A Major Histocompatibility Class I Locus Contributes to Multiple Sclerosis Susceptibility Independently from HLA-DRB1*15:01

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Abstract

Background: In Northern European descended populations, genetic susceptibility for multiple sclerosis (MS) is associated with alleles of the human leukocyte antigen (HLA) Class II gene DRB1. Whether other major histocompatibility complex (MHC) genes contribute to MS susceptibility is controversial.

Methodology/Principal Findings: A case control analysis was performed using 958 single nucleotide polymorphisms (SNPs) spanning the MHC assayed in two independent datasets. The discovery dataset consisted of 1,018 cases and 1,795 controls and the replication dataset was composed of 1,343 cases and 1,379 controls. The most significantly MS-associated SNP in the discovery dataset was rs3135391, a Class II SNP known to tag the HLA-DRB1*15:01 allele, the primary MS susceptibility allele in the MHC (O.R. = 3.04, p<1x10^-78). To control for the effects of the HLA-DRB1*15:01 haplotype, case control analysis was performed adjusting for this HLA-DRB1*15:01 tagging SNP. After correction for multiple comparisons (false discovery rate = 0.05) 52 SNPs in the Class I, II and III regions were significantly associated with MS susceptibility in both datasets using the Cochran Armitage trend test. The discovery and replication datasets were merged and subjects carrying the HLA-DRB1*15:01 tagging SNP were excluded. Association tests showed that 48 of the 52 replicated SNPs retained significant associations with MS susceptibility independently of the HLA-DRB1*15:01 as defined by the tagging SNP. 20 Class I SNPs were associated with MS susceptibility with p-values ≤1x10^-6. The most significantly associated SNP was rs4959039, a SNP in the downstream un-translated region of the non-classical HLA-G gene (Odds ratio 1.59, 95% CI 1.40, 1.81, p=8.45x10^-13) and is in linkage disequilibrium with several nearby SNPs. Logistic regression modeling showed that this SNP’s contribution to MS susceptibility was independent of the Class II and Class III SNPs identified in this screen.

Conclusions: A MHC Class I locus contributes to MS susceptibility independently of the HLA-DRB1*15:01 haplotype.

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Introduction

The autoimmune disease multiple sclerosis (MS) is one of the leading causes of neurological disability in young adults. Pathologically the disease is characterized by focal areas of inflammation and demyelination (plaques) within the central nervous system with ensuing axonal damage. Although the etiology is not fully understood, MS is a complex genetic disorder and whole genome studies indicate that the major histocompatibility complex (MHC) on chromosome 6p21 represents the strongest genome-wide MS susceptibility locus [1,2].

In both Northern European and African descended populations, MS susceptibility is associated with alleles of the HLA Class II gene DRB1 [2–5] whereas the contribution of other genes within the extended MHC has been controversial [6–8]. Extensive linkage disequilibrium (LD) operating in the region [9–11], as well as marked polymorphism and high gene density, have complicated efforts to fully resolve the roles of HLA and non-HLA genes in MS susceptibility. Due to these inherent challenges, a comprehensive approach is needed to refine the contributions of the MHC to genetic risk for MS that includes a large and well-characterized dataset, dense concentration of markers, and appropriate methods to control for the extensive LD across the region.

A panel of single nucleotide polymorphisms (SNPs) selected for moderate LD across the 29 to 34 Mb region of the MHC was employed to map both HLA and non-HLA disease susceptibility signals [12]. Here we present the results of an analysis of two independent case control MS datasets using 958 SNPs adjusting for rs3135391 using the 958 SNPs (FDR = .05) and the HLA-DRB1*15:01 haplotype, all subjects carrying at least one copy of this allele, as defined by the tagging SNP rs3135391, were dropped from the merged dataset to create a “HLA-DRB1*15:01(-)” dataset. This excluded a total of 2088 subjects (1277 cases and 811 controls) leaving a HLA-DRB1*15:01(-) dataset that consisted of 1075 cases and 2363 controls. Association tests were performed in this merged HLA-DRB1*15:01(-) dataset and significant associations were found for 48 of the 52 SNPs identified in the case control screens including all previously identified Class I and Class III SNPs (Table S4).

Using the genotype test for association in the HLA-DRB1*15:01(-) dataset, 20 Class I SNPs had \( p \)-values \( \leq 10^{-8} \) (Table 1). The HLA-G linked rs4959039:A>G allele (rs4959039) continued to have the strongest association in this HLA-DRB1*15:01(-) dataset (odds ratio 1.59, 95% confidence intervals 1.40, 1.81, \( p < 8.45 \times 10^{-18} \)). Importantly, rs4959039 and the other Class I SNPs associated with MS susceptibility are poorly correlated with the SNPs in the Class III and Class II regions as illustrated by the LD map (Figure 2). For example, the average (range) \( r^2 \) for rs4959039 with the Class III SNPs was .081 (.014 to .149) and for the Class II SNPs was .024 (.002 to .085).

In contrast to the poor correlations with the Class III and Class II SNPs, the LD map (Figure 2) shows that some of the associated Class I SNPs are closely linked. SNPs in the Class I region with \( p \)-values \( \leq 1 \times 10^{-6} \) that are in moderate to strong LD with each other (as defined by LD-\( R^2 \geq 0.5 \)) include: rs2523822, rs2517701 (HLA-\( \beta \)-A), rs4713270 (HCG26), rs4713274 (HICD), rs2523946 (MICD), rs3823355 (MICD), rs4959039 (in between HLA-G and HLA-A), rs4713281 (HLA-J), rs9357092 (HCGB), and rs9393989 (RNF39). Using an algorithm to define haplotype blocks by LD-\( R^2 \geq 0.5 \) an apparently separate Class I SNP cluster (rs1362126, rs2523393, rs2743951) emerges that includes a tagging SNP for the HLA-B*44:02 allele (rs2523393), a recently identified MS protective allele [12,14].

Tests for independent association using logistic regression models

To confirm that the contribution to MS susceptibility of the rs4959039 SNP was independent of any residual Class II associations, logistic regression models were constructed. Because many of the 48 SNPs associated with MS susceptibility in the HLA-
Figure 1. Association test results for 958 SNPs spanning the MHC in the discovery dataset are shown. The location of the SNPs is depicted on the X-axis and the statistical significance of the association is depicted on the Y-axis. A: Discovery dataset (1018 cases and 1795 controls), 958 common SNP subset, FDR = .05, adjusted for sex and center (US versus UK), trend test. B: Discovery dataset, 958 common SNP subset, FDR = .05, adjusted for the HLA-DRB1*15:01 tagging SNP rs3135391), sex and center (US versus UK), trend test.
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**Table 1.** SNPs associated with MS susceptibility with genome-wide statistical significance in the merged dataset excluding all subjects who carry the HLA-DRB1*15:01 allele listed in order of highest to lowest statistical significance using the Cochran-Armitage trend test for association.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Position</th>
<th>Class</th>
<th>Gene</th>
<th>Allele</th>
<th>MS Associated</th>
<th>Trend</th>
<th>Odds</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4959039</td>
<td>30065047</td>
<td>Class I</td>
<td>HLA-G</td>
<td>A</td>
<td>8.45×10⁻¹³</td>
<td>1.59</td>
<td>1.40</td>
<td>1.81</td>
</tr>
<tr>
<td>rs9393989</td>
<td>30148062</td>
<td>Class I</td>
<td>RNF39</td>
<td>A</td>
<td>9.84×10⁻¹³</td>
<td>0.63</td>
<td>0.56</td>
<td>0.72</td>
</tr>
<tr>
<td>rs9357092</td>
<td>30092230</td>
<td>Class I</td>
<td>HCG9</td>
<td>A</td>
<td>1.17×10⁻¹²</td>
<td>0.63</td>
<td>0.56</td>
<td>0.72</td>
</tr>
<tr>
<td>rs4713281</td>
<td>30086330</td>
<td>Class I</td>
<td>HLA-I</td>
<td>A</td>
<td>3.19×10⁻¹²</td>
<td>0.63</td>
<td>0.56</td>
<td>0.72</td>
</tr>
<tr>
<td>rs4713274</td>
<td>30045471</td>
<td>Class I</td>
<td>MICD</td>
<td>C</td>
<td>5.11×10⁻¹²</td>
<td>1.56</td>
<td>1.38</td>
<td>1.77</td>
</tr>
<tr>
<td>rs1736936</td>
<td>29902295</td>
<td>Class I</td>
<td>HCG4P8</td>
<td>C</td>
<td>2.22×10⁻¹¹</td>
<td>0.70</td>
<td>0.63</td>
<td>0.78</td>
</tr>
<tr>
<td>rs2523822</td>
<td>29936638</td>
<td>Class I</td>
<td>A</td>
<td>2.99×10⁻¹¹</td>
<td>1.51</td>
<td>1.34</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td>rs4713270</td>
<td>30042675</td>
<td>Class I</td>
<td>HCG2P6</td>
<td>A</td>
<td>4.26×10⁻¹¹</td>
<td>0.66</td>
<td>0.58</td>
<td>0.75</td>
</tr>
<tr>
<td>rs3823335</td>
<td>30050061</td>
<td>Class I</td>
<td>MICD</td>
<td>C</td>
<td>7.74×10⁻¹¹</td>
<td>1.50</td>
<td>1.33</td>
<td>1.70</td>
</tr>
<tr>
<td>rs2734971</td>
<td>29942427</td>
<td>Class I</td>
<td>3.8–1.4</td>
<td>C</td>
<td>2.07×10⁻¹⁰</td>
<td>1.42</td>
<td>1.27</td>
<td>1.58</td>
</tr>
<tr>
<td>rs2239530</td>
<td>30260093</td>
<td>Class I</td>
<td>TRIM26</td>
<td>C</td>
<td>2.96×10⁻¹⁰</td>
<td>0.65</td>
<td>0.57</td>
<td>0.74</td>
</tr>
<tr>
<td>rs2523393</td>
<td>29813637</td>
<td>Class I</td>
<td>FLJ35429</td>
<td>C</td>
<td>6.04×10⁻¹⁰</td>
<td>0.72</td>
<td>0.65</td>
<td>0.80</td>
</tr>
<tr>
<td>rs1541268</td>
<td>30211372</td>
<td>Class I</td>
<td>TRIM40</td>
<td>C</td>
<td>1.40×10⁻⁹</td>
<td>0.66</td>
<td>0.58</td>
<td>0.76</td>
</tr>
<tr>
<td>rs2256266</td>
<td>29740296</td>
<td>Ext Cls I</td>
<td>MOG</td>
<td>A</td>
<td>2.67×10⁻⁹</td>
<td>0.66</td>
<td>0.58</td>
<td>0.76</td>
</tr>
<tr>
<td>rs2743951</td>
<td>29817212</td>
<td>Class I</td>
<td>FLJ35429</td>
<td>C</td>
<td>3.55×10⁻⁹</td>
<td>1.37</td>
<td>1.24</td>
<td>1.52</td>
</tr>
<tr>
<td>rs1611710</td>
<td>29936894</td>
<td>Class I</td>
<td>C</td>
<td>5.06×10⁻⁹</td>
<td>0.74</td>
<td>0.66</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>rs2517701</td>
<td>30033950</td>
<td>Class I</td>
<td>HLA-B8</td>
<td>A</td>
<td>5.62×10⁻⁹</td>
<td>1.41</td>
<td>1.26</td>
<td>1.58</td>
</tr>
<tr>
<td>rs2523946</td>
<td>30049921</td>
<td>Class I</td>
<td>MICD</td>
<td>C</td>
<td>8.69×10⁻⁹</td>
<td>1.36</td>
<td>1.23</td>
<td>1.51</td>
</tr>
<tr>
<td>rs2256543</td>
<td>30045811</td>
<td>Class I</td>
<td>MICD</td>
<td>C</td>
<td>9.15×10⁻⁹</td>
<td>1.36</td>
<td>1.22</td>
<td>1.51</td>
</tr>
<tr>
<td>rs1362126</td>
<td>29798997</td>
<td>Class I</td>
<td>HLA-F</td>
<td>A</td>
<td>6.99×10⁻⁸</td>
<td>0.75</td>
<td>0.67</td>
<td>0.83</td>
</tr>
</tbody>
</table>

The area under the receiver operator curve modeling the rs4959039 SNP alone was .617 showing that the contribution of these Class II SNPs is modest. Importantly, during the backward stepwise selection process all other Class I SNP clusters were dropped from the model suggesting that the Class I contribution to MS susceptibility is driven by the SNP cluster tagged by rs4959039.

Logistic regression was used to determine whether the association of the rs4959039 SNP was dependent on the rs2523393 SNP (tags HLA-B*44:02). Despite their close physical proximity, the association of the rs4959039 SNP remained highly significant (p = 6.10×10⁻⁹, odds ratio = 1.43) after adjusting for the effect of the rs2523393 SNP whereas the association of the rs2523393 SNP (that tags the MS protective allele HLA-B*44:02) was attenuated (p = .015, odds ratio = .85).

**Two-locus Class I haplotypes**

To further understand the contributions of these loci to MS susceptibility two-locus haplotypes were constructed for SNPs rs2523393 (the HLA-B*44:02 tagging SNP) and rs4959039 (Table S6). This analysis defined a MS risk haplotype as rs2523393:T>rs4959039:A>G and the converse MS protective haplotype as rs2523393:C>T with rs4959039:G>A. Due to LD this analysis could not definitively prove that the influence of these loci on MS risk was independent. However, the heterozygous haplotype appears to be protective for MS risk (odds ratio = .71, p < 9.73×10⁻⁵) indicating that the protective haplotype is dominant.
Transmission disequilibrium test in HLA-DRB1*15:01(-) trio families

As an additional test of association, the rs4959039 was assessed using the transmission disequilibrium test in a subset of the discovery dataset for whom parental genotyping was available. 347 trio families (affected individual plus both parents) that did not carry the HLA-DRB1*15:01 allele were genotyped for the rs4959039 SNP. The chromosome carrying the allele of rs4959039:A>G was transmitted 112 times and not transmitted 81 times in heterozygous trio families. Despite the small size of this...
family based dataset, a borderline level of statistical significance was observed ($p = .046$) supporting the validity of this SNP as an MS susceptibility locus using a family-based association test.

To determine whether the rs4959039:A$\rightarrow$G allele adds to the risk of MS in HLA-DRB1*15:01 subjects, bi-allelic haplotypes for rs3155991:T$\rightarrow$G (the SNP that tags HLA-DRB1*15:01) and rs4959039:A$\rightarrow$G individuals were constructed in the merged dataset (Table 2). Each bi-allelic haplotype was treated as a dichotomous variable in this analysis. The presence of the rs4959039:A$\rightarrow$G allele contributed to MS susceptibility both in subjects who carry the HLA-DRB1*15:01 allele as well as those that do not. In addition, the rs4959039:A$\rightarrow$G allele appears to be additive to the effect of HLA-DRB1*15:01 increasing the odds ratio for MS from 5.89 to 6.46, although the confidence intervals for the odds ratios of these haplotypes overlap.

**HLA-G SNP associations from a meta-analysis genome-wide association study**

Depending on the reference sequence the SNP rs4959039 maps to non-coding regions centromeric to HLA-G or HLA-A. The chromosome 6 $\alpha$ ox reference sequence places this SNP in the intergenic non-coding region centromeric to HLA-G whereas the chromosome 6 $\beta$1 reference sequence maps the SNP centromeric to HLA-A. It appears that this SNP tags a possible ancestral duplication near both genes [16]. This observation raises the question as to whether the MS susceptibility signal associated with this SNP arises from alleles of HLA-G, HLA-A, or other nearby genes. Indeed, as presented above, many of the Class I SNPs identified in this study are in moderate to strong LD with each other.

A panel of different SNPs in the HLA-G locus was assessed using a dataset described in a recent genome wide association scan (GWAS) meta-analysis [14]. Although the published GWAS meta-analysis included subjects from the discovery dataset, these subjects were excluded from the following analysis to create an independent dataset consisting of 1606 MS cases and 5425 controls. In the GWAS meta-analysis 167 SNPs mapped to the HLA-G locus. After adjusting for HLA-DRB1*15:01 using a tagging SNP and sex 63 of the 167 SNPs were associated with MS susceptibility with $p$-values $\leq .01$ (Table S7). The majority of the SNPs mapped to the untranscribed region centromeric to HLA-G, some with $p$-Values $\leq 1 \times 10^{-6}$ (rs1611715, rs3115627, rs2734982, rs2975033). 6 SNPs map within the HLA-G gene itself with $p$-Values $\leq 1 \times 10^{-4}$. SNPs rs1611627, rs915668, rs 1736920 and rs1639293 are intronic SNPs whereas SNP rs1063320 maps to the 3’ end of the last exon of HLA-G and is transcribed but not translated. These data are consistent with the proposition that a MHC Class I MS susceptibility locus that is transcribed but independent of the extended HLA-DRB1*15:01 haplotype maps to the region of the HLA-G gene.

**Summary**

This comprehensive SNP based analysis spanning the 29 to 34 kb region of the MHC shows that 52 SNPs in Class I, II and III regions of the MHC were associated with MS susceptibility in two independent datasets. Moreover, 20 of these SNPs were associated with MS susceptibility with $p$-values $<1 \times 10^{-6}$ in a dataset that does not carry the extended HLA-DRB1*15:01 haplotype. The most significant association was with rs4959039, a class I SNP near HLA-G. The association of this SNP with MS susceptibility appears to be independent of the effects of the other identified Class II and Class III SNPs.

**Discussion**

Using two case control datasets and a panel of SNPs specifically selected to capture the genetic variation within the MHC region we found that the MHC locus contributes to MS susceptibility, not only through the well recognized effect of HLA-DRB1*15:01, but also through independent contributions from a Class I locus. This study proves that, after the HLA-DRB1*15:01 extended haplotype, the Class I region is the most significant contributor to MS susceptibility within the MHC. Importantly, these observations contrast with an earlier publication of a Canadian cohort which concluded that all Class I associations with MS susceptibility were due to LD with HLA-DRB1*15:01 [5]. Although genetic heterogeneity might account for these differences, it is more likely that the structure of the current study, specifically the large dataset and denser set of informative markers, made possible the detection of independent effects of Class I and Class III genes.

**Class I genes and MS susceptibility**

The strongest HLA-DRB1*15:01 independent MS association was with rs4959039, a SNP near the non-classical HLA-G gene. Several other SNPs in neighboring pseudogenes HLA-80, HCG2P6, MICD and HLA-J were also associated with MS susceptibility and are in LD with the rs4959039 SNP. These SNPs are not strongly linked to the SNP that tags the recently identified Class I MS protective allele HLA-K*0402 [12,14] and are independent of the major MS susceptibility allele HLA-DRB1*15:01. Because of the prohibitive cost we were unable to genotype classical HLA alleles in these large datasets to control for the possible contributions of HLA-DRB1*0301 [17] or other HLA-DRB1 alleles. Nevertheless, logistic regression models that controlled for the 10 most statistically significant Class II SNPs, as well as the 8 Class III SNPs identified in this study,

### Table 2. Paired marker analysis for HLA-DRB1*15:01 and rs4959039 haplotypes in the merged dataset.

<table>
<thead>
<tr>
<th>HLA-DRB1</th>
<th>rs4959039</th>
<th>N</th>
<th>O.R.</th>
<th>95 C.I. lower</th>
<th>95 C.I. upper</th>
<th>$p$-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DRB1*15:01</td>
<td>rs4959039</td>
<td>3226</td>
<td>5.89</td>
<td>3.62</td>
<td>9.59</td>
<td>1.036 x 10^-12</td>
</tr>
<tr>
<td>HLA-DRB1*15:01</td>
<td>rs4959039</td>
<td>129</td>
<td>6.46</td>
<td>4.57</td>
<td>9.13</td>
<td>4.95 x 10^-26</td>
</tr>
</tbody>
</table>

Two locus haplotypes were constructed and the odds ratio for association with MS susceptibility for each haplotype was tested in a logistic regression model treating each haplotype as a categorical variable. The odds ratio for the HLA-DRB1*15:01 allele in the merged dataset was 3.50 ($p<1.46 \times 10^{-10}$). All results are adjusted for stratification effects caused by sex, location (US versus UK) and dataset (discovery versus replication). DRB1*15:01 X refers to subjects who do not carry the HLA-DRB1*15:01 allele.

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demonstrated an independent allelic contribution of rs4959039 to MS susceptibility.

Although this association study cannot exclude the possibility that another closely linked MHC Class I gene, or genes, gives rise to the MS susceptibility signal detected by the rs4959039 SNP it is clearly of interest that this SNP is in the 3’ un-translated region of HLA-G. We conclusively demonstrated that this SNP’s association with MS susceptibility is independent of HLA-DRB1*15:01 and provided evidence that this SNP is not tightly linked to any of the Class III or Class II associations identified in this screen.

However, the rs4959039 SNP also maps to a duplication that is near HLA-A. HLA-A alleles were previously associated with MS susceptibility; the HLA-A*03 allele is thought to increase MS risk in HLA-DRB1*15:01 subjects [18,19] whereas the HLA-A*02 allele is thought to reduce MS risk [20]. Several lines of evidence suggest that the rs4959039 SNP’s association with MS might be through HLA-G rather than HLA-A*03. First, the HLA-A*03 allele is part of the extended HLA-DRB1*15:01 haplotype that was effectively excluded in this study. Second, the HLA-A*03 allele that was imputed in the discovery dataset is not tightly correlated with the rs4959039 SNP (r² = .002). Third, SNPs in HLA-A were not identified as disease-associated in either the discovery or the replication datasets. Lastly, using a different panel of HLA-G imputed SNPs from a genome wide meta-analysis in an independent dataset, multiple SNPs in the HLA-G locus were significantly associated with MS susceptibility after adjusting for HLA-DRB1*15:01. Thus we interpret our results as suggesting that the rs4959039 SNP association with MS risk is not through HLA-A*03. However, because typing of class I genes was unavailable for nearly the entire dataset we were unable to further analyze the relationship between the rs4959039 SNP and HLA-A*02, or other HLA-A alleles. Given that SNP rs4959039 tags a large haplotype block that includes HLA-A, mapping the class I susceptibility gene, or genes, will not only require classical typing of HLA-A but also could require an even larger dataset that excludes HLA-G.

HLA-G is a biologically interesting candidate gene because of its prominent function in immune tolerance. HLA-G is a non-classical, HLA Class I molecule characterized by relatively limited polymorphism and alternate splice sites that result in several membrane bound and soluble isoforms [21]. The HLA-G gene includes 42 alleles at the DNA level, 14 alleles at the protein level, and 2 null alleles based on sequence variation in exons 2–4 (the _1 to _3 domains) [22]. In theory, polymorphisms affecting the HLA-G primary sequence, differences in alternate splicing and expression pattern, could promote or reduce immune tolerance and in this manner influence MS susceptibility. Prior genetic studies of HLA-G in MS susceptibility found conflicting results. One study found no association of three HLA-G alleles and MS susceptibility [23] whereas another found an association of an HLA-G promoter polymorphism with MS susceptibility by the transmission distortion test [8]. Both studies were limited by relatively small sample sizes and few genetic markers.

In contrast to the ubiquitous expression of HLA-A, HLA-B and HLA-C, HLA-G is found primarily in extravillous trophoblasts: fetal cells that invade the maternal decidua during placenta formation [24,25]. These fetal trophoblasts are thought to play a role in inducing maternal tolerance for the fetus. HLA-G probably does not function in antigen presentation to HLA class restricted T cells [26]. Rather, HLA-G binds to and stimulates signaling via the leukocyte immunoglobulin-like receptors (LILRB1/ILT2/CD85j) as well as LILRB2/ILT4/CD85d and KIR2DL4 (CD158d) [25]. These cell surface receptors are expressed on antigen presenting cells such as dendritic cells, macrophages and B cells and are also found on natural killer (NK) cells, T cells, eosinophils, and osteoclasts. Although not well understood, LILRB signaling inhibits co-stimulation of T cell responses during antigen presentation [27]. When expressed on target cells HLA-G inhibits NK cell killing of the target cell by stimulation of inhibitory pathways [28]. These observations suggest that HLA-G has an important role in inducing maternal-fetal tolerance. Additional support for role of HLA-G in immune tolerance comes from murine allogenic tissue graft experiments in which HLA-G expression prolongs graft survival [29].

Whether HLA-G is involved in induction of immune tolerance in other body tissues, or disease states, is somewhat controversial. Some authors challenge the idea that HLA-G is expressed anywhere other than the trophoblast [30]. However, a growing body of evidence suggests that HLA-G has an important role in preventing immunological targeting of malignant cells [31]. Furthermore, HLA-G may have important roles in inflammatory skin conditions [32] and myopathies [33].

A role for HLA-G in multiple sclerosis pathogenesis was first proposed based on the observation that sHLA-G levels were elevated in MS patients relative to healthy controls [34]. Furthermore, sHLA-G is down-regulated in patients who have actively inflamed MS plaques as evidenced by gadolinium-DPTA enhancement on brain MRI imaging [35]. HLA-G is known to be strongly expressed in brain specimens from MS patients where it is present in acute inflammatory demyelinating plaques, chronic active plaques, peri-plaque areas and normal appearing white matter [36]. In MS, HLA-G is expressed primarily on microglia, macrophages, and endothelial cells. In addition to HLA-G, one of its receptors, LILRB1/ILT2, is also found in MS brain tissue suggesting that HLA-G expression in MS brain is functionally relevant, possibly through an inhibitory feedback pathway directed at down regulating pro-inflammatory T cells. Recently, HLA-G<sup>trans</sup> T<sub>reg</sub> cells were identified in MS cerebrospinal fluid, as well as in inflammatory brain tissue, and these cells are thought to function as suppressor cells, counterbalancing the tissue destructive effects of autoimmune inflammation [37]. Taken together, these observations suggest that HLA-G may have a fundamental role in limiting tissue injury in MS by regulating auto-reactive immune cells within the central nervous system.

Thus, it is possible that a HLA-G associated haplotype could contribute to MS risk by influencing signaling via LILRB1/ILT2 or the KIR2DL4 natural killer (NK) receptors [38]. Polymorphisms in HLA-G or KIR2DL4 could influence CD56<sup>bright</sup> NK cell function whose corresponding immunoregulatory pathway involves the already established MS susceptibility genes, the interleukin 2 receptor (IL2RA) and interleukin 7 receptor (IL7R) [2].

Other Class I loci

In addition to the rs4959039 (near HLA-G) association, several other Class I SNPs associated with MS susceptibility were identified, replicated and shown to have HLA-DRB1*15:01 independent effects. One group of SNPs tags the HLA-B*44:02 allele. Tagging SNPs for the closely linked HLA-C*0501 allele [7] did not survive the stringent criteria for association used in this study. These SNPs narrowly missed the cutoff for inclusion as candidates in the discovery dataset screen but were associated with MS susceptibility in the replication dataset screen. When these SNPs were included in the merged HLA-DRB1*15:01(-) dataset, tagging SNPs for HLA-C*0501 [7] were significantly associated with MS susceptibility (data not shown). Logistic regression modeling suggested that the primary signal in the Class I region
arises from the locus identified by SNP rs4595039 although it remains possible that there could be independent contributions from other Class I loci.

Both HLA-C*05:01 and HLA-B*44:02 are reportedly protective alleles for MS susceptibility [7,12,14]. These neighboring alleles are in tight LD making discrimination between the effects of each allele challenging. In addition, different alleles of HLA-A may influence MS susceptibility in opposite directions. HLA-A*0201 may increase MS risk; however, this allele is part of the expanded haplotype shared by HLA-DRB1*15:01 and its proposed influence on MS susceptibility may be confounded by linkage to HLA-DRB1*15:01 [6]. In contrast, HLA-A*0201 appears to have a protective effect [20]. This allele is also linked to the SNP identified in the present study, rs4595039. Functional studies, or fine mapping studies in populations with different patterns of LD, will be needed to determine whether the protective effect proposed for HLA-A*0201 is mediated by linkage to an allele of HLA-G or other neighboring genes.

In summary, we found MHC SNP associations with MS susceptibility, independent from the primary influence of HLA-DRB1*15:01, in the Class I, Class II and Class III regions. The most significant contribution arises from the Class I region in the vicinity of the HLA-G gene. HLA-G, or another closely linked gene such as HLA-A, contributes to MS risk independently from the recently identified Class I allele HLA-B*44:02, as well as other Class II and Class III SNPs identified in the present study. Thus a Class I locus near HLA-G/HLA-A is a replicated locus within the MHC that contributes to MS risk independently of HLA-DRB1*15:01. The possible HLA-G association is particularly interesting because HLA-G is thought to function in induction of immune tolerance and is highly expressed in MS brain tissue. Further studies of functional polymorphisms in HLA-G, classical HLA typing, as well as studies in populations with different patterns of LD within the MHC, will help further define this locus’s contribution to MS risk.

Methods

All study subjects signed written informed consent forms approved by the following local institutional review boards in accordance with the Declaration of Helsinki: Committee on Human Research (UCSF), CERDNT (MHI), Human Subjects Research Office (University of Miami), Partners Healthcare IB/ Human Research Office, North Thames MREC, The North Shore - LIJ Health System IRB, Vanderbilt HRPP and Berkshire Research Ethics Committee.

The MS discovery dataset consists of 1018 cases (520 from the US and 498 from the UK) and 1795 controls (1049 from the US and 746 from the UK). All MS subjects met International Panel criteria for multiple sclerosis [39]. The control population was composed of samples from the United Kingdom 1958 birth cohort (N = 1343 cases and 1379 controls from the US and UK). Of the 1337 Illumina SNPs, 958 passed QC in both datasets.

Marker Trait Association

Associations with MS susceptibility with SNPs and imputed alleles were assessed by the Cochran Armitage trend tests using the false discovery rate method to control for multiple comparisons [13]. SAS, JMP® genomics (Cary, NC) and STATA 9 (North Fork, TX) were used to perform statistical analyses. Population stratification caused by differences in markers between the sexes, the country of the subject’s origin (United States versus United Kingdom) and dataset (discovery versus replication) was controlled for by inclusion of these covariates as fixed effects in the regression analyses.

Following identification of SNPs that were significantly associated with MS susceptibility in both datasets the discovery and replication datasets were merged and subjects carrying the rs135391:T>C SNP that tags the HLA-DRB1*15:01 allele were excluded. MS associated SNPs where (Hardy Weinberg Equilibrium) HWE <0.01 in the control population of the merged dataset were dropped. 52 SNPs were significantly associated with MS susceptibility in both datasets.

In the discovery dataset previous 2- or 4-digit typing of HLA-DRB1 was available for 27.6% of the dataset (N = 777). [14] In this subset, the tagging SNP rs135391 was 100% sensitive and 100% specific for correctly calling HLA-DRB1*15:01. HLA typing was performed by different methodologies, including PCR-based sequence-specific oligonucleotide probe reverse-line blot assay, sequence-specific oligonucleotide (LABType) typing, and exons 2/3 sequence based typing.

Supporting Information

Figure S1 Study design summary. The 958 SNPs spanning the MHC used in the initial screens are listed in Supplemental Table 2. The 48 SNPs associated with MS in both datasets are listed in
Supplemental Table 3 and the 48 SNPs with p-values ≤1 × 10^{-8} in the merged HLA-DRB1*15:01(-) dataset are listed in Table 1. Found at: doi:10.1371/journal.pone.0011296.s001 (0.10 MB TIF)

Table S1  Table S1. Case control datasets: The proportion of women to men in the control populations was well matched at the two study centers. However, the proportion of women to men in the MS subjects was significantly increased in the UK dataset. Found at: doi:10.1371/journal.pone.0011296.s002 (0.07 MB DOC)

Table S2  Table S2: 958 SNPs genotyped in both discovery and replication datasets. Ext = extended. Found at: doi:10.1371/journal.pone.0011296.s003 (0.17 MB DOC)

Table S3  Table S3: 52 SNPs significantly associated with MS susceptibility in the discovery and replication datasets using Cochran Armitage trend test, FDR = .05, adjusted for sex, center (US versus UK) and HLA-DRB1*15:01. The SNPs are listed in order of chromosomal position from telomere to centromere. The p-values for the merged dataset are unadjusted. rs2523393 is a tagging SNP for HLA-B*44:02 [12,14]. Found at: doi:10.1371/journal.pone.0011296.s004 (0.17 MB DOC)

Table S4  Table S4: 48 SNPs significantly associated with MS susceptibility in the merged HLA-DRB1*15:01(-) dataset, using the trend test and adjusting for sex, center (US versus UK) and dataset (discovery versus replication). SNPs are listed in order of most to least statistical significance. Four class II SNPs identified in the discovery and replication datasets were no longer significantly associated with MS susceptibility in the HLA-DRB1*15:01(-) dataset; rs3129961, rs3135352, rs3135391, and rs3135388. Found at: doi:10.1371/journal.pone.0011296.s005 (0.14 MB DOC)

Table S5  Table S5: 48 SNPs that are associated with MS susceptibility in the HLA-DRB1*15:01(-) dataset are grouped together using an algorithm to define SNP clusters based on LD-R^2>0.5 (moderate to strong LD) [15]. The 48 SNPs can be grouped into 20 SNP clusters and tagging SNPs for each cluster are designated by an asterisk. The SNPs are listed in order of cluster size with the largest cluster including 10 SNPs and the smallest SNP clusters include only single SNPs. Found at: doi:10.1371/journal.pone.0011296.s006 (0.09 MB DOC)

Table S6  Table S6: Two locus haplotypes for the SNPs rs2523393 (tags HLA-B*44:02) and SNP rs459039 (near HLA-G). A MS risk haplotype is rs2523393:T>G and rs459039:A>G and a protective haplotype is rs2523393:C>T and rs459039:G>A. The heterozygous haplotype is appears to be protective suggesting a dominant effect of the protective haplotype. The p-values and odds ratios are adjusted for the covariates sex (men versus women) and cohort (discovery versus replication) to control for stratification. Found at: doi:10.1371/journal.pone.0011296.s007 (0.05 MB DOC)

Table S7  Table S7: SNPs significantly associated with MS susceptibility in the HLA-G locus typed in an independent dataset used for a genome wide meta-analysis. Found at: doi:10.1371/journal.pone.0011296.s008 (0.12 MB DOC)

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Author Contributions

Conceived and designed the experiments: BACC JDR JLM PLDJ TJV PG IMAGEN IMGEC DBM DAH HLJ MAPV AC SS JRO SLH. Performed the experiments: BACC JDR JLM PG IMAGEN IMGC DBM JRO SLH. Analyzed the data: BACC JDR JLM PAG JPM PLDJ AS SS JRO SLH. Contributed reagents/materials/analysis tools: BACC JDR JLM PG JPM PLDJ AS SS JRO SLH. Performed the experiments: BACC JDR JLM PG IMAGEN IMGC DBM JRO SLH. Wrote the paper: BACC JDR JLM PG JPM PLDJ AS SS JRO SLH. Acknowledgments

[References listed here]


16. www.sanger.ac.uk/HGP/Chr6/MHC/.


