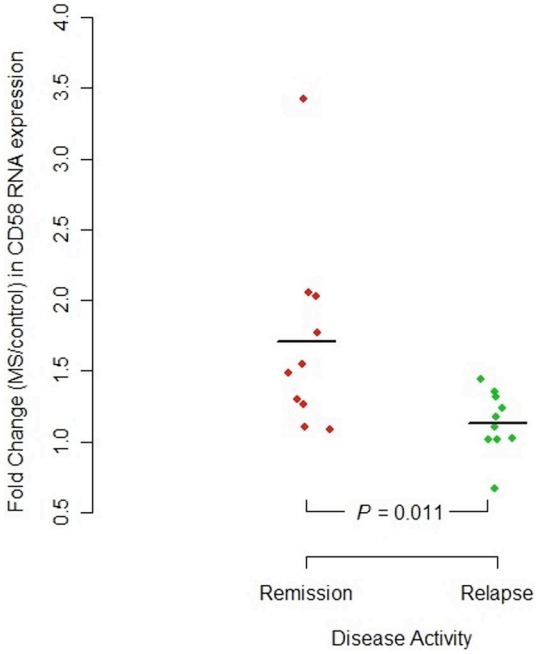


Fig. 3. Expression of *CD58* RNA is enhanced during the remission phase of MS in untreated subjects. We compare the expression of *CD58* RNA found in whole blood during the clinically defined remission and relapse phases of MS. For each phase, 10 unique untreated subjects with MS were sampled, and individual values of *CD58* RNA expression were normalized to the mean *CD58* RNA expression of 20 healthy control subjects. On average, the expression of *CD58* increases 1.7-fold in whole blood during a relapse relative to the expression found in 20 healthy control subjects, and the distribution of the relative change observed in the remission phase is significantly different from that seen during the relapse phase ($P = 0.011$). This difference remains significant after removing the extreme outlier of each category (relapse and remission) ($P = 0.040$).

These data suggest that an enhanced level of *CD58* RNA expression is correlated with a clinically quiescent state and may therefore have a role in limiting inflammation in MS.

Activation of the CD58/CD2 Pathway Enhances FoxP3 Expression in Regulatory T Cells. We examined one potential mechanism by which alterations in CD58 expression may influence immune function. CD58 engagement of its receptor, CD2, provides an activation signal for human T cells, including $CD4^+CD25^{high}$ regulatory T cells. While the precise mechanism of action of human regulatory T cells has not been established, the transcription factor FoxP3 is associated with regulatory T cell activity. However, FoxP3 expression alone does not necessarily confer suppressor activity to human T cells (16). Nonetheless, the importance of FoxP3 in mice and humans is highlighted by the association of mutations in *FOXP3* with the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (17) and by the observations that murine null alleles of *FOXP3* or even attenuated expression of FoxP3 in murine regulatory T cells are associated with aggressive autoimmune disease (18, 19).

Recent experiments have implicated CD2 in regulatory T cell activity, including the binding of FoxP3 to the CD2 promoter region in chromatin immunoprecipitation experiments (20) and the induction, by CD2 coactivation, of suppressor function in human T cells characterized by $CD4^+CD25^{high}DR^+CD62L^{high}$ expression (21). Our group demonstrated that while the proportion of regulatory T cells is normal in subjects with MS, these cells are dysfunctional (4). Subsequent studies have both confirmed these observations (22) and demonstrated that the expression of FoxP3 is diminished in regulatory T cells from patients with MS, suggesting a central role for this transcription factor in regulatory T cell dysfunction (23, 24).

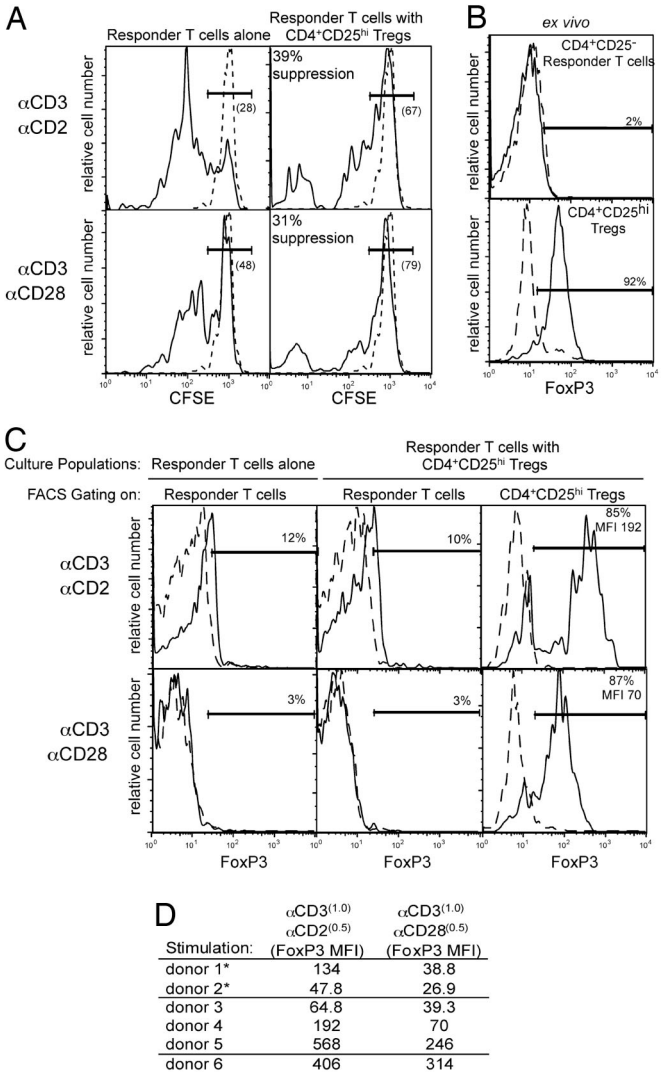
Based on these observations, we compared the induction of FoxP3 by the costimulatory signal provided through the CD58 receptor, CD2, to the effect of the strong CD28 costimulatory signal in regulatory T cells; both costimulatory signals were given in the context of T cell receptor (TCR) activation using cross-linking of the TCR by anti-CD3 monoclonal antibody. Of note, T cells express CD58, and thus exogenous CD28 costimulation occurs in the context of endogenous interaction between CD58 on T responder cells and CD2 on regulatory T cells. Both costimulatory signals are capable of triggering substantial suppression of T cell proliferation in vitro by activation of $CD4^+CD25^{high}$ regulatory T cells: 39% suppression with anti-CD2 and 30% suppression with anti-CD28 (Fig. 4*A*). Both stimuli result in enhanced FoxP3 expression in regulatory T cells when these cells are compared to regulatory T cells analyzed ex vivo (Fig. 4 *B* and *C*) and the effect of anti-CD2 is dose-dependent (Table S6). There is significantly more induction of FoxP3 with CD2 as compared to CD28 engagement in $CD4^+CD25^{high}$ regulatory T cells after 4 days of culture (Fig. 4*C*). On average, FoxP3 expression is 2.1-fold greater (log scale) following CD2 as compared to CD28 engagement (Fig. *4D*). Functionally, we have previously reported that in vitro anti-CD2 costimulation of this regulatory population results in suppression of T cell proliferation within 3 days of stimulation, as compared to 5 days with anti-CD28 (22). Thus, it appears engagement of the CD58 receptor, CD2, has a significant effect on FoxP3 expression and a more rapid impact on regulatory T cell function as compared to engagement of the CD28 costimulatory pathway. Of note, the higher proliferation of the T responder cell population in response to anti-CD2 vs. anti-CD28 stimulation (Fig. 4*A*) suggests that the costimulatory effect of anti-CD2 is broad and not specific to FoxP3 expression.

Discussion

Here, we report the detailed characterization of the *CD58* locus that affects susceptibility to MS and propose a mechanism of action for its putative protective effect. After studying 5,326 subjects with MS, we have discovered a better susceptibility marker (rs2300747) within the *CD58* locus, and, by adding 1,530 new subjects with MS to the previously published analysis (5), the evidence for this locus affecting disease risk in MS has been enhanced ($P = 1.1 \times 10^{-6}$). Although the magnitude of this allelic variant's effect on susceptibility to MS is modest, functional characterization of this polymorphism uncovers compelling evidence that the protective allele has an effect on the level of CD58 RNA expression both in vitro and ex vivo. We also show that enhanced *CD58* expression, which is associated with protection from MS, is further associated with a clinically quiescent disease state.

While CD58 is widely expressed in immune and non-immune cells, we propose that its role in the pathogenesis of MS is related to alterations in immune function. This is consistent with the pronounced inflammatory lesions associated with CNS demyelination. Moreover, enhanced CD58 expression may both mediate protection from the onset of MS and moderate acute attacks of inflammatory demyelination once the disease has begun.

Our analyses of *CD58* RNA expression led us to examine whether the protective effect of the *CD58* locus on CNS inflammation may be mediated in part through the function of regulatory T cells. Functional investigations indeed suggest a potential mechanism whereby the *CD58* risk allele leads to decreases in CD58 expression, with consequent down-regulation of FoxP3 leading to the dysfunction of regulatory T cells observed in subjects with MS (4, 22–24). Nonetheless, the ubiquitous ex-



D

Stimulation:	αCD3(1.0) αCD2(0.5) (FoxP3 MFI)	αCD3(1.0) αCD28(0.5) (FoxP3 MFI)
donor 1*	134	38.8
donor 2*	47.8	26.9
donor 3	64.8	39.3
donor 4	192	70
donor 5	568	246
donor 6	406	314

Fig. 4. CD2 costimulation strongly induces FoxP3 expression in regulatory T cells (Treg). (*A*) Cultures of CFSE-labeled responder T cells with or without $CD4^+CD25^{high}$ regulatory T cells, stimulated with αCD3/αCD2 (*Top*) or αCD3/αCD28 (*Bottom*) mAbs demonstrated 39% and 31% suppression at day 4, respectively. The percentage of cells not diluting CFSE is shown in parentheses. (*B*) Shown here is ex vivo staining for intracellular FoxP3of $CD4^+CD25^-$ responder T cells and the $CD4^+CD25^{high}$ regulatory T cells to demonstrate the purity of the initial pool of cells; 92% of cells are $FoxP3^+$. (*C*) Cultures of responder T cells only (only $CFSE^{high/low}$) or regulatory T cell cocultures ($CFSE^{neg}$ $CD4^+CD25^{high}$ regulatory T cells and $CFSE^{high/low}$ target responder T cells) were stained for intracellular FoxP3 after 4 days of culture. Expression of FoxP3 is shown on the gated responder T cells ($CFSE^{high/low}$) or regulatory T cells ($CFSE^{neg}$). Percent positive cells as compared to isotype control antibodies and mean fluorescence activity (MFI) are shown. These data are representative of 8 independent experiments performed. (*D*) On average, the expression of FoxP3 in $CFSE^{neg}$ $CD4^+CD25^{high}$ regulatory T cells after 4 days of culture is 2.1-fold greater following anti-CD2 costimulation when compared to anti-CD28 costimulation. The mean fluorescence intensity of FoxP3 expression is shown for each tested individual under each stimulation condition. Additional details are provided in Table S6 which demonstrates that the expression of FoxP3 increases in a dose-dependent manner following anti-CD2 costimulation.

pression of CD58 and its role as both an adhesion and signaling molecule necessitate caution in a single interpretation of the effect of CD58 expression in the pathogenesis of MS.

The association of allelic variants in the *CD58* gene with MS susceptibility may open new avenues of investigation into the pathogenesis of MS and offer another pathway and set of targets for the development of novel therapies. In particular, these studies suggest that manipulation of the CD58/CD2 pathway, perhaps with the CD58:IgG1 fusion protein (Alefacept) approved for the treatment of psoriasis, may be of utility (25). The in vivo immune effects associated with infusions of this fusion protein are pleiotropic and may be cell-type specific: its use in patients with psoriasis is associated with reduced numbers of memory T cells (26, 27). However, it may also have important agonistic properties that are evidenced by changes of in vivo and in vitro peripheral blood mononuclear cell gene expression within 6 hours of infusing the CD58 fusion protein (28). Finally, further characterization of immune effects driven by *CD58* gene variants in the context of other immune genes associated with susceptibility to develop MS may provide direct insight into the pathogenesis of the disease.

Materials and Methods

Subjects. All subjects (MS and healthy controls) were enrolled under study protocols approved by the Institutional Review Board of each institution. Subjects with MS all meet McDonald criteria for MS (29). Details of the clinical composition of each collection of subjects are presented in Table S1. For the trio samples, only complete trio families were used in transmission disequilibrium test (TDT) analyses. Control subjects in the Brigham and Women's Hospital collection are spouses or friends of subjects with MS. The control subjects for the Belgian, United Kingdom and University of California, San Francisco, CA, collections have been previously described (4), and the Finnish controls are anonymized individuals who underwent a blood count at the University hospital.

Genotyping and Sequencing. Details of the genotyping and sequencing platforms used in these experiments are presented in the *SI Methods* section as are descriptions of the resequencing strategy and SNP selection approach.

Disease Association Analyses. For the case/control analyses, we used a standard χ^2 calculation to establish the level of significance of an observation. For the trio analysis, we used only complete trio families and performed a TDT analysis (30). Both were implemented using the Haploview software (31), which was also used to estimate the number of each category of haplotypes used in our study. A Mantel-Haenszel approach was used for the pooled analysis of the replication data (32). Before performing a combined analysis, we performed a Pearson χ^2 goodness-of-fit test to assess the validity of combining our different replication samples; the result of this analysis ($P = 0.17$) suggests that differences in allele frequencies between the sample sets are not significant.

The analyses conditional on the genotype of rs12044852 or rs2300747 were performed using logistic regression as implemented in the PLINK toolkit v0.99r by S. Purcell (13).

Analyses of Cell Line and ex Vivo RNA Data. Details of the source and processing of the cell line and ex vivo RNA data are provided in the *SI Methods* section.

Cell Isolation, Culture, and Cytometric Analysis. Cell isolation: $CD4^+$ T cells, isolated by negative selection (Miltenyi Biotec) from whole blood mononuclear cells after Ficoll-Hypaque (Amersham Pharmacia) gradient centrifugation of heparinized blood, were FACS-sorted on a FACS ARIA (BD Biosciences) after staining for HLA-DR (PerCP, clone L243), CD62L (APC, clone Dreg 56), CD32 (FITC, clone 3D3), CD14 (FITC, clone M5E2) and CD116 (FITC, clone M5D12) (all from BD PharMingen) and CD25 (Pacific Blue, clone BC96 from BioLegend) to typically greater than 98% purity in postsort analyses. The FITC labeled mAbs were used as a combined mixture to ensure that no accessory cells were isolated in the responder T cell population ($CD4^+DR^-CD25^-CD62L^{high}$), or regulatory T cell populations ($CD4^+DR^+CD25^{high}CD62L^{high}$). Responder T cells were plated at 2.5×10^3 cells/well, while the regulatory T cells were plated at 1.25×10^3 cells/well.

Cells were cultured in RPMI 1640 media supplemented as previously described (5) with 5% human AB serum (MediaTech, Inc.) in 96 well U-bottom plates (CoStar, Corning). To be able to discriminate responder $CD4^+CD25^-$ responder T cells and regulatory T cells after their coculture, responder T cells were labeled with 0.25 μM CFSE directly after FACS isolation as we previously described in detail (21). The sorted, CFSE labeled responder $CD4^+CD25^-$ cells were then plated at 2.5×10^3 cells/well with or without $CD4^+CD25^{high}$ regulatory T cells at 1.25×10^3 cells/well to generate responder T cell only cultures or regulatory T cell cocultures at a 2:1 ratio in the previously described in vitro accessory cell-free micro coculture assay (22). The in vitro assay was modified

to include the different plate-bound stimuli of αCD3 (UCHT1, BD Biosciences, 0.5 μg/ml) with αCD2 (RPA-2.10, BD PharMingen, 0.125 μg/ml) or αCD28 (28.2, BD PharMingen, 0.5 μg/ml). The cells were harvested on day 4, stained for FoxP3 using the eBioscience FoxP3 staining buffers and the αFoxP3-PE (PCH101, eBioscience), acquired on a FACS Calibur using CellQuest Software (BD Biosciences), and analyzed with FlowJo software (TreeStar Industries).

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