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High Resolution HLA Analysis Reveals Independent Class I Haplotypes and Amino-Acid Motifs Protective for Multiple Sclerosis

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Abstract

We investigated association between *HLA* class I and class II alleles and haplotypes, and *KIR* loci and their HLA class I ligands, with multiple sclerosis (MS) in 412 European-American MS patients and 419 ethnically-matched controls, using next generation sequencing. The *DRB1*15:01~DQB1*06:02* haplotype was highly predisposing (odds ratio (OR) = 3.98; 95% confidence interval (CI) = 3−5.31; p-value (p) = 2.22E−16), as was *DRB1*03:01~DQB1*02:01* (OR = 1.63; CI = 1.19–2.24; p = 1.41E−03). Hardy-Weinberg (HW) analysis in MS patients revealed a significant *DRB1*03:01~DQB1*02:01* homozyote excess (15 observed, 8.6 expected; p = 0.016). The OR for this genotype (5.27; CI = 1.47–28.52; p = 0.0036) suggests a recessive MS risk model. Controls displayed no HW deviations. The *C*03:04~B*40:01* haplotype (OR = 0.27; CI = 0.14–0.51; p = 6.76E−06) was highly protective for MS, especially in haplotypes with *A*02:01* (OR = 0.15; CI = 0.04–0.45; p = 6.51E−05). By itself, *A*02:01* is moderately protective, (OR = 0.69; CI = 0.54–0.87; p = 1.46E−03), and haplotypes of *A*02:01* with the HLA-B Thr80 Bw4 variant (Bw4T) more so (OR = 0.53; CI = 0.35–0.78; p = 7.55E−04). Protective associations with the Bw4 KIR ligand resulted from linkage disequilibrium (LD) with *DRB1*15:01*, but the Bw4T variant was protective (OR = 0.64; CI = 0.49–0.82; p = 3.37E−04) independent of LD with *DRB1*15:01*. The Bw4I variant was not associated with MS. Overall, we find specific class I *HLA* polymorphisms to be protective for MS, independent of the strong predisposition conferred by *DRB1*15:01*.

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Conflicts of Interest

All authors declare that they have no competing financial interests in relation to the work described.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system with well-documented genetic contributions to its pathogenesis[1]. Genome wide association studies have implicated >100 loci in MS risk[1]. The strongest genetic associations with MS are with specific alleles at the HLA loci, in the Major Histocompatibility Complex (MHC) on chromosome 6p21. In particular, *HLA-DRB1*15:01* is the strongest genetic determinant of MS; this association has been very well-established in a variety of studies and populations[2–4]. *HLA-DRB1* allelic heterogeneity in MS risk has been described[5–16], but the role of genetic variation at the other HLA loci has been less clearly defined, due, in part, to the extensive linkage disequilibrium (LD) among the alleles at these loci. The MHC includes ~165 closely-linked genes, roughly half of which have immune-related functions[17], and large-scale SNP screening of the MHC has identified at least one non-HLA MS association in the so-called class III region[14]. Using recently developed next generation sequencing (NGS) assays, we investigated the association of HLA class I and class II alleles with MS. NGS also facilitates the association analysis of the DRB3, DRB4 and DRB5 loci (DRB3/4/5). These loci display strong LD with specific DRB1 allele families[18], and may modulate autoimmune disease associations attributed to the DRB1 locus[19] and display DRB1-independent associations[20, 21].

HLA disease associations are typically interpreted in terms of peptide binding and presentation driving specific adaptive immune responses, but class I epitopes serve as ligands for the killer immunoglobulin-like receptors (KIR) on natural killer (NK) cells, key elements in innate immunity[22, 23] and possible contributors to MS pathogenesis. While the precise role of innate immunity in MS pathogenesis is unclear, NK cells may contribute to MS indirectly via immunoregulatory activity, or directly through cytotoxicity of self-tissues[24–27].

KIR epitope ligands are encoded by class I amino acid positions 77 and 80; variants at these positions define the HLA-C C1 and C2 ligands[28, 29], the HLA-A A3/A11 ligand, and the Bw4 ligand of HLA-B and some HLA-A molecules[30, 31]. Encoded by genes on 19q13.4, inhibitory and stimulatory KIRs regulate the cytolytic killing and cytokine secretion of NK cells. The *KIR* gene complex is characterized by extensive gene content variation and allelic diversity; *KIR* haplotypes have been classified into two broad categories: *KIR A* (nine genes with primarily inhibitory functions) and *KIR B* (14 genes with inhibitory and stimulatory functions). The C1 ligand is recognized by the inhibitory KIR2DL2 and KIR2DL3 receptors, C2 by KIR2DL1[29], Bw4 by KIR3DL1[32], and A3/11 by KIR3DL2[33]. The stimulatory KIR2DS1[34, 35] and KIR2DS2 receptors are thought to bind to C2 and C1, respectively[36]; KIR2DS4 receptors bind strongly to A11 and weakly to C1 and C2[37].

KIR polymorphism has also been implicated in predisposition to many diseases, including MS[38–43]. The presence of Bw4, the ligand for KIR KIR3DL1, was protective for MS in a Norwegian cohort[38] and, more recently, the combination of KIR3DL1 and Bw4 was protective in a study of African-American patients and controls[44]. Disease association analyses of KIR variation in the context of the HLA ligand require adjustment for LD

between the HLA ligands, and specific disease associated HLA alleles. Using a MALDI-TOF mass spectrophotometer assay for *KIR* locus presence/absence and a NGS assay for HLA class I and class II alleles, we explored the association of specific KIR/HLA ligand combinations in a group of 412 patients of non-Hispanic European ancestry and 419 ethnically matched controls. We address the confounding issue of LD in these association analyses using the strategy of stratification, analyzing those strata of the data in which an associated allele is present separately from those in which it is absent.

2. Results

We initially examined the association of alleles at individual *HLA* loci. Due to the very high LD between the *DRB1* and *DQB1* loci, and the *HLA–C* and *–B* loci, each locus pair (*DRB1~DQB1* and *C~B*) haplotype was analyzed as a "super-locus" (Tables 1, 2, 3 and Supplementary Table S1). With the exception of *DPB1*, all loci and super-loci displayed significant locus level heterogeneity between MS patients and controls (Table 1).

2.1. HLA Class II Associations

Table 2 shows the association of *DRB1~DQB1* haplotypes and of *DPB1* alleles. As extensively documented in previous studies[4, 45, 46], *DRB1*15:01~DQB1*06:02* confers very high disease risk in this population (OR = 3.98; p-value (p) = <2.22E−16). We note that the other relatively common *DR2* (including *DR15* and *DR16* alleles) haplotype in this population, *DRB1*16:01~DQB1*05:02*, does not confer MS risk (OR = 1.0; p = 0.95) in this dataset. Association studies of African-American populations, in which the LD patterns differ and the *DQB1*06:02* allele is often found on non-*DRB1*15* haplotypes, indicate that it is *DRB1*15:01* and not *DQB1*06:02* that confers MS risk[44, 47]. Given the strength of the *DRB1*15:01* association with MS, all observed associations (class I alleles or HLA ligands) should be examined in light of potential LD with *DRB1*15:01*.

The other significantly associated susceptible *DRB1~DQB1* haplotype in this dataset is *DRB1*03:01~DQB1*02:01* (OR = 1.63; p = 1.41E−03), as previously reported[5, 6]. The *DRB1*04:05*, **08:01*, and **13:03* alleles, previously reported to be associated with MS [7–16] were not associated in this data set. *DRB1*04:05* and **08:01* are found on haplotypes with different *DQB1* alleles in European and East Asian populations. The low frequency of the *DRB1*13:03~DQB1*03:01* haplotype in this data set (f = 0.014 in controls and 0.02 in cases) may explain the lack of statistical significance for this association (OR = 1.37; CI = 0.6–3.19; p = 0.412). The frequency of *DRB1*04:05* haplotypes was very low, and these haplotypes were "binned" (Supplementary Table S1). Counts, frequencies and summary statistics for all detected alleles and haplotypes are included in Supplementary Table S2.

*DRB1*01:01~DQB1*05:01* (OR = 0.41; p = 9.57E−06), *DRB1*04:01~DQB1*03:01* (OR = 0.4; p = 1.24E−03), *DRB1*14:01~DQB1*05:03* (OR = 0.42; p = 0.038) and *DRB1*07:01~DQB1*02:02* (OR = 0.55; p = 0.0014) were significantly protective for MS. The *DRB1*01:01~DQB1*05:01* haplotype is known to include the *DQA1*01:01* allele[48–52], which, along with *DRB1*01:01*, was recently shown to be protective for MS in the presence of *DRB1*15:01*[53]. While no *DRB1~DQB1* haplotypes in our study displayed MS associations in the *DRB1*15:01*-positive stratum (Supplementary Table S3),

*DRB1*01:01~DQB1*05:01* remained protective in the *DRB1*15:01*-negative stratum (OR = 0.57; p = 1.9E−02). The *DRB1*14:01* protective effect has been previously reported[54–56]. No individual *DPB1* alleles were associated with MS in this data set.

The clonal nature of NGS allows the analysis of the secondary *DRB* loci (*DRB3/4/5*). Because all *DRB1*15:01~DQB1*06:02* haplotypes carried the *DRB5*01:01* allele, and all *DRB1*16:01~DQB1*05:02* haplotypes carried the *DRB5*02:02* allele, the role of allelic variation at DRB5 could not be assessed in this dataset. However, the predisposing *DRB1*03:01~DQB1*02:01* haplotype carries either *DRB3*01:01 or *02:02*. A recent study of type 1 diabetes[19] showed that the *DRB1*03:01* haplotypes carrying *DRB3*02:02* conferred greater risk than did those carrying *DRB3*01:01*. For MS, the allelic variation in *DRB3*, appeared to affect the risk conferred by *DRB1*03:01* haplotypes based on this modest sample set (15 MS patients, 3 controls) but this effect was not significant. The OR for *DRB1*03:01* homozygotes homozygous for *DRB3*01:01* was 3.64 (CI = 0.69–36.1), whereas the OR for *DRB1*03:01* homozygotes that carried *DRB3*02:02* was 8.36 (CI = 1.1–371.2). Testing whether the point estimates for these ORs are significantly different will require a larger sample set. We note that *A*30:02* and *B*18:01*, alleles in strong LD with *DRB1*03:01~DRB3*02:02* haplotypes[19], are associated with MS (Table 3).

2.2. Protective Association of A*02:01

In the association analyses of the class I loci (Table 3), *HLA-A*02:01* appears protective (OR = 0.69; p = 1.46E−03), as previously reported[57–62]. After stratifying the data to account for negative LD with *DRB1*15:01* (Table 4), *A*02:01* on haplotypes lacking *DRB1*15:01* remains protective (OR = 0.48; p = 1.1E−08).

Further, the ORs of three common extended *A~C~B~DRB1~DQB1~DPB1* haplotypes, all bearing *DRB1*15:01* and differing only in the *HLA-A* allele, indicate that the presence of *A*02:01* can reduce the risk conferred by *DRB1*15:01* (Table 5). The OR conferred by the extended *C*07:02~B*07:02~DRB1*15:01~DQB1*06:02~DPB1*04:01* haplotype bearing *A*02:01* is lower (OR = 1.65) than the OR for the same haplotype bearing *A*03:01* (OR = 2.83) or *A*24:02* (OR = 4.48). This protective effect of *A*02:01* is not simply a haplotype effect. The modification of *DRB1*-mediated risk by *A*02:01* can also be assessed by stratifying the data based on the presence of *A*02:01* (Table 6); these observations suggest that *A*02:01* in *cis* or in *trans* can decrease the OR of other *DRB1~DQB1* haplotypes.

2.3. Associated C~B haplotypes

For *C~B* haplotypes (Table 3), *C*07:02~B*07:02* is associated strongly with MS (OR = 1.99; p = 8.8E−07); however, this association reflects the strong LD between this haplotype and the predisposing *DRB1*15:01* allele (d'_{ij} = 0.71 in MS patients, and 0.52 in controls). Two different *C~B* haplotypes display a protective association in this data set. As previously reported[60, 63, 64], *C*05:01~B*44:02* is modestly protective (OR = 0.65; p = 0.043). *B*44:02* is rarely found with any other *HLA-C* allele, while the *C*05:01~B*18:01* haplotype is clearly not protective (OR = 2.07; CI = 0.88–5.25; p = 0.71), suggesting that *B*44:02* may be responsible for the observed modest association for this haplotype.

In addition, the *C*03:04~B*40:01* haplotype (OR = 0.27; p = 6.76E−06) shows a strong protective association. This protective *C~B* haplotype is in LD with the protective *A*02:01* allele, and this three-locus haplotype (Table 4) is even more strongly protective (OR = 0.15; CI = 0.04–0.45; p = 6.5E−05).

The cALD measures $W_{HLA\text{-}A/HLA\text{-}C\sim HLA\text{-}B}$ and $W_{HLA\text{-}C\sim HLA\text{-}B/HLA\text{-}A}$ are 0.6 and 0.42 in cases, and 0.6 and 0.39 in controls, respectively, indicating more variation of *C~B* haplotypes relative to *HLA-A* alleles, than in *HLA-A* alleles relative to *C~B* haplotypes; the intermediate level of LD between *A*02:01* and the *C*03:04~B*40:01* haplotype (d'_{ij} = 0.11 in MS patients and 0.34 in controls) suggests that the strong protective association for the *A*02:01~C*03:04~B*40:01* haplotype results from the combination of these three alleles, and not LD with a single protective locus. *C*03:04~B*40:01* remains protective in the absence of *A*02:01* (OR = 0.42; p = 0.018), and *A*02:01* is modestly protective in the absence of *C*03:04~B*40:01* (OR = 0.79; p = 0.048), suggesting that the observed protective association for the *A*02:01* allele is not due entirely to LD with *C*03:04~B*40:01*.

2.3.1. Impact of DRB1*15:01 Predisposition on C*03:04~B*40:01 Association—

The highly protective *C*03:04~B*40:01* haplotype is in negative LD with the highly predisposing *DRB1*15:01* allele (d'_{ij} = -1); no *C*03:04~B*40:01*-bearing haplotypes carry *DRB1*15:01*. In principle, this negative LD with *DRB1*15:01* might account for the protective associations observed for *A*02:01* and *C*03:04~B*40:01*. We applied stratification analyses (Table 4) to determine if this LD pattern could account for the observed protective association of this *C~B* haplotype. In the stratum lacking *DRB1*15:01*, the protective association of *C*03:04~B*40:01* is even stronger (OR = 0.29; CI = 0.15–0.55; p = 2.45E−05), so the protective association cannot be attributed simply to negative LD with the highly predisposing *DRB1*15:01*. In individuals carrying *DRB1*15:01*, the presence of the *C*03:04~B*40:01* haplotype on the other chromosome reduces MS risk (OR = 1.37; p = 0.57) compared to all other *C-B* haplotypes (OR = 5.06; p = 3.21E−13) (Table 6). The only other significant associations in this *DRB1*15:01*-negative stratum are *C*05:01~B*18:01* (OR = 2.87; p = 0.01) and *C*07:01~B*08:01* (OR = 1.98; p = 0.0004) but these are both due to LD with the predisposing *DRB1*03:01* (d'_{ij} = 0.87 and 0.72 in cases, and 0.51 and 0.69 in controls, respectively). *C*03:04~B*40:01* remained protective in the *DRB1*03:01*-negative stratum (OR = 0.32) (data not shown).

2.4. Hardy-Weinberg Equilibrium Analyses

The analysis of Hardy-Weinberg equilibrium (HWE) among controls can serve as a test of genotyping and sampling validity, while deviations from HWE among cases can, potentially, reveal patterns of disease association. Adherence to HWE expectations is a requirement for control groups in case-control studies. Among those loci that showed a significant MS association (*HLA-A*, *-B*, *-C*, *DRB1*, *DQB1*), no deviation from HWE was observed among controls (data not shown), including HWE analysis for *DRB1~DQB1* haplotypes. While studies of HLA diversity in the US population have identified varying degrees of population stratification among non-Hispanic European Americans[65, 66], these Hardy-Weinberg analyses reveal no significant population stratification in this cohort.

Among MS patients, highly significant deviations from HWE were seen for genotypes of *DR~DQ* haplotypes (p = 0.0027). The two most common genotypes of *DRB1~DQB1* haplotypes that contributed to this deviation were *DRB1*03:01~DQB1*02:01* homozygotes (15 observed, 8.6 expected; p = 0.016) and *DRB1*07:01~DQB1*03:03*+*DRB1*15:01~DQB1*06:02* heterozygotes (10 observed, 5.3 expected; p = 0.0078), both observed more often than expected among cases. The excess of *DRB1*03:01* homozygotes among cases suggests a recessive model for MS risk. Consistent with this interpretation of the HWE deviation, the OR for the homozygous *DRB1*03:01~DQB1*02:01* genotype is 5.27 (p = 0.0037) compared to *DRB1*03:01~DQB1*02:01*+*DR~DQ*X* (OR = 0.74; p = 0.13), where *DR~DQ*X* is any haplotype that does not include *DRB1*15:01* or *DRB1*03:01*. The OR for this *DRB1*03:01~DQB1*02:01* homozygote is close to that for *DRB1*03:01~DQB1*02:01*+*DRB1*15:01~DQB1*06:02* (OR = 5.55; p = 1.32E−06) and *DRB1*15:01~DQB1*06:02*+*DRB1*15:01~DQB1*06:02* homozygote (OR = 7.6; p = 1.13E−05).

The excess of observed *DRB1*07:01~DQB1*03:03*+*DRB1*15:01~DQB1*06:02* genotypes among cases suggests that the susceptibility conferred by the *DRB1*15:01* haplotype may be “dominant” over the protection conferred by the *DRB1*07:01* haplotype. The expected number of cases in the HWE analysis is based on the protective effect of the *DRB1*07:01~DQB1*03:03* haplotype over **all** genotype combinations.

2.5. Association analysis of KIR and HLA ligands

2.5.1 HLA Ligands—Association analyses for the presence/absence of the KIR loci and their HLA ligands are shown in Table 7 and Supplementary Table S4. As previously reported[38] the HLA ligand Bw4 (Thr or Ile at HLA-B amino-acid position 80) is negatively associated with MS (Table 7A; OR = 0.62; p = 5.95E−04). The OR for Bw4/Bw4 is 0.63 and for Bw6/Bw6 is 1.61. The observed protective effect of Bw4+ alone, however, may be attributed, in part, to negative LD with the highly predisposing *DRB1*15:01*; when the data are stratified on the presence of *DRB1*15:01* (Table 7B), the statistical significance of the Bw4+ effect is diminished in the stratum missing *DRB1*15:01* (OR = 0.72; p = 0.08). This interpretation suggests that the observed Bw4 protective association with MS is not necessarily due to the Bw4 signaling via its inhibitory receptor KIR3DL1, but may simply reflect LD patterns between *HLA-B* and *DRB1*.

In the association analysis of individual amino acid residues (see below, Table 8C), the Bw4 epitope with Thr at position 80 (Bw4T) shows a protective association (OR = 0.64; p = 0.0003) but the stronger-binding Bw4 epitope with Ile (Bw4I) does not (OR = 0.92; p = 0.56), consistent with the Bw4 association reflecting LD and not ligand mediated KIR signaling. Association analyses of the Bw4 epitope on some HLA-A molecules (ABw4) reveal no protective effect (data not shown). The frequency of *DRB1*15:01* in Bw4+ individuals is 48% in MS patients and 16% in controls, while it is 51% in Bw4- patients and 26% in Bw4- controls, suggesting that the disease risk associated with *DRB1*15:01* is not reduced in the Bw4 positive stratum. However, subdividing Bw4 does reveal a difference in the association pattern, and this difference cannot be attributed simply to LD. Both Bw4T

and Bw4I are in negative LD with *DRB1*15:01* (d'_{ij} = -0.45 and -0.64 in cases, and -0.79 and -0.92 in controls, respectively) but the negative association of Bw4T with MS remains nominally significant (OR = 0.071; p = 0.032) even in the *DRB1*15:01* negative stratum (Table 7C).

2.5.2 HLA Ligand with KIR—Since the interaction of specific receptors and their HLA ligands is functional, we analyzed specific combinations of HLA ligands and KIR genotypes (Supplementary Table S4A). To address the issue of LD with *DRB1*15:01*, we also examined these combinations in the stratum lacking *DRB1*15:01* (Supplementary Table S4B). The combination of Bw4 and *KIR3DL1* has been reported to be protective in the recent study of MS in African-Americans[44] including the *DRB1*15:01*-lacking stratum. In our dataset, Bw4 is protective in the presence of *KIR3DL1*, a gene present on virtually all *KIR* haplotypes (OR = 0.62; p = 6.12E−04) but also protective in the presence of *KIR2DL3* (OR = 0.58; p = 9.12E−05). However, following stratification on *DRB1*15:01*, Bw4 and *KIR3DL1* are no longer significantly protective (OR = 0.75; p = 0.11) in the *DRB1*15:01* negative stratum. The protective association with Bw4 and *KIR2DL3*, however, is still nominally significant (OR = 0.62; p = 0.010). At the KIR genotype level, one specific combination in this *DRB1*15:01* negative stratum (Bw4+ and *KIR2DL2*/*KIR2DL3*) shows a nominally significant protective association (OR = 0.59; p = 0.017), but Bw4+ with *KIR2DL2*/*KIR2DL2* (OR = 2.17; p = 0.051), or with *KIR2DL3*/*KIR2DL3* (OR = 0.91; p = 0.63) do not. Given the multiple comparisons in this association analysis, replication in another cohort will be critical in validating this observation.

2.6. Association Analyses of Individual Amino Acids

The association analyses of individual amino acids in the *HLA class I* and *class II* genes can potentially reveal functionally important aspects of disease associations. Several statistically significant associations are shown in Table 8A and dissected in Tables 8B and 8C.

Table 8B shows the individual *DRB1* exon 2-encoded amino acid residues associated with MS. Pro at DRβ position 11 and Arg at position 13 are significantly associated with MS (OR = 3.23; p = 2.22E−16, each) but these specific residues are unique to *DRB1*15* and **16* alleles and reflect the association of *DRB1*15:01*. The less common *DRB1*15:02* and *DRB1*16:01* alleles found in this population share this amino acid motif but do *not* confer risk to MS. Position 86 Val is also associated with MS (OR = 2.15; p = 1.56E−14). Many *DRB1* alleles that are not associated with MS also encode Val-86 but the Val-Gly dimorphism at position 86 is the only difference between highly susceptible *DRB1*15:01* and neutral *DRB1*15:02*. Position 86 contributes to peptide binding pocket 1, underscoring the role of position 86 dimorphism in determining peptide specificity.

Association analyses of individual HLA class I-encoded amino acid residues that constitute the KIR ligand epitopes are shown in Table 8C. As noted above, the HLA-B position 80 Bw4T subtype is protective while Bw4I, thought to be a stronger binding ligand of KIR3DL1, is not. The modest protective association of Bw4T is not due to negative LD with *DRB1*15:01*, as it remains nominally significant even in the *DRB1*15:01*-negative stratum (Table 7C).

The HLA-C positions 77 and 80, which encode the C1 and C2 KIR ligands, are not associated with MS but, interestingly, amino acid positions 73–90, which influence the strength of KIR ligand binding[67] are significantly associated. The OR for the 73-77-80-99 motif (A~S~N~D) for the C2 epitope is 1.63 (p = 2.3E−06). This motif, however is in LD with *HLA-C*07:02* (OR = 1.9; p = 2.11E−06), the HLA-C allele in LD with *DRB1*15:01*. Thus, the observed association of the A~S~N~D C2 motif probably reflects LD rather than KIR signaling. The same motif is present in C*07:01 and *07:04, alleles not associated with MS, consistent with this interpretation.

2.7 HLA-A*02:01 and Bw4

The strong protective associations of *C*03:04~B*40:01* and *A*02:01* do not appear to reflect LD with *DRB1*15:01* or the Bw4 ligand group. The *A*02:01* protective association with MS has been previously reported in various populations[14, 57–61]. In a recent study of African-American MS, the combined presence of KIR3DL1 and Bw4, its ligand, was protective, and the protective association for A*02 was attributed to LD with Bw4[44]. This interpretation suggests that innate immunity and NK cell function, regulated by the Bw4 ligand, account for the observed negative association with *A*02:01*.

Our data suggest that *A*02:01* is associated with protection from MS in European Americans, and that the protection conferred by *A*02:01* in combination with *C*03:04~B*40:01* (OR = 0.15; p = 6.51E−05) is stronger than the observed negative association with Bw4 presence (OR = 0.62; p = 5.95E−04). In our study, LD is modest between *A*02:01* and the Bw4 epitope (d'_{ij} = 0.17 in MS patients and 0.18 in controls), but much lower than LD of *C*07:02~B*07:02* with *DRB1*15:01* in MS patients (0.71) or *A*02:01* with *C*03:04~B*40:01* in controls (0.34). The *A*02:01*~Bw4 haplotype is as protective as Bw4 presence (OR = 0.62; p = 1.69E−03) (Table 9), but *A*02:01*~Bw4T haplotypes are more protective (OR = 0.53; p = 7.55E−04), while *A*02:01*~Bw4I haplotypes are not, consistent with Table 8C.

LD between *A*02:01* and *Bw4T* is comparable to that between *A*02:01* and *Bw4* (d'_{ij} 0.16 in MS patients and controls), whereas LD is much stronger between *A*02:01* and *C*05:01~B*44:02* (0.59 in MS patients and 0.62 in controls). Of the *HLA-B* alleles in protective *C~B* haplotypes, *B*40:01* encodes Bw6, while *B*44:02* encodes Bw4T; the protection associated with Bw4T may reflect, in part, the protective *C*05:01~B*44:02* haplotype (and perhaps other Bw4T-encoding *HLA-B* alleles).

3. Discussion

We have identified multiple *HLA* class I and class II alleles and haplotypes associated with MS. Strong LD is a characteristic of the HLA region, and we investigated allele-pair LD and conditional asymmetric LD, and applied stratification analysis to adjust for LD in order to dissect and interpret these associations. In addition to standard case-control association analyses, we applied Hardy-Weinberg equilibrium analyses to cases and controls to validate our association findings. Many immune-related genes in the MHC were not analyzed in this study; given the LD known for the MHC, our analyses do not exclude these genes as potentially playing roles in MS susceptibility. However, association analysis, following

stratification, proved effective at identifying the independent effects of specific HLA alleles and haplotypes. As reported in many previous studies, the *DRB1*15:01~DQB1*06:02* haplotype is most strongly associated with MS risk; *DRB1*03:01~DQB1*02:01* is also significantly associated with a recessive effect on MS risk and, as expected, is very strongly associated with MS in the *DRB1*15:01*-negative stratum. NGS HLA typing allowed the analysis of the *DRB3* allelic diversity on *DRB1*03:01* haplotypes, and our analyses suggest that *DRB3*02:02* may confer higher risk than *DRB3*01:01*, but this observation must be tested in a larger study.

*A*02:01, C*03:04~B*40:01* and the haplotype carrying all three alleles show very strong protective associations (OR = 0.15 for the three-locus haplotype) with MS, independent of LD with *DRB1*15:01*. The protective association of the *A*02:01~C*03:04~B*40:01* haplotype displays the strongest effect size of the observed *HLA* associations in this study.

For the HLA ligands of the KIR, the presence of Bw4 was negatively associated with MS in the unstratified dataset, as noted in previous reports, but was no longer significant in the stratum lacking *DRB1*15:01.* While this observed association may simply reflect negative LD between *Bw4* and *DRB1*15:01* in this population, the two Bw4 subtypes, Bw4T and Bw4I, showed different association patterns. The protective association of Bw4T remained nominally significantly even in the *DRB1*15:01*-negative stratum, while Bw4I was not associated in either stratum. The Bw4 motif on HLA-A molecules (all of which are Bw4I) was also not significantly protective. From the available data, we cannot distinguish between a potential effect on peptide binding mediated by this Thr/Ile polymorphism in HLA-B pocket F, differential signaling via the KIR3DL1 receptor, or a combination of the two. A recent study of HIV infection indicates that the binding of a specific HIV peptide can influence the interaction of the Bw4 epitope with the KIR3DL1 receptor[68]. The difference between an uncharged, polar side chain (Thr) and an aliphatic side chain (Ile) may influence peptide binding, and through differential peptide binding, KIR3DL1 signaling.

In investigating different HLA ligand/KIR genotype combinations in the *DRB1*15:01*-negative stratum, the strongest protective Bw4 association we observed was in combination with *KIR2L2/KIR2DL3*, which is stronger than Bw4 in combination with 3DL1. This protective association was nominally significant but, given the number of comparisons, validation of this observation requires testing in another large cohort. The immunological mechanism underlying the Bw4 T protective association remains unclear.

Many other amino acid positions were implicated in our analyses, but, as in all HLA related association studies, they must be considered in the context of LD. Some disease associated amino acid residues simply “tag” an allele, recapitulating an already well-established allele association. These associations, the report of Raychaudhuri and colleagues notwithstanding[69], do not increase our functional understanding of HLA-related disease association. However, other individual amino acid associations that do *not* correspond uniquely to specific alleles may provide some functional insights, although the peptide binding properties of HLA molecules are obviously determined by *multiple* amino acid residues. In general, the potential role of individual amino acids in disease associations can

be best evaluated by comparing alleles that differ in disease risk, and differ in only one amino acid position.

For example, *DRB1* alleles encode either Gly or Val at DRβ position 86; this position contributes to peptide binding pocket 1[70], which anchors the N-terminal end of the bound peptide[71]. Positions 82 and 89 also contribute to pocket 1, but are invariant in this dataset and in most HLA alleles. Neither 86G nor 86V tag a specific allele, but the predisposing *DRB1*15:01* allele (86V) and the neutral *DRB1*15:02* allele (86G) differ only at encoded position 86. In Table 8B, position 86V (OR = 2.15; p = 1.56E−14) was implicated as potentially being functionally related to the observed association of *DRB1* with MS.

Finally, the non-Hispanic European-American cohort in this study represents a “pan-European” population, and as such may be subject to population stratification. However, our Hardy-Weinberg analyses revealed no significant population stratification in this cohort. In addition, the frequencies of key alleles and haplotypes (e.g., *HLA-B*18:01* and the *A1~B8~DR3* haplotype) in our cohort are consistent with those observed across Europe[72], as opposed to the very high-frequencies observed for these variants in specific European populations, again suggesting that stratification in this cohort is minimal.

3.1 Conclusions

Some associations of specific HLA alleles, e.g., the strong protective effect of the *C*03:04~B*40:01* haplotype, remain highly significant following stratification on *DRB1*15:01*. In general, the results of these analyses indicate that a careful consideration of LD patterns among HLA alleles is essential in the interpretation of MS association data. Overall, we conclude that specific *HLA* class I polymorphisms are protective for MS, independent of the strong MS predisposition conferred by the *DRB1*15:01* allele.

4. Materials and *Methods*

4.1 Samples

Blood samples were collected for 412 MS patients of self-identified non-Hispanic European ancestry, and 419 healthy, ethnically matched controls. MS patients were diagnosed by neurologists specialized in demyelinating diseases in accordance with well-established diagnostic and study inclusion criteria[73]. Controls were of self-identified non-Hispanic European ancestry and reported no history of chronic diseases for themselves or their nuclear family. De-identified genomic DNA was extracted using a standard desalting method and quantitated in duplicate using the PicoGreen dsDNA quantitation reagent. Coded DNA aliquots are stored at −80°C. Study protocols were approved by the UCSF Committee on Human Research and informed consent was obtained from all participants.

4.2 Genotyping

Locus-specific genotyping for the 14 *KIR* loci was performed as previously described[74, 75]. Next-generation sequencing of *HLA*-class I exons 2, 3 and 4, *HLA* class II exon 2, and *HLA* class II exon 3 (for the *DQB1* locus) on the Roche (Pleasanton, CA, USA) 454 GS FLX instrument was used to genotype *HLA-A*, *-C*, *-B*, *DRB1*, *DRB3/4/5*, *DQA1*, *DQB1* and

DPB1 alleles[76–79]. NGS *HLA* sequences were assigned to *HLA* alleles on the basis of reference sequences in IMGT/HLA Database version 3.1.0 (July 17, 2010) using Conexio (Fremantle, Australia) Assign ATF version 1.1.0.35.

HLA genotyping was blinded with respect to MS patients and controls, with 15% of specimens retyped for quality assurance purposes; our NGS HLA genotypes were verified independently via HistoGenetics (Ossning, NY, USA)[80] with >98% concordance. Discordant typings were reviewed and re-typed, and the final dataset was 100% concordant between the two NGS methods. Subject disease-status (case/control) was only released for analysis after the genotyping was completed.

4.3 Data Analysis

4.3.1. Tests of Association—We applied locus-level tests of heterogeneity and variant-level chi-squared (χ^2) tests of association at the genotype, haplotype, locus and individual amino acid levels using BIGDAWG (v1.8.1)[81]. In these tests, each multi-gene group (e.g., *HLA-C~HLA-B*), individual gene (e.g., *HLA-DRB1*) and inferred polymorphic amino acid position (e.g., DRβ position 86) was treated as a locus, and individual haplotypes (e.g., *HLA-C*07:02~HLA-B*07:02*), alleles (e.g., *DRB1*03:01*) and amino-acid residues (e.g., DRβ position 86V) were treated as variants. For each comparison, variants with expected counts less than 5 in cases or controls were combined into a common "binned" category for analyses[82].

We measured interaction between *KIR* and *HLA* loci by applying a χ^2 test to contingency tables that crossed disease phenotype with genotype, where genotype was defined as a given *KIR-HLA* combination. Specifically, we tested dominant and additive effects of *KIR* genes and their ligands at all biallelic loci in the overall cohort in addition to sub-cohorts defined by presence of *DRB1*15:01*. From these contingency tables, we calculated odds ratio with 95% confidence intervals, and *p*-values.

4.3.2. Test of Hardy-Weinberg Equilibrium—We performed tests for deviations from Hardy-Weinberg equilibrium (HWE) proportions using BIGDAWG and PyPop (v0.7.0)[83], assessing genotyping proportions for both individual loci and specific haplotypes (using haplotypes assigned to individuals in BIGDAWG on the basis of posterior probabilities). We identified significant locus-level HWE deviations using Guo and Thompson's exact method[84], and identified individual genotypes deviating significantly from HWE expectations using Chen's method[85, 86], using a threshold of significance of 0.05.

4.3.3. Evaluation of Linkage Disequilibrium—We calculated normalized LD values (d'_{ij})[87] for individual haplotypes with PyPop, and calculated conditional asymmetric LD (cALD) values, evaluating LD between sets of loci, using the "asymLD" R package (v0.1)[88]. Values of d'_{ij} range from -1, when the haplotype is never observed, to 1, describing the maximum possible LD based on the frequencies of the constituent alleles. The cALD measure $W_{A/B}$ is the correlation coefficient for alleles at locus *A* conditioned on the alleles at locus *B*, and describes the overall variation of alleles at locus *A*, given specific alleles at locus *B*. $W_{B/A}$ is the correlation coefficient for alleles at locus *B*, conditioned on the alleles at locus *A*, and describes the overall variation of alleles at locus *B*, given specific alleles at

locus A[89]. When there are equal numbers of alleles and complete allele correlation between both loci, the value of $W_{A/B}$ and $W_{B/A}$ is 1, indicating no variation of alleles between loci.

4.3.4. Corrections for Multiple Comparisons—For locus-level χ^2 tests of heterogeneity involving individual loci (i.e., *HLA-A* and *DPB1*) and haplotypes of loci (e.g., *C~B* and *DRB1~DQB1*), the threshold of significance was calculated as 0.05/n, where n is the number of comparisons. We note that these comparisons are not necessarily independent (e.g., the *HLA-A* locus is included in four comparisons), so that these estimates can be considered overly conservative.

Our tests of the specific hypothesis that the protective effect of *HLA-A*02:01* is due to LD with the Bw4 motif[44] are addressed separately from other locus-level and haplotype-level comparisons. These tests pertained to the *A*~Bw4/Bw6 and *A*~HLA-B Position 80 amino-acid variant haplotypes. Similarly, our tests of Bw4 Thr and Ile subtypes in *DRB1*15:01*-positive and –negative strata address our observation that HLA-B position 80T is associated with MS, whereas position 80I is not, and our tests of *DRB1* alleles in *DRB1*15:01*-positive and –negative strata address the observation that *DQA1*01:01* (found on the *DRB1*01:01~DQB1*05:01* haplotype) is protective only in the presence of *DRB1*15:01*[53]. The threshold of significance for both of these pairs of locus-level χ^2 tests of heterogeneity was calculated as 0.05/2 (0.025E−2).

For χ^2 tests of heterogeneity of amino-acid positions, the threshold of significance was calculated for each individual locus as 0.05/n, where n is the number of variant amino-acid positions at that locus. Results are not presented for positions that did not display significant position-level heterogeneity.

In cases where locus-level tests of heterogeneity were not significant (p-value > the threshold of significance), the threshold of significance for the χ^2 tests of association was calculated as 0.05/n, where n equals the number of variants at that locus.

4.3.5. Statistical Power Analysis—We used the pwr.chisq.test function in the R “pwr” package (version 1.2–0) to evaluate the size of an effect detectable in our dataset with the recommended statistical power (1-β) of 0.8 with an α of 0.05[90]. For association tests of alleles and haplotypes, with 31 allele categories, we expect to detect small effect sizes (0.121). For tests of locus presence, motifs and amino acid positions, with 2–5 categories, we expect to detect very small effect sizes (0.068 – 0.085).

4.4. Data Access

The HLA and KIR genotype data used for the analyses described here have been deposited into ImmPort (http://www.immport.org), the public data-sharing resource of the National Institute of Allergy and Infectious Disease’s (NIAID) Division of Allergy, Immunology, and Transplantation (DAIT) and Division of Microbiology and Infectious Diseases (DMID), and can be accessed under the ImmPort Study Accession Number SDY1045 (doi:10.21430/M3QW34U2SG).

4.5 Code Availability

The source code for BIGDAWG is available online at https://cran.r-project.org/web/packages/BIGDAWG/index.html and https://github.com/IgDAWG/BIGDAWG, with version 1.8.1 code at https://github.com/IgDAWG/BIGDAWG/tree/eb0b4140ec3fb85b1a4fba5826ffc9f9e3239d10.

The source code for asymLD v0.1 is available online at https://cran.r-project.org/web/packages/asymLD/index.html.

The source code for PyPop is available online at https://github.com/alexlancaster/pypop, with version 0.70 code at https://github.com/alexlancaster/pypop/tree/3f29d4b53548ce4deb60a5960368627999396653.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Locus-level Heterogeneity between Multiple Sclerosis Patients and Controls

Locus	χ^2	d.f.	p-value	Significance [1]
A	43.3012	17	4.34E−04	*
A~DRB1	169.5478	38	< 2.22E−16	*
A~DRB1~DQB1	161.7085	39	< 2.22E−16	*
A~C~B~DRB1~DQB1~DPB1	42.5118	11	1.3205E−05	*
C~B	81.8819	33	4.9007E−06	*
C~B~DRB1	100.52	24	2.45E−11	*
DRB1~DQB1	159.1034	26	< 2.22E−16	*
DPB1	21.061	15	1.35E−01	NS

χ^2: Chi-squared value.

d.f.: Degrees of freedom.

[1] After correcting for eight comparisons, significance was evaluated at the 6.25E−03 level. Significant p-values are indicated with asterisks. NS: Not Significant.

Table 2

Significant Association of DRB1~DQB1 Haplotypes and DPB1 Alleles with Multiple Sclerosis

Locus	Allele	Controls (N)	Patients (N)	OR	95% CI Lower	95% CI Upper	p-value	Significance [1]
DRB1~DQB1	01:01~05:01	84	36	0.41	0.27	0.63	9.5866E−06	*
DRB1~DQB1	03:01~02:01	79	119	1.63	1.19	2.24	0.0014115	*
DRB1~DQB1	04:01~03:01	42	17	0.4	0.21	0.73	0.0012401	*
DRB1~DQB1	07:01~02:02	86	49	0.56	0.38	0.81	0.001417	*
DRB1~DQB1	11:01~03:01	58	38	0.65	0.42	1.01	0.0462	*
DRB1~DQB1	14:01~05:03	19	8	0.42	0.16	1.02	0.037742	*
DRB1~DQB1	15:01~06:02	79	240	3.98	3	5.31	< 2.22e−16	*

OR: Odds Ratio

CI: Confidence Interval

Binned: Alleles with expected counts less than five in cases or controls were combined into a common “binned” category for analysis, as described in section 4.3.1. Results for all comparisons are included in Supplementary Table S1. Counts and frequencies for all detected alleles and haplotypes are included in Supplementary Table S2.

[1] For each locus, the evaluation of significance was informed by the significance of locus-level heterogeneity as shown in Table 1. P-values were not corrected for loci that displayed significant locus-level heterogeneity. P-values for loci that did not display significant locus-level heterogeneity were corrected for the number of analyzed categories; for the *DPB1* locus, significance was evaluated at the 0.003125 level, and no *DPB1* alleles displayed a significant association. Significant p-values are indicated with asterisks.

NS: Not Significant.

NA: Not Applicable. These haplotypes were included in the Binned category, and are shown only for purposes of comparison.

Table 3

Significant Association of HLA-A alleles and B~C haplotypes with Multiple Sclerosis

Locus	Allele	Controls (N)	Patients (N)	OR	95% CI Lower	95% CI Upper	p-value	Significance [1]
A	02:01	223	164	0.69	0.54	0.87	0.0014642	*
A	03:01	114	141	1.32	1	1.74	0.0427	*
A	30:02	5	16	3.32	1.15	11.62	0.013665	*
A	33:01	11	3	0.28	0.05	1.05	0.035161	*
C~B	03:04~40:01	50	14	0.27	0.14	0.51	6.7591E−06	*
C~B	05:01~44:02	57	37	0.65	0.41	1.01	0.043834	*
C~B	07:01~08:01	76	101	1.41	1.02	1.96	0.032259	*
C~B	07:02~07:02	91	160	1.99	1.5	2.66	8.8881E−07	*
C~B	12:03~18:01	12	24	2.08	0.99	4.59	0.036793	*

Details of each comparison are included in the legend to Table 2.

Table 4

Association of HLA-A*02:01 and C*03:04~B*40:01 with Multiple Sclerosis in the presence and absence of DRB1*15:01

Locus	Haplotype	Controls (N)	Patients (N)	OR	95% CI Lower	95%CI Upper	p-value	Significance [1]
A~C~B	02:01~03:04~40:01	26	4	0.15	0.04	0.45	6.51E−05	*
A~C~B	02:01~XXXX~XXXX	197	160	0.79	0.62	1.01	4.78E−02	*
A~C~B	YYYY~03:04~40:01	24	10	0.42	0.18	0.92	1.82E−02	*
C~B~DRB1	03:04~40:01~WWWW	47	14	0.29	0.15	0.55	2.45E−05	*
C~B~DRB1	ZZZZ~ZZZZ~15:01	85	253	3.95	3.00	5.23	1.24E−25	*
A~DRB1	02:01~15:01	21	56	2.85	1.68	5.01	2.885E−05	*
A~DRB1	02:01~UUUU	202	108	0.48	0.37	0.62	1.13E−08	*
A~DRB1	VVVV~15:01	65	197	3.76	2.77	5.15	1.09E−19	*

UUUU: Any DRB1 allele other than *DRB1*15:01*

VVVV: Any HLA-A allele other than *A*02:01*

WWWW: Any *DRB1* allele (no *C*03:04~B*40:01~DRB1*15:01* haplotypes were observed)

XXXX~XXXX: Any *C~B* haplotype other than *C*03:04~B*04:01*

YYYY: Any *HLA-A* allele other than *A*02:01*

ZZZZ~ZZZZ: Any *C~B* haplotype (no *C*03:04~B*40:01~DRB1*15:01* haplotypes were observed)

OR: Odds Ratio

CI: Confidence Interval

[1]For each locus, the evaluation of significance was informed by the significance of locus-level heterogeneity as shown in Table 1. P-values were not corrected for loci that displayed significant locus-level heterogeneity. Significant p-values are indicated with asterisks.

Table 5

Association of Specific A~C~B~DRB1*15:01~DQB1~DPB1 haplotypes with Multiple Sclerosis

Locus	Haplotype [1]	Controls (N)	Patients (N)	OR	95% CI Lower	95% CI Upper	p-value	Significance [2]
A~C~B~DRB1~DQB1~DPB1	02:01~ 07:02~07:02~15:01~06:02~04:01	10	16	1.65	0.7	4.09	2.14E−01	NS
A~C~B~DRB1~DQB1~DPB1	03:01~ 07:02~07:02~15:01~06:02~04:01	20	53	2.83	1.64	5.04	5.22E−05	*
A~C~B~DRB1~DQB1~DPB1	24:02~ 07:02~07:02~15:01~06:02~04:01	3	13	4.48	1.22	24.6	1.06E−02	*

OR: Odds Ratio

CI: Confidence Interval

[1] *HLA-A* alleles encoding the A3/A11 KIR ligand, *HLA-C* alleles encoding the C1 KIR ligand (none shown) and *HLA-B* alleles encoding the Bw4 KIR ligand are highlighted in grey.

[2] For each locus, the evaluation of significance was informed by the significance of locus-level heterogeneity as shown in Table 1. P-values were not corrected for loci that displayed significant locus-level heterogeneity. Significant p-values are indicated with asterisks. NS: Not Significant.

Table 6

Association of DRB1~DQB1 genotypes in the presence and absence of HLA-A*02:01 and C~B genotypes in the presence and absence of DRB1*15:01 with Multiple Sclerosis

Locus	Genotype	Controls (N)	Cases (N)	OR	95% CI Lower	95% CI Upper	p-value	Significance [1]
C~B~DRB1	03:04~ 40:01~UUUU+VVVV~VVVV~DRB1*15:01	6	8	1.37	0.41	4.83	5.68E−01	NS
C~B~DRB1	WWWW~WWWW~UUUU+VVVV~VVVV~DRB1*15:01	75	215	5.06	3.65	7.04	3.21E−13	*
A~DRB1~DQB1	02:01~15:01~06:02+02:01~YYYY~YYYY	5	9	1.86	0.55	7.12	2.63E−01	NS
A~DRB1~DQB1	02:01~15:01~06:02+XXXX~YYYY~YYYY	17	36	2.28	1.22	4.40	5.45E−03	*
A~DRB1~DQB1	02:01~YYYY~YYYY+02:01~YYYY~YYYY	16	7	0.44	0.15	1.14	6.42E−02	NS
A~DRB1~DQB1	02:01~YYYY~YYYY+XXXX~15:01~06:02	14	26	1.96	0.97	4.12	4.39E−02	*
A~DRB1~DQB1	02:01~YYYY~YYYY+XXXX~YYYY~YYYY	147	55	0.29	0.20	0.41	3.70E−13	*
A~DRB1~DQB1	XXXX~15:01~06:02+XXXX~15:01~06:02	2	22	11.82	2.87	104.21	2.70E−05	*
A~DRB1~DQB1	XXXX~15:01~06:02+XXXX~YYYY~YYYY	35	111	4.07	2.67	6.32	1.50E−12	*

OR: Odds Ratio

CI: Confidence Interval

UUUU: Any *DRB1* allele (no *C*03:04~B*40:01~DRB1*15:01* haplotypes were observed)

VVVV~VVVV: Any *C~B* haplotype (no *C*03:04~B*40:01~DRB1*15:01* haplotypes were observed)

WWWW~WWWW: Any non-*C*03:04~B*40:01* haplotype.

XXXX: Any non-*A*02:01* allele

YYYY~YYYY: Any non-*DRB1*15:01~DQB1*06:02* haplotype.

[1] For each locus, the evaluation of significance was informed by the significance of locus-level heterogeneity. The p-value of locus-level heterogeneity for *C~B~DRB1* genotype evaluations is 7.07E−25, and that of locus-level heterogeneity for *A~DRB1~DQB1* genotype evaluations is 3.50E−23. P-values were not corrected for loci that displayed significant locus-level heterogeneity. Significant p-values are indicated with asterisks. NS: Not Significant.

Table 7

Association of KIR loci and HLA ligands with Multiple Sclerosis

A. Association of HLA Ligand and KIR Loci with Multiple Sclerosis						
Molecule	**Genotype**	**Controls (N)**	**Cases (N)**	**OR**	**p-value**	**Significance**
HLA Ligands	C1C1	153 (0.365)	164 (0.398)	1.15	3.29E−01	NS
HLA Ligands	C1C2	201 (0.480)	200 (0.485)	1.02	8.69E−01	NS
HLA Ligands	C2C2	65 (0.155)	47 (0.114)	0.70	8.30E−02	NS
HLA Ligands	Bw4+	263 (0.628)	210 (0.510)	0.62	5.95E−04	*
HLA Ligands	Bw4Bw4	55 (0.131)	36 (0.087)	0.63	4.30E−02	*
HLA Ligands	Bw4Bw6	208 (0.496)	174 (0.422)	0.74	3.20E−02	*
HLA Ligands	Bw6Bw6	156 (0.372)	201 (0.488)	1.61	7.67E−04	*
HLA Ligands	Bw6+	364 (0.869)	375 (0.910)	1.53	5.70E−02	NS
KIR	KIR2DL2	206 (0.492)	206 (0.500)	1.03	8.10E−01	NS
KIR	KIR2DL3	386 (0.921)	369 (0.896)	0.73	2.00E−01	NS
KIR	KIR3DL1	397 (0.947)	391 (0.949)	1.03	9.20E−01	NS
KIR	KIR3DS1	182 (0.434)	173 (0.420)	0.94	6.73E−01	NS
KIR	KIR2DL2/2	33 (0.079)	43 (0.104)	1.36	2.00E−01	NS
KIR	KIR2DL2/3	173 (0.413)	163 (0.396)	0.93	6.12E−01	NS
KIR	KIR2DL3/3	213 (0.508)	206 (0.500)	0.97	8.10E−01	NS
KIR	KIR3DL1/L1	237 (0.566)	238 (0.578)	1.05	7.26E−01	NS
KIR	KIR3DL1/S1	160 (0.382)	153 (0.371)	0.96	7.55E−01	NS
KIR	KIR3DS1/S1	22 (0.053)	20 (0.049)	0.92	7.94E−01	NS

B. Association of HLA Ligand and KIR Loci with Multiple Sclerosis in the absence of DRB1*15:01.						
Molecule	**Genotype**	**Controls (N)**	**Cases (N)**	**OR**	**p-value**	**Significance**
HLA Ligands	C1C1	113 (0.335)	54 (0.286)	0.79	2.41E−01	NS
HLA Ligands	C1C2	167 (0.496)	101 (0.534)	1.17	3.93E−01	NS
HLA Ligands	C2C2	57 (0.169)	33 (0.175)	1.04	8.73E−01	NS
HLA Ligands	Bw4+	222 (0.659)	110 (0.582)	0.72	8.00E−02	NS
HLA Ligands	Bw4Bw4	51 (0.151)	20 (0.106)	0.66	1.43E−01	NS

B. Association of HLA Ligand and KIR Loci with Multiple Sclerosis in the absence of DRB1*15:01.						
Molecule	**Genotype**	**Controls (N)**	**Cases (N)**	**OR**	**p-value**	**Significance**
HLA Ligands	Bw4Bw6	171 (0.507)	90 (0.476)	0.88	0.492	NS
HLA Ligands	Bw6Bw6	115 (0.341)	78 (0.413)	1.36	0.103	NS
HLA Ligands	Bw6+	286 (0.849)	168 (0.889)	1.43	0.198	NS
KIR	KIR2DL2	173 (0.513)	98 (0.519)	1.02	9.09E−01	NS
KIR	KIR2DL3	311 (0.923)	167 (0.884)	0.63	1.34E−01	NS
KIR	KIR3DL1	319 (0.947)	180 (0.952)	1.13	7.73E−01	NS
KIR	KIR3DS1	148 (0.439)	69 (0.365)	0.73	9.77E−02	NS
KIR	KIR2DL2/2	26 (0.077)	22 (0.116)	1.58	1.34E−01	NS
KIR	KIR2DL2/3	147 (0.436)	76 (0.402)	0.87	4.48E−01	NS
KIR	KIR2DL3/3	164 (0.487)	91 (0.481)	0.98	9.09E−01	NS
KIR	KIR3DL1/L1	189 (0.561)	120 (0.635)	1.36	0.0977	NS
KIR	KIR3DL1/S1	130 (0.386)	60 (0.317)	0.74	0.1177	NS
KIR	KIR3DS1/S1	18 (0.053)	9 (0.048)	0.89	0.7727	NS

C. Association of Bw Motif Subgroups with Multiple Sclerosis in the Presence and Absence of DRB1*15:01.								
Stratum	**HLA-B Position 80 Sequence**	**Control (N)**	**Case (N)**	**OR**	**95% CI Lower**	**95% CI Upper**	**p-value**	**Significance** [1]
DRB1*15:01 Negative	T	161 (0.239)	68 (0.182)	0.71	0.51	0.98	3.23E−02	NS
	N	401 (0.595)	244 (0.652)	1.28	0.97	1.68	6.70E−02	NS
	I	112 (0.166)	62 (0.166)	1.00	0.70	1.42	9.87E−01	NS
DRB1*15:01 Positive	T	29 (0.177)	61 (0.137)	0.74	0.45	1.24	2.16E−01	NS
	N	119 (0.726)	330 (0.740)	1.08	0.70	1.64	7.22E−01	NS
	I	16 (0.098)	55 (0.123)	1.3	0.71	2.51	3.79E−01	NS

Frequencies are shown in parentheses in the Controls and Cases columns.

Bw4: *HLA-B* alleles encoding either Threonine at codon 80 (80T) or Isoleucine at codon 80 (80I).

Bw6: *HLA-B* alleles encoding Asparagine at codon 80 (80N).

[1]For each stratum, the evaluation of significance was informed by the significance of overall heterogeneity between cases and controls in that stratum. The p-value of locus-level heterogeneity is 8.75E−02 in the *DRB1*15:01*-negative stratum, and 3.64E−01 in the *DRB1*15:01*-positive stratum. For each stratum, the threshold of significance was calculated as 0.05/3 (1.67E−02). Significant p-values are indicated with asterisks. NS: Not Significant.

Table 8

Association of HLA Amino Acid Positions with Multiple Sclerosis

A. Association of Variant *DRB1* Exon-2 encoded Amino Acid Positions, HLA-B Amino Acid position 80, and HLA-C Amino Acid positions 73, 77, 80 and 90 with Multiple Sclerosis.					
Locus	**Variant Amino Acid Position**	**X.square**	**d.f.**	**p-value**	**Significance [1]**
DRB1	10	0.8016	2	6.70E−01	NS
DRB1	11	110.594	5	< 2.22e−16	*
DRB1	12	0.6384	1	4.24E−01	NS
DRB1	13	113.1725	5	< 2.22e−16	*
DRB1	14	11.6544	1	6.41E−04	*
DRB1	16	3.5177	1	6.07E−02	NS
DRB1	25	11.6544	1	6.41E−04	*
DRB1	26	24.945	2	3.83E−06	*
DRB1	30	35.0811	5	1.45E−06	*
DRB1	31	15.869	2	3.58E−04	*
DRB1	32	1.3433	1	2.46E−01	NS
DRB1	33	15.2457	1	9.44E−05	*
DRB1	37	63.6089	4	5.05E−13	*
DRB1	38	1.5824	2	4.53E−01	NS
DRB1	40	0	1	1.00E+00	NS
DRB1	47	70.1772	1	< 2.22e−16	*
DRB1	57	18.4418	3	3.57E−04	*
DRB1	58	6.8992	1	8.62E−03	NS
DRB1	60	17.2657	2	1.78E−04	*
DRB1	67	30.5091	2	2.37E−07	*
DRB1	70	32.3764	2	9.32E−08	*
DRB1	71	121.9146	3	< 2.22e−16	*
DRB1	73	0	1	1.00E+00	NS
DRB1	74	25.6389	4	3.74E−05	*
DRB1	77	10.5184	1	1.18E−03	*

A. Association of Variant *DRB1* Exon-2 encoded Amino Acid Positions, HLA-B Amino Acid position 80, and HLA-C Amino Acid positions 73, 77, 80 and 90 with Multiple Sclerosis.					
Locus	**Variant Amino Acid Position**	**X.square**	**d.f.**	**p-value**	**Significance** [1]
DRB1	78	11.3048	1	7.73E−04	*
DRB1	85	2.17	1	1.41E−01	NS
DRB1	86	58.2623	1	2.29E−14	*
HLA-B	80	14.6302	2	6.65E−04	*
HLA-C	73	20.4422	1	6.15E−06	*
HLA-C	77	2.1921	1	1.39E−01	NS
HLA-C	80	2.1921	1	1.39E−01	NS
HLA-C	90	14.7985	1	1.20E−04	*
HLA-C	73~90	20.7641	2	3.10E−05	*
HLA-C	77~80	2.3061	1	1.29E−01	NS
HLA-C	73~77~80~90	33.3303	5	3.24E−06	*

B. Association of DRB1 Exon-2 encoded Amino Acid Residues with Multiple Sclerosis									
Locus	**Position**	**Residue**	**Controls (N)**	**Cases (N)**	**OR**	**95% CI Lower**	**95% CI Upper**	**p-value**	**Significance** [2]
DRB1	11	L	108	57	0.5	0.35	0.71	5.41E−05	*
DRB1	11	S	339	315	0.92	0.75	1.12	0.39573	NS
DRB1	11	V	155	95	0.58	0.43	0.77	8.43E−05	*
DRB1	11	G	117	70	0.58	0.41	0.79	0.00048	*
DRB1	11	D	3	3	1.02	0.14	7.65	0.97874	NS
DRB1	11	P	116	280	3.23	2.51	4.15	< 2.22e−16	*
DRB1	13	F	119	67	0.54	0.39	0.75	0.0001	*
DRB1	13	S	296	289	1	0.81	1.23	0.97339	NS
DRB1	13	H	147	88	0.57	0.42	0.76	7.04E−05	*
DRB1	13	Y	117	70	0.58	0.41	0.79	0.00048	*
DRB1	13	G	43	26	0.61	0.35	1.02	0.04566	*
DRB1	13	R	116	280	3.23	2.51	4.15	< 2.22e−16	*
DRB1	14	E	721	750	1.74	1.26	2.41	0.00048	*
DRB1	14	K	117	70	0.58	0.41	0.79	0.00048	*
DRB1	25	R	721	750	1.74	1.26	2.41	0.00048	*

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B. Association of DRB1 Exon-2 encoded Amino Acid Residues with Multiple Sclerosis

Locus	Position	Residue	Controls (N)	Cases (N)	OR	95% CI Lower	95% CI Upper	p-value	Significance [2]
DRB1	25	Q	117	70	0.58	0.41	0.79	0.00048	*
DRB1	26	L	130	72	0.52	0.38	0.72	2.79E−05	*
DRB1	26	Y	83	125	1.64	1.21	2.23	0.001032	*
DRB1	26	F	625	623	1.08	0.86	1.36	0.5109	NS
DRB1	30	C	108	57	0.5	0.35	0.71	5.41E−05	*
DRB1	30	Y	588	675	1.98	1.56	2.52	6.39E−09	*
DRB1	30	L	117	70	0.58	0.41	0.79	0.00048	*
DRB1	30	G	3	3	1.02	0.14	7.65	0.97874	NS
DRB1	30	R	8	7	0.89	0.27	2.83	0.82809	NS
DRB1	30	H	14	8	0.58	0.21	1.49	0.21624	NS
DRB1	31	I	111	60	0.52	0.37	0.73	7.23E−05	*
DRB1	31	F	719	753	1.86	1.34	2.59	0.0001	*
DRB1	31	V	8	7	0.89	0.27	2.83	0.82809	NS
DRB1	33	N	691	732	1.77	1.32	2.38	7.04E−05	*
DRB1	33	H	147	88	0.57	0.42	0.76	7.04E−05	*
DRB1	37	S	224	337	1.91	1.55	2.37	6.34E−10	*
DRB1	37	N	167	201	1.3	1.03	1.66	0.024731	*
DRB1	37	Y	297	195	0.57	0.46	0.71	2.03E−07	*
DRB1	37	F	136	79	0.55	0.4	0.75	6.43E−05	*
DRB1	37	L	14	8	0.58	0.21	1.49	0.21624	NS
DRB1	47	Y	466	287	0.43	0.35	0.53	< 2.22e−16	*
DRB1	47	F	372	533	2.33	1.9	2.85	< 2.22e−16	*
DRB1	57	D	643	692	1.64	1.27	2.12	8.24E−05	*
DRB1	57	S	42	38	0.92	0.57	1.48	0.71967	NS
DRB1	57	V	134	81	0.58	0.42	0.78	0.000212	*
DRB1	57	A	19	9	0.48	0.19	1.12	0.06458	NS
DRB1	60	Y	685	729	1.79	1.34	2.39	3.88E−05	*
DRB1	60	S	134	82	0.58	0.43	0.79	0.000291	*
DRB1	60	H	19	9	0.48	0.19	1.12	0.06458	NS

B. Association of DRB1 Exon-2 encoded Amino Acid Residues with Multiple Sclerosis									
Locus	**Position**	**Residue**	**Controls (N)**	**Cases (N)**	**OR**	**95% CI Lower**	**95% CI Upper**	**p-value**	**Significance** [2]
DRB1	67	L	342	265	0.69	0.56	0.85	0.000331	*
DRB1	67	I	347	450	1.72	1.41	2.1	4.05E−08	*
DRB1	67	F	149	105	0.68	0.51	0.9	0.004919	*
DRB1	70	Q	404	509	1.76	1.44	2.15	1.4E−08	*
DRB1	70	D	404	292	0.59	0.49	0.73	2.02E−07	*
DRB1	70	R	30	19	0.64	0.34	1.18	0.12898	NS
DRB1	71	R	457	280	0.43	0.35	0.53	< 2.22e−16	*
DRB1	71	E	113	99	0.88	0.65	1.19	0.38958	NS
DRB1	71	K	174	180	1.07	0.84	1.37	0.55523	NS
DRB1	71	A	94	261	3.7	2.83	4.85	< 2.22e−16	*
DRB1	74	A	578	588	1.14	0.92	1.42	0.22315	NS
DRB1	74	R	80	122	1.66	1.21	2.27	0.000906	*
DRB1	74	E	33	22	0.67	0.37	1.2	0.15367	NS
DRB1	74	Q	117	70	0.58	0.41	0.79	0.00048	*
DRB1	74	L	30	18	0.6	0.31	1.13	0.092675	NS
DRB1	77	T	758	698	0.6	0.44	0.82	0.000906	*
DRB1	77	N	80	122	1.66	1.21	2.27	0.000906	*
DRB1	78	Y	718	747	1.71	1.24	2.36	0.000584	*
DRB1	78	V	120	73	0.58	0.42	0.8	0.000584	*
DRB1	86	G	483	318	0.47	0.38	0.57	1.56E−14	*
DRB1	86	V	355	502	2.15	1.76	2.63	1.56E−14	*

C. Association of HLA class I Amino Acids that Form KIR Ligand Epitopes with Multiple Sclerosis.									
Locus	**Position**	**Residue(s)**	**Controls (N)**	**Cases (N)**	**OR**	**95% CI Lower**	**95% CI Upper**	**p-value**	**Significance** [3]
HLA-B	80	N	520	574	1.43	1.16	1.76	6.38E−04	*
HLA-B	80	T	190	129	0.64	0.49	0.82	3.37E−04	*
HLA-B	80	I	128	117	0.92	0.7	1.22	5.64E−01	NS
HLA-C	73	T	361	264	0.63	0.51	0.77	4.83E−06	*
HLA-C	73	A	477	556	1.59	1.3	1.96	4.83E−06	*

C. Association of HLA class I Amino Acids that Form KIR Ligand Epitopes with Multiple Sclerosis.									
Locus	**Position**	**Residue(s)**	**Controls (N)**	**Cases (N)**	**OR**	**95% CI Lower**	**95% CI Upper**	**p-value**	**Significance [3]**
HLA-C	90	A	416	329	0.68	0.56	0.83	9.77E−05	*
HLA-C	90	D	422	491	1.47	1.21	1.8	9.77E−05	*
HLA-C	73~90	A~A	55	65	1.22	0.83	1.81	2.90E−01	NS
HLA-C	73~90	A~D	422	492	1.47	1.2	1.79	1.01E−04	*
HLA-C	73~90	T~A	361	265	0.63	0.51	0.77	5.21E−06	*
HLA-C	73~77~80~90	A~N~K~A	6	16	2.75	1.02	8.63	2.84E−02	*
HLA-C	73~77~80~90	A~N~K~D	179	163	0.91	0.71	1.16	4.41E−01	NS
HLA-C	73~77~80~90	A~S~N~A	49	49	1.02	0.66	1.57	9.22E−01	NS
HLA-C	73~77~80~90	A~S~N~D	243	329	1.63	1.33	2.02	2.28E−06	*
HLA-C	73~77~80~90	T~N~K~A	146	115	0.77	0.59	1.01	5.48E−02	NS
HLA-C	73~77~80~90	T~S~N~A	215	150	0.65	0.51	0.82	2.69E−04	*

χ^2: Chi-squared value.

d.f.: Degrees of freedom.

OR: Odds Ratio.

[1]After correcting for 28 comparisons, *DRB1* significance was evaluated at the 1.79E−03 level. *HLA-C* significance was evaluated at the 7.14E−03 level (7 comparisons). Significant p-values are indicated with asterisks. NS: Not Significant.

[2]For each amino acid residue, the evaluation of significance was informed by the significance of heterogeneity for that position, as shown in Table 8A. Results are shown for positions that displayed significant heterogeneity.

[3]For each amino acid residue or combination of residues, the evaluation of significance was informed by the significance of heterogeneity for that position or positions, as shown in Table 8A. Results are shown for positions that displayed significant heterogeneity.

Table 9

Association of HLA-A*02:01 and Bw4 with Multiple Sclerosis

A. Locus Level Heterogeneity among Tests of Association between HLA-A and Bw4/Bw6 Related Polymorphisms in MS Patients and Controls				
Locus	**χ^2**	**d.f.**	**p-value**	**Significance [1]**
HLA-A~Bw4/Bw6	46.0751	24	4.34E−03	*
HLA-A~HLA-B Position 80	43.2836	27	2.45E−02	*

B. Association of HLA-A*02:01 and Bw4/Bw6-Related Polymorphism in MS Patients and Controls								
Locus	**Haplotype**	**Controls (N)**	**Cases (N)**	**OR**	**95% CI Lower**	**95% CI Upper**	**p-value**	**Significance [2]**
A~Bw4/Bw6	A*02:01~Bw4	123	79	0.62	0.45	0.85	1.69E−03	*
A~Bw4/Bw6	A*02:01~Bw6	100	85	0.85	0.62	1.17	3.11E−01	NS
A~HLA-B Position 80	A*02:01~80I	45	34	0.76	0.47	1.23	2.42E−01	NS
A~HLA-B Position 80	A*02:01~80T	83	45	0.53	0.35	0.78	7.55E−04	*

Bw4: *HLA-B* alleles encoding either Threonine at codon 80 (80T) or Isoleucine at codon 80 (80I).

Bw6: *HLA-B* alleles encoding Asparagine at codon 80 (80N).

d.f.: Degrees of Freedom.

[1] After correcting for two comparisons, significance was evaluated at the 2.5E−02 level. Significant p-values are indicated with asterisks.

[2] For each locus, the evaluation of significance was informed by the significance of locus-level heterogeneity as shown in Table 9A. P-values were not corrected for loci that displayed significant locus-level heterogeneity. P-values for loci that did not display significant locus-level heterogeneity were corrected for the number of analyzed categories.

The complete set of *HLA-A*~Bw4/Bw6 and *A*~*HLA-B* Position 80 comparisons is included in Supplementary Table S5.