**HLA-A Confers an HLA-DRB1 Independent Influence on the Risk of Multiple Sclerosis**

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A recent high-density linkage screen confirmed that the HLA complex contains the strongest genetic factor for the risk of multiple sclerosis (MS). In parallel, a linkage disequilibrium analysis using 650 single nucleotide polymorphisms (SNP) markers of the HLA complex mapped the entire genetic effect to the HLA-DR-DQ subregion, reflected by the well-established risk haplotype HLA-DRB1*15, DQB1*06. Contrary to this, in a cohort of 1,084 MS patients and 1,347 controls, we show that the HLA-A gene confers an HLA-DRB1 independent influence on the risk of MS (P = 8.4 × 10⁻¹⁰). This supports the opposing view, that genes in the HLA class I region indeed exert an additional influence on the risk of MS, and confirms that the class I allele HLA-A*02 is negatively associated with the risk of MS (OR = 0.63, P = 7 × 10⁻¹²) not explained by linkage disequilibrium with class II. The combination of HLA-A and HLA-DRB1 alleles, as represented by HLA-A*02 and HLA-DRB1*15, was found to influence the risk of MS 23-fold. These findings imply complex autoimmune mechanisms involving both the regulatory and the effector arms of the immune system in the triggering of MS.

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**INTRODUCTION**

The human leukocyte antigen (HLA) gene complex on chromosome 6 has been linked to and associated with several supposedly autoimmune diseases. This highly variable region harbors over 420 genes[1] and has been unambiguously associated with multiple sclerosis (MS, MIM 126200) susceptibility since 1972[2]. Extensive polymorphism and linkage disequilibrium (LD) complicates the efforts to precisely map associations with MS in this region but most evidence has pointed to the class II region and the HLA-DRB1 (GeneID: 3123) and -DQB1 genes, and specifically to the haplotype DRB1*1501, DRB5*0101, DQA1*0102, DQB1*0602[3].

Recently, Lincoln and colleagues reported data on 4,203 individuals from 1,185 Canadian and Finnish MS families genotyped for approximately 650 SNP markers spanning the HLA complex and its flanking regions, in all 13 Mb[4]. Strong associations were observed with several LD blocks within the HLA class II subregion. Conditioning for HLA-DRB1 revealed that all block or SNP associations were dependent on the HLA class II gene, implying that the HLA associated MS susceptibility is determined by HLA class II alleles or closely located variants [4]. However, this study did not assess any functional gene variants besides HLA-DRB1, such as the HLA-A gene (GeneID: 3105). Also, most likely due to the difficulty of designing assays against SNP markers in highly polymorphic genes, no SNP within or close to HLA-A were genotyped. The original reports on HLA associations with MS were indeed focused on the HLA class I specificities HLA-A and HLA-B [2,5]. Somewhat later, when class II specificities were discovered and the strong association between HLA-DRB1 and MS emerged, the class I associations were regarded as secondary [6]. More recently the possible influence of HLA class I genes in MS susceptibility has been re-investigated by us and others [7–9]. Fougell-Hahn and co-workers examined the role of classical HLA genes including both class I (HLA-A, -B, -C) and class II (HLA-DRB1, -DQ, -DP), indicating that HLA-A*03 confers a risk for MS while HLA-A*02 has a protective effect [7]. This primary study, as well as that of Harbo and co-workers [8], was too limited in size to establish a possible independence from the influence of class II alleles due to LD. Further, in addition to the classical association with the HLA-DRB1*15-carrying haplotype, recent studies have identified a more complex situation where several HLA-DRB1 alleles interact in determining the risk of MS [10,11]. In the present study, we set out to investigate how alleles of the HLA-DRB1 and HLA-A genes interact in determining the risk of MS. In order to distinguish the importance of two multiallelic loci, in addition to a sufficiently large sample size, a statistic strategy needs to be applied where the effect of one allele can be evaluated in the presence of other effects. Here, we use multiple logistic regression models enabling simultaneous analysis of all included alleles while adjusting for the potential confounding effect due to LD [12]. Thus, we were able to establish an HLA-DRB1 independent influence of HLA-A in MS susceptibility.

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**MATERIALS AND METHODS**

The 1,084 MS patients included in this study were diagnosed by neurologists at the Karolinska University Hospital and fulfilled the McDonald criteria of MS[13], their mean age was 51.5 years. The control group consisted of 1,347 consecutively collected blood donors with a mean age of 45.6. The study population was
**Table 1.** Allele frequencies (at a resolution corresponding to serological specificities) of HLA-A and HLA-DRB1 in MS patients (n = 1084) and healthy controls (n = 1347).

| HLA-  | MS frequency (%) | HC frequency (%) |
|-------|------------------|------------------|
| A*01  | 14.4             | 13.6             |
| A*02  | 26.6             | 35.9             |
| A*03  | 21.2             | 17.5             |
| A*11  | 5.7              | 5.1              |
| A*24  | 9.8              | 8.2              |
| AX    | 22.4             | 19.8             |
| DRB1*01 | 6.8          | 11.3             |
| DRB1*03 | 11.5         | 12.5             |
| DRB1*04 | 16.0         | 18.9             |
| DRB1*08 | 5.1           | 5.4              |
| DRB1*13 | 10.9          | 14.3             |
| DRB1*15 | 35.5          | 15.6             |
| DRB1X | 22.2             | 14.4             |

| DRB1 includes all observed alleles at the HLA-DRB1 locus with frequencies of less than 5% in cases; A*23, A*25, A*26, A*29, A*30, A*31, A*32, A*33, A*66, A*68, A*69, A*74 and A*210. DRB1X includes all observed alleles at the HLA-DRB1 locus with frequencies of less than 5% in cases; DRB1*02, DRB1*09, DRB1*10, DRB1*11, DRB1*12, DRB1*14, DRB1*16 and DRB1*103.

| Model | Model terms | Deviance | Df | Model comparison | ΔDeviance | ΔDf | P-value |
|-------|-------------|----------|----|------------------|-----------|-----|---------|
| 0     |             | 3336.4   | 2426 |                  |           |     |         |
| 1     | HLA-A<sup>a</sup> | 3286.1   | 2421 | Model 1 vs. 0    | 50.3      | 5   | 1.2x10^-9 |
| 2     | HLA-DRB1<sup>b</sup> | 3054.1   | 2420 | Model 2 vs. 0    | 282.3     | 6   | 5.1x10^-58 |
| 3     | HLA-DRB1+HLA-A<sup>a</sup> | 3003.1   | 2415 | Model 3 vs. 2    | 51.05     | 5   | 8.4x10^-10 |
| 4     | HLA-DRB1+HLA-A<sup>0</sup> | 3008.05  | 2422 | Model 5 vs. 4    | 0.19      | 3   | 0.98   |
| 5     | HLA-DRB1+HLA-A<sup>b</sup> | 3007.87  | 2419 |                  |           |     |         |

<sup>a</sup> AX includes all observed alleles at the HLA-A locus with frequencies of less than 5% in cases; A*23, A*25, A*26, A*29, A*30, A*31, A*32, A*33, A*66, A*68, A*69, A*74 and A*210. DRB1X includes all observed alleles at the HLA-DRB1 locus with frequencies of less than 5% in cases; DRB1*02, DRB1*09, DRB1*10, DRB1*11, DRB1*12, DRB1*14, DRB1*16 and DRB1*103.

<sup>b</sup> The Δ Deviance is the difference in deviance between the compared models. A large value (small P-value) indicates that the smaller models do not predict the outcome well, thus the smaller model is rejected.

<sup>c</sup> P-value is the probability of observing a larger Δ Deviance by chance. The P-value is adjusted for multiple testing using a Bonferroni correction.

To pinpoint the specific alleles contributing to MS susceptibility, we applied stepwise logistic regression on an allele level. Starting from a model with all HLA-A and HLA-DRB1 alleles, the least significant allele was removed one at the time, until the effect size of all remaining alleles were significant. Finally, the last model comparison assessed whether an interaction between HLA-DRB1 and HLA-A would improve the prediction. For a complete description of all the models used in the analysis see Supporting Information Table S1. It should be noted that odds ratios obtained in a logistic regression framework are adjusted for the overall significance level, chosen to be 0.05, was divided by 11, i.e. the total number of global tests (4) plus the total number of steps in the stepwise allele selection (7).

**RESULTS AND DISCUSSION**

A model including the main effects of both HLA-A and HLA-DRB1 (model 3 in Table 2) predicted the subjects' disease status significantly better than the model including only HLA-DRB1 (model 2). The P-value for this comparison was 8x10^-10, illustrating a strong additional effect of HLA-A on MS susceptibility (Table 2). It should be noted that the P-value refers to the independent effect of HLA-A, since the LD between the two loci is accounted for by including (conditioning on) HLA-DRB1 in the logistic regression model.

In order to pinpoint the alleles that confer an effect on MS susceptibility, a stepwise logistic regression was performed on all.

Table 2. Comparison of the nested logistic regression models of HLA-DRB1 and HLA-A

| Model | Model terms | Deviance | Df | Model comparison | ΔDeviance | ΔDf | P-value |
|-------|-------------|----------|----|------------------|-----------|-----|---------|
| 0     |             | 3336.4   | 2426 |                  |           |     |         |
| 1     | HLA-A<sup>a</sup> | 3286.1   | 2421 | Model 1 vs. 0    | 50.3      | 5   | 1.2x10^-9 |
| 2     | HLA-DRB1<sup>b</sup> | 3054.1   | 2420 | Model 2 vs. 0    | 282.3     | 6   | 5.1x10^-58 |
| 3     | HLA-DRB1+HLA-A<sup>a</sup> | 3003.1   | 2415 | Model 3 vs. 2    | 51.05     | 5   | 8.4x10^-10 |
| 4     | HLA-DRB1+HLA-A<sup>0</sup> | 3008.05  | 2422 | Model 5 vs. 4    | 0.19      | 3   | 0.98   |
| 5     | HLA-DRB1+HLA-A<sup>b</sup> | 3007.87  | 2419 |                  |           |     |         |
the alleles with frequency above 5%. The excluded alleles are reported in Supporting Information Table S2, whereas the alleles in the final model are reported in Table 3 along with corresponding statistics. As expected, our results showed that the DRB1*15 allele increased the risk of developing MS (OR = 2.9, P = 2 × 10^{-16}) (Table 3). The described heterogeneity of effects on MS susceptibility at the HLA-DRB1 locus [10,11] was partly confirmed, since several DRB1 alleles show significant association (Table 3). In contrast, we observed a protective effect of HLA-DRB1*01 (OR = 0.69, P = 1 × 10^{-5}), independently of HLA-DRB1*15. Similarly, a pool of less common HLA-DRB1 alleles (DRB1*07, DRB1*09, DRB1*10, DRB1*11, DRB1*12, DRB1*14, DRB1*16 and DRB1*10) was negatively associated with the risk of MS (OR = 0.77, P = 2 × 10^{-5}). This was most likely an effect of one or several of the included alleles. All DRB1X alleles had low frequency and were combined into one entity due to expected lack of power.

At the HLA-A locus, the A*02 allele decreased the risk of MS (OR = 0.63, P = 7 × 10^{-12}). No other HLA-A allele had an effect that differed significantly from that of the baseline.

Our results do not confirm an independent role of HLA-A*03 in MS susceptibility. In Table 4 we have compared our results, using logistic regression, with Fishers exact test and Cochran-Armitage test for trend of genotype distribution in our material. Both unadjusted approaches showed significant p-values for HLA-A*03 (3.8 × 10^{-3} and 7 × 10^{-4} respectively), whereas adjusting for other alleles generated a p-value of 0.95. This may seem contradictory to previous studies [7,8], but in fact shows the strength of the current statistical approach; the A*03 association previously detected is presumably a reciprocal effect of the decreased A*02 frequency or caused by confounding from the DRB1 locus. At the HLA-DRB1 locus, DRB1*03, DRB1*04 and DRB1*13 show significant p-values in the unadjusted analysis, not confirmed by logistic regression, thus most likely a reciprocal effect of the strong DRB1*15 association.

We proceeded by fitting a model with possible interactions between the HLA-A and HLA-DRB1 loci. When comparing the model including interaction terms (model 5) with model 4 we found no improvement in the ability to predict the subjects’ disease status (Table 2); the global p-value was 0.98 and none of the included interaction terms were significant. We could therefore conclude that the HLA-A*02 association was independent of the HLA-DRB1 locus and specifically the DRB1*15 allele; neither LD nor interaction with HLA-DRB1 had influenced the association of HLA-A*02 with MS. So in contrary to the recent SNP-based LD mapping study [4], we clearly show a HLA class I effect independent of HLA-DRB1.

It has previously been reported that HLA-DRB1*15 exerts an allele dose effect on the risk of MS [16,17]. Figure 1 illustrates how the genotypes of HLA-DRB1 and HLA-A jointly modified the empirical OR’s of MS, the most susceptible genotypes (homozygosity for DRB1*15 but no A*02) and most resistant (homozygosity for A*02 but no DRB1*15) genotypes differed 23 fold in the risk of MS. The magnitude of the risk jointly conferred by these loci greatly exceeds the relative risk of 3.5 typically seen for carriage of HLA-DRB1*15. This indicates that genes in the HLA region may confer a larger fraction of the genetic aetiology of MS than previously thought. This notion is further supported by the results of recent MS linkage analyses; in these studies, with increasing power of the analysis, the HLA locus has gradually obtained higher LOD scores, eventually exceeding 11, while all other candidate loci have remained insignificant [18,19]. Recently, Yeo and co-workers reported an association with several alleles of HLA-DRB1, together with a negative association with the HLA class I allele HLA-C*0501 in patients lacking DRB1 risk alleles, a finding which supports the importance of HLA class I genes in MS [9]. However, it remains to be studied whether the HLA-C locus contributes to MS susceptibility in our population, and, if so, whether there may be a confounding effect of LD between HLA-A*02 and HLA-C*0501.

The HLA complex is reputed for strong LD. Therefore, a newly reported genetic association could be expected to be secondary to a previously known association. As explained above, this was clearly not the case for the HLA-A association. The two global measures used (Cramer’s V and Kendall’s tau-b) showed low levels of LD (0.25 and -0.030 respectively). However weak, an LD of this magnitude could potentially affect allele distributions at the linked locus and induce a secondary association, but in the case of the HLA-A*02 allele this possibility is rejected by the results of the logistic regression analysis (P = 8.4 × 10^{-10}). Pairwise LD has been estimated and is reported in Supporting Information Tables S3 and S4. While it is principally possible that the HLA-A association in MS is due to another genetic variant in LD with HLA-A*02, data from the HapMap project shows that HLA-A is not part of any sizable LD block.

Frequencies of HLA alleles are known to vary considerably between different populations, presumably due to genetic drift and
different environmental challenges. Therefore a correct random sample of the population is important for the evaluation of HLA associations within that population. We therefore compared the distribution of \textit{HLA-A} and \textit{HLA-DR} alleles in our controls to the HLA genotype data available from the Swedish bone marrow registry (n = 40,162 and 11,006 respectively) and found no significant deviation. In particular, the \textit{HLA-A*02} frequencies are highly comparable (allele frequency of 35.9\% in our controls compared to 35.3\% in the Swedish bone marrow registry). Thus we feel confident that our control group is a suitable random sample of the Swedish population.

The HLA gene complex is different from other genomic regions by harbouring the most polymorphic genes we know; isotypic and allotypic polymorphism is in fact central to the physiological role of the class I and class II molecules. Therefore, it is not surprising that genetic association studies applying anonymous markers such as microsatellites and SNPs [4] are less efficient in detecting associations than the functional variants themselves.

With the present results in mind, it is reasonable to speculate that the genetic risk is indeed influenced by a function of an HLA class I molecule, possibly HLA-A. In contrast to the principle role of HLA-class II molecules in the triggering of an adaptive immune response, HLA class I antigens instead typically interact with cytotoxic CD8\(^+\) T cells. In fact, CD8\(^+\) T cells outnumber CD4\(^+\) T cells in MS lesions and may be of central importance in lesion pathogenesis [reviewed in [20]]. In addition, HLA class I molecules also interact with NK cells, and are in this way important for innate immunity.

Further mapping efforts to locate genetic effects on MS susceptibility within this region should preferably employ functional variants along with other markers. In addition, functional studies of HLA class I molecules in MS are motivated.

To conclude; using a large case control material and simple, straightforward statistical analysis we have shown that \textit{HLA-A} confers an additional influence on MS susceptibility in the Swedish population, not attributable to the known \textit{HLA-DRB1} association. This role of HLA class I genes may have important implications for the disease triggering mechanisms in MS.

**SUPPORTING INFORMATION**

| Table S1 | Model description. | Found at: doi:10.1371/journal.pone.0000664.s001 (0.03 MB DOC) |
| Table S2 | Sequential exclusion of alleles in the stepwise logistic regression procedure. At each step, the least significant allele was removed until all remaining alleles in the model were significant (Table 3) at \(\alpha = 0.05/11\). | Found at: doi:10.1371/journal.pone.0000664.s002 (0.03 MB DOC) |
| Table S3 | Pair-wise LD measures, D’ and R\(^2\), and two global (multi-allelic) measures of LD: Cramer’s V and Kendall’s tau-b for alleles of \textit{HLA-A} and \textit{HLA-DRB1} among controls. | Found at: doi:10.1371/journal.pone.0000664.s003 (0.05 MB DOC) |
| Table S4 | Pair-wise LD measures, D’ and R\(^2\), and two global (multi-allelic) measures of LD: Cramer’s V and Kendall’s tau-b for alleles of \textit{HLA-A} and \textit{HLA-DRB1} among cases. | Found at: doi:10.1371/journal.pone.0000664.s004 (0.05 MB DOC) |

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**Figure 1.** Empirical odds ratios (ORs) for combinations of the HLA-DRB1*15 and HLA-A*02 alleles. A genotype of two HLA-A*02 alleles but no HLA-DRB1*15 allele was used as baseline in the calculation of ORs. P values are reported above the bars. doi:10.1371/journal.pone.0000664.g001
Author Contributions
Conceived and designed the experiments: KD JH. Performed the experiments: BB. Analyzed the data: GJ. Wrote the paper: BB KD. Other: Contributed with reagents and samples: JH. Revised the manuscript: GJ JH EA IR KD BB. Contributed to the analysis of data: JP. Wrote the statistical sections in the manuscript: GJ JP. Collected samples and evaluated the patients: EA. Contributed to genotyping: IR. Contributed to the statistical design: GJ KD BB.

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