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The CD6 Multiple Sclerosis Susceptibility Allele Is Associated with Alterations in CD4+ T Cell Proliferation

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Genome-wide association studies have revealed a large number of genetic associations with autoimmune diseases. Despite this progress, the mechanisms underlying the contribution of allelic variants to the onset of immune-related diseases remain mostly unknown. Our recent meta-analysis of genome-wide association studies of multiple sclerosis (MS) identified a new susceptibility locus tagged by a single nucleotide polymorphism, rs17824933 (p = 3.8 × 10−9), that is found in a block of linkage disequilibrium containing the CD6 gene. Because CD6 plays an important role in maintenance of T cell activation and proliferation, we examined the biologic phenotypes of the risk-associated allele. In this article, we report that the MS susceptibility allele in CD6 is associated with decreased expression of full-length CD6 in CD4+ and CD8+ T cells. As a consequence, proliferation is diminished during long-term activation of CD4+ T cells from subjects with the risk allele. Selective knockdown of full-length CD6 using exon 5-specific small interfering RNA induces a similar proliferation defect of CD4+ T cells from subjects homozygous for the protective allele. Exon 5 encodes for the extracellular binding site of the CD6 ligand ALCAM, which is required for CD6 stimulation. In CD4+ T cells from subjects with the risk allele, exon 5 is consistently underexpressed, thereby providing a mechanism by which the allele affects proliferation of CD4+ T cells. These findings indicate that the MS risk allele in the CD6 locus is associated with altered proliferation of CD4+ T cells and demonstrate the influence of a disease-related allelic variant on important immunological characteristics. The Journal of Immunology, 2011, 187: 3286–3291.

Multiple sclerosis (MS) is an inflammatory disease of the CNS that shares elements of its genetic architecture with other autoimmune diseases (1). Pathologically, there are perivenular infiltrates of CD4+ and CD8+ T cells in the CNS white matter and meninges with demyelinating lesions and loss of axons in both white and gray matter (2, 3). There are also marked changes in systemic immune function with loss of regulatory T cell (Treg) function and increase in myelin-reactive CD4+ T cells (4–6). Interestingly, unbiased genome-wide association scans have identified susceptibility loci almost exclusively in regions containing genes with immune function, including CD6, CD25, CD40, CD58, HLA A, HLA B, HLA-DRB1, IL2RA, IL7R, IL12A, IRF8, STAT3, and TLR3 (7–11). The identification of susceptibility loci is a major step forward in MS, but we do not yet understand how allelic variation influences immune function. This transition remains a major challenge in the understanding of MS and of other human autoimmune diseases.

CD6 is a 130-kDa type 1 transmembrane glycoprotein expressed on the surface of CD4+ and CD8+ T cells and, to a lesser extent, on B cells. CD6 stimulation plays an important role in the maintenance of T cell activation (12–19) because blocking interactions between CD6 and its ligand activated leukocyte cell adhesion molecule (ALCAM) result in diminished proliferation of T cells (12). The ALCAM-binding site is located at the membrane-proximal scavenger receptor cysteine-rich (SRCR) domain 3 of CD6 and is present in most of the five intracellular and three extracellular isoforms of CD6 that have been described (20–22). The CD6Δ3 splice variant, created by excluding exon 5, is lacking the membrane-proximal SRCR domain 3 with the ALCAM-binding site (23). The MS risk allele rs17824933G is located in intron 1 of the CD6 gene (7) and is found at a frequency of 44% of subjects of European ancestry from the PhenoGenetic Project, a cohort of >1200 healthy subjects recruited from the Greater Boston Metropolitan area that supports the functional characterization of genetic variation; subjects homozygous for the risk allele (with an rs17824933GG genotype) are found at 7.6% frequency in this population.

Materials and Methods

Subjects

The Brigham and Women’s Hospital PhenoGenetic Cohort with a collection of 1200 healthy control subjects served as basis for subject recruitment and sample collection. These subjects were genotyped and had the following characteristics: female/male sex ratio was 60/40%; race distribution was 14% African American, 12% Asian American, 68% white, and 6% Hispanic; smoking history was 82% never smokers, 9% former smokers, and 9% smokers; mean age was 24.3 y (range, 18–50 y); mean body mass index was 22.5 (range, 13–30); and MHC was 51% HLA A2, 20% HLA A3, 23% HLA-DR2, and 20% HLA-DR1 and -DR4. All subjects have been comprehensively genotyped for allelic variants related to susceptibility to autoimmune diseases. The study was conducted in compliance with the Declaration of Helsinki. Approval was obtained from the local ethics committee before study initiation, and written informed consent was obtained from all patients before performing any study procedures.

Received for publication March 3, 2011. Accepted for publication July 18, 2011. The online version of this article contains supplemental material. Abbreviations used in this article: ALCAM, activated leukocyte cell adhesion molecule; MS, multiple sclerosis; rhALCAM-Fc, recombinant human ALCAM-Fc fusion protein; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; SRCR, scavenger receptor cysteine-rich; Treg, regulatory T cell.
Primary human lymphocytes were isolated from peripheral blood of healthy donors by Ficoll gradient, and CD4+ and CD8+ T cells were purified by MACS separation. CD4+ and CD8+ T cells were cultured in RPMI 1640 medium, 10% (v/v) FCS (Life Technologies, Paisley, U.K.).

Genotyping
Healthy control subjects of the Brigham and Women’s Hospital PhenoGenetic cohort were genotyped on the Affymetrix Genome-wide Human SNP Array 6.0 (Genechip 6.0) at the Broad Institute’s Center for Genotyping and processed for quality control using the PLINK software suite. We applied its standard quality-control pipeline for subjects (genotype success rate >95%, sex concordant, excess interheterozygosity/infra-heterozygosity) and for single nucleotide polymorphisms (SNPs) (Hardy-Weinberg equilibrium $p > 1 \times 10^{-5}$, minor allele frequency $> 0.01$, genotype call rate > 0.95, mis hap test $> 1 \times 10^{-5}$) to these data. Searchable genome-wide association

The complete results of the genome-wide association analysis are available to search by SNP id. chromosome, or base pair location on the International Multiple Sclerosis Genetics Consortium Web site (http://www.imsgc.org).

Real-time PCR
Full-length CD6 and the CD6d3 isoform were amplified by PCR as previously described (13). The following primers were used for amplification of full-length CD6: 5'-TAGTAGTCTGAGGCCAGACGACGTC-CCAGAC-3' (forward) and 5'-TTAGTAGCTGCAGGAGATTCTGCTC-3' (reverse). The CD6d3 isoform was amplified using the following primers: 5'-TAGTAGTCTGAGGCCAGACGACGTC-CC-3' (forward) and 5'-TTAGTAGCTGCAGGAGATTCTGCTC-3' (reverse). Total CD6 was analyzed using commercial probes (Hs00198752_m1; Applied Biosystems) specific for the exon 1/2 binding primers: 5'-CCAGCAGTGCAGAGAGTGAGCTGGCTTCCGGTCC-3' (Hs00198752_m1; Applied Biosystems) for the exon 1/2 boundary (5’-AGGTCTACCATCTTCAGCCACCTAGCGAGGTGAGTTCTGCTC-3’). Relative fold changes of mRNA expression were calculated by the $\Delta$ACt cycle threshold method, and the amount of target gene was normalized to GAPDH (Applied Biosystems).

Small interfering RNA
Full-length CD6 expression in CD4+ T cells was selectively knocked down using a transient direct small interfering RNA (siRNA) delivered by electroporation nucleofection (Amaxa), according to the manufacturer’s instructions. Nonspecific siRNA was used as control. Expression levels of full-length CD6 and the CD6d3 isoform were analyzed by real-time PCR. 

$[^3]H$thymidine incorporation
CD4+ T cells were cultured in 96-well, round-bottom tissue culture plates at a density of $2 \times 10^5$ cells/well in RPMI 1640 medium, 10% (v/v) FCS (Life Technologies), and stimulated with 4.5-mm polylysine beads (Dynal/Invitrogen) covalently linked to anti-CD3 Abs (0.5 μg/ml), anti-CD28 Abs (0.5 μg/ml), and recombinant human ALCAM-Fc fusion protein (rhALCAM-Fc; 1 μg/ml). Cell proliferation was assessed at day 4 by $[^3]H$thymidine incorporation.

Flow cytometry
The PE-conjugated and FITC-conjugated anti-CD3, anti-CD4, anti-CD8, and anti-CD6 mAbs were purchased from BD Pharmingen (San Diego, CA). The allopolycoylin-conjugated anti-CD4 mAb was purchased from Dako North America (Carpinteria, CA). The goat anti-human IgG Ab and its biotin-, FITC-, and PE-conjugated F(ab')2 fragment derivatives were purchased from Dako North America. Living cells were gated using Live/Dead fixable dead cell stain kit (Molecular Probes, Eugene, OR). Labeled cells were analyzed on a FACSCanto cytometer equipped with the FACS Diva research software (BD Bioscience, San Diego, CA). ELISA assays
T cells were cultured in the presence of the anti-CD3 mAb OKT3 (0.5 μg/ml) and anti-CD8 mAb 15E8 (0.3 μg/ml). Where indicated, cells were incubated with 1 μg/ml anti-CD6 mAb M-T605 or 1 μg/ml isotype control. After 3 or 6 d, supernatants were collected and cytokine production was determined by ELISA as previously described (24). Matched pair capture and biotinylated Detection Abs for determination of IFN-γ (NIB42, BI133), IL-10 (JES3-19F1, JES3-12G8), and IL-13 (JES10-5A2, B69-2) were purchased from BD Bioscience. Recombinant human IFN-γ, IL-10, and IL-13 were purchased from Chiron (Ratingen, Germany).

CFSE proliferation assay
Freshly isolated CD4+ T cells were labeled with 5 μM CFSE (Invitrogen, Karlsruhe, Germany) for 10 min and washed five times with PBS/FBS. CFSE-labeled CD4+ T cells (5 × 106 cells/well) were cultured in the presence of the anti-CD3 mAb OKT3 (0.5 μg/ml) and anti-CD8 mAb 15E8 (0.5 μg/ml). Where indicated, cells were incubated with 1 μg/ml anti-CD6 mAb M-T605 or 1 μg/ml isotype control. After 3 and 6 d, CFSE dilution was analyzed by flow cytometry, and the number of cycling and noncycling CFSE-labeled T cells was determined.

Statistical analysis
Unpaired Student t test was used to compare RNA expression, cell proliferation, and cytokine production between T cells from donors with CC, CG, or GG genotype. Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA).

Results
In this study, we examined the correlation of the CD6 rs17824933G allele with changes in the immune function of healthy subjects. We use healthy subjects to investigate correlations between MS susceptibility alleles and immunophenotype because the phenotype and function of immune cells in patients with autoimmune diseases are likely to be confounded by immunotherapies or by effects of the inflamed state of autoimmune disease. PhenoGenetic Project subjects are self-reported to be free of autoimmune diseases or chronic infectious diseases; they were genotyped for rs17824933, and subjects were recalled for blood donation based on their genotype. The characteristics of the donors who were recalled for the functional assays are summarized in Supplemental Table I. Using real-time PCR, we analyzed the expression pattern of CD6 isoforms in primary CD4+ and CD8+ T cells obtained from the donors. The relative expression of the CD6d3 isoform is significantly higher on CD4+ and CD8+ T cells isolated ex vivo from donors with CC, CG, or GG genotype.
subjects homozygous for the risk allele (rs17824933CC) when compared with the same cells extracted from subjects homozygous for the protective allele (rs17824933CC). Although total CD6 expression does not significantly differ between the different groups of subjects, the relative expression of full-length CD6 is diminished in CD4+ and CD8+ T cells from rs17824933GG subjects (Fig. 1). A decrease in full-length CD6 mRNA correlates with increased levels of CD6Δd3 mRNA (Supplemental Fig. 1). Furthermore, the overall expression of CD6 on the cell surface was analyzed by flow cytometry and does not differ between the two groups of subjects (Supplemental Fig. 2). The expression patterns of other known CD6 isoforms are not affected by the risk allele (Supplemental Fig. 3).

It has been previously shown that the CD6 ligand, ALCAM, is induced on CD4+ T cells after activation, and blocking this interaction with inhibitory anti-CD6 mAb inhibits sustained entry into cell cycle (12, 25–30). Thus, we activated highly purified CD4+ T cells from donors with each of the three rs17824933 genotypes for 3 and 6 d with anti-CD3 and anti-CD28 mAbs. The interaction between CD6 on CD4+ T cells and its ligand ALCAM on activated PBMCs results in obstruction of the access to the ALCAM-binding site (23). To directly explore this mechanism, we established a model system with CD4+ T cells exclusively expressing the risk CD6Δd3 isoform without expression of full-length CD6. Expression of isoforms containing exon 5 in CD4+ T cells of subjects with rs17824933CC genotype was specifically inhibited using exon 5-specific siRNA (Fig. 3A), thus increasing the relative proportion of CD6Δd3 to CD6 isoforms; as a control, CD4+ T cells from the same subjects were transfected with randomly generated control siRNA, allowing the comparison of identical CD4+ T cells differing only in the expression level of CD6 isoforms containing exon 5. Using this model system, we found that stimulation with a rhALCAM-Fc induced significant lower entry into cell cycle of CD4+ T cells in the context of the exon 5-specific siRNA construct versus CD4+ T cells derived from the same donor and transfected with control siRNA (p = 0.0111; Fig. 3B). These data demonstrate that increasing the relative proportion of the CD6Δd3 isoform in vitro recapitulates the diminished proliferative capacity of T cells seen in rs17824933GG subjects who also have an increased proportion of the CD6Δd3 isoform.

We hypothesized that the decreased proliferative response in rs17824933GG subjects was due to the relative increase of the CD6Δd3 splice variant lacking the membrane-proximal SRCR domain 3 with the ALCAM-binding site (23). To directly explore this mechanism, we established a model system with CD4+ T cells exclusively expressing the risk CD6Δd3 isoform without expression of full-length CD6. Expression of isoforms containing exon 5 in CD4+ T cells of subjects with rs17824933CC genotype was specifically inhibited using exon 5-specific siRNA (Fig. 3A), thus increasing the relative proportion of CD6Δd3 to CD6 isoforms; as a control, CD4+ T cells from the same subjects were transfected with randomly generated control siRNA, allowing the comparison of identical CD4+ T cells differing only in the expression level of CD6 isoforms containing exon 5. Using this model system, we found that stimulation with a rhALCAM-Fc induced significant lower entry into cell cycle of CD4+ T cells in the context of the exon 5-specific siRNA construct versus CD4+ T cells derived from the same donor and transfected with control siRNA (p = 0.0111; Fig. 3B). These data demonstrate that increasing the relative proportion of the CD6Δd3 isoform in vitro recapitulates the diminished proliferative capacity of T cells seen in rs17824933GG subjects who also have an increased proportion of the CD6Δd3 isoform.

We then examined the proliferative response of CD4+ T cells to anti-CD3 and anti-CD28 in the context of inhibition of the CD6-ALCAM costimulatory pathway, postulating that inhibitory effects would be observed only in subjects expressing high relative amounts of CD6 isoforms with the ALCAM-binding domain. Specifically, we activated highly purified CD4+ T cells from donors with each of the three rs17824933 genotypes for 3 and 6 d with anti-CD3 and anti-CD28 mAbs. The interaction between CD6 on CD4+ T cells and its ligand ALCAM on activated PBMCs was blocked with the CD6-specific Ab M-T605 as previously described (12). Although the M-T605 binding domain is located in the extracellular SRCR domain 1 and is not close to the ALCAM-binding domain in the extracellular SRCR domain 3, the assembly of the CD6 molecule provides an explanation for the prevention of ALCAM binding by M-T605: binding of M-T605 to domain 1 results in obstruction of the access to the ALCAM-binding site (Fig. 4A) (31). Inhibitory effects were observed only in subjects expressing high relative amounts of the CD6 isoforms with the ALCAM-binding domain. Blocking CD6 reduced the proliferation of CD4+ T cells from rs17824933GG subjects by 27.3%, whereas no effect was seen in CD4+ T cells from subjects with one (0.6% inhibition; p = 0.0001) or two (0.4% inhibition; p = 0.0001)
rs17824933G alleles, indicating that proliferation of CD4+ T cells with CG or GG genotype is not influenced by CD6 stimulation (Fig. 4B, 4C). Similar differences were seen in IFN-γ production after blocking CD6 on CD4+ T cells from subjects of the different groups of genotype (Fig. 4D).

**Discussion**

MS is an inflammatory neurodegenerative disease with complex causative factors. The mechanisms underlying the pathogenesis of MS are not yet completely known. Infiltrations of CD4+ and CD8+ T cells in the CNS white matter are involved in the...
autoinflammatory process. Recent data suggest a possible role for autoreactive Th1 and Th17 cells in mediating tissue inflammation and demyelination in the CNS (32). Although there is a potential paradox in that the risk allele results in less activation of CD4+ cells, this may be explained by the presence of CD4+ T cell populations that both induce and suppress immune responses. Specifically, there are two major regulatory CD4+ T cell populations: Tregs (FOXP3+/CD127+/CD25hi) and the type 1 Tregs cell population expressing IL-10. This may be explained by the presence of CD4+ T cell populations that both induce and suppress immune responses. Specifically, there are two major regulatory CD4+ T cell populations: Tregs (FOXP3+/CD127+/CD25hi) and the type 1 Tregs cell population expressing IL-10. These two populations differ in their ability to suppress immune responses. Tregs are known to play a crucial role in maintaining immune tolerance and preventing autoimmune disease.

We currently do not know what the causal variant may be; rs17824933 is simply the best marker for the causal variant in our MS susceptibility analysis. A review of SNPs in linkage disequilibrium with rs17824933 (r² > 0.5 in HapMap Caucasians of European origin in Utah) did not reveal a variant with a clear effect to affect splicing of exon 5. Dedicated fine-mapping efforts will be needed to identify the site of genetic variation whose effect could be captured by rs17824933. Nevertheless, it is clear that this rs17824933 tags a haplotype of CD6 that is associated not only with susceptibility to MS but also with evidence of variation in the expression of CD6 and downstream function.

The genetic component in MS is indicated by a 20–40% increased relative risk for monozygotic twins of affected individuals (36). Moreover, siblings of affected individuals exhibit a 15–20% increased relative risk for development of MS (37). Although autoimmune diseases are more likely to be triggered by complex genetics than by a single genetic variation, not all genes associated with autoimmune diseases might be causal genes contributing to the onset of disease. Deeper insights into the functional consequences of genetic variations are crucial for the elucidation of how genes contribute to the development of autoimmune diseases. To our knowledge, the results are the first report of a direct relationship between disease-associated CD6 SNP and T cell function. Extension of this approach to other susceptibility alleles is required to enable the elucidation of pathways contributing to the onset of inflammatory diseases.

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Disclosures
The authors have no financial conflicts of interest.

References
The authors have no financial conflicts of interest.

Some of the funding sources were not included in the grant footnote. The corrected footnote is shown below.

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Supplementary Figure 1
Supplementary Figure 2

CD4+ T cells

rs17824933
Supplementary Figure 3
## Supplementary Table I

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Supplemental Material

**Supplementary Figure 1.** Differences in mRNA levels of total CD6, full-length CD6, and CD6Δd3. (A) Relative expression of total CD6 \( (p = 0.6418) \), (B) full-length CD6 \( (p = 0.0021) \), and (C) CD6Δd3 \( (p = 0.001) \) were compared in CD4+ T cells from subjects with rs17824933CC \( (n = 18) \) and rs17824933GG \( (n = 16) \) genotype.

**Supplementary Figure 2.** Expression of CD6 on CD4+ T cells. The overall expression of CD6 on the cell surface of CD4+ T cells was analyzed by flow-cytometry and does not differ between subjects with rs17824933CC \( (n = 19) \) and rs17824933GG \( (n = 17) \) genotype \( (p = 0.3256) \). The intensity of fluorescence is depicted as *Molecules of Equivalent Fluorescence* (MESF).

**Supplementary Figure 3.** Expression of the isoforms CD6b, CD6c, CD6d, and CD6e in CD4+ T cells. The overall mRNA level of full-length CD6 and CD6Δd3 together was analyzed using primers specific for the Exon 8/9 boundary, which is only expressed in those two isoforms. The mRNA level of total CD6 was analyzed with primers specific for the Exon 1/2 boundary, which is expressed in all known CD6 isoforms. The amount of CD6b, CD6c, CD6d, and CD6e was calculated by the arithmetic equation \( \Delta\Delta CT = (\Delta CT \text{ Exon 1/2} - \Delta CT \text{ Exon 8/9}) \). Overall expressions of CD6b, CD6c, CD6d, and CD6e together are compared between CD4+ T cells with rs17824933CC \( (n = 4) \), rs17824933CG \( (n = 4) \), and rs17824933GG \( (n = 3) \) genotype \( (p = 0.3185) \).
Supplementary Table I. Study subjects. Characteristics of the donors who were recalled for the functional assays.