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LETTERS

Functional epistasis on a common MHC haplotype associated with multiple sclerosis

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Genes in the major histocompatibility complex (MHC) encode proteins important in activating antigen-specific immune responses. Alleles at adjacent MHC loci are often in strong linkage disequilibrium; however, little is known about the mechanisms responsible for this linkage disequilibrium. Here we report that the human MHC HLA-DR2 haplotype, which predisposes to multiple sclerosis[1–3], shows more extensive linkage disequilibrium than other common caucasian HLA haplotypes in the DR region and thus seems likely to have been maintained through positive selection. Characterization of two multiple-sclerosis-associated HLA-DR alleles at separate loci by a functional assay in humanized mice indicates that the linkage disequilibrium between the two alleles may be due to a functional epistatic interaction, whereby one allele modifies the T-cell response activated by the second allele through activation-induced cell death. This functional epistasis is associated with a milder form of multiple-sclerosis-like disease. Such epistatic interaction might prove to be an important general mechanism for modifying exuberant immune responses that are deleterious to the host and could also help to explain the strong linkage disequilibrium in this and perhaps other HLA haplotypes.

The HLA-DR2 haplotype in the human MHC contains the DR alleles *DRB1*1501* (*DR2b*) and *DRB5*0101* (*DR2a*) at the respective *DRB1* and *DRB5* loci, located 85 kb apart. In all ethnic groups studied so far, these alleles show almost complete linkage disequilibrium with haplotype frequencies varying across populations[4–10]. Previous data suggest that this haplotype is exceptional in the extent of its linkage disequilibrium[11]. Using extensive single nucleotide polymorphism (SNP) genotyping data available for this genomic region[11], we analysed the extent and length of linkage disequilibrium in caucasian samples. We found that the HLA-DR2 haplotype shows the most extensive preservation relative to all other common HLA-DR haplotypes (Fig. 1a–c). These data thus suggest that strong positive selection is operating on this haplotype and that these DR loci may confer a particular selective advantage.

In theory, the inseparability of *DR2a* from *DR2b* could imply the persistence of a founder haplotype; however, its ubiquity in widely variant ethnic groups[4–10] argues against this being the only explanation. Another possibility might be an advantageous epistatic interaction between these alleles. As in Bateson's original definition[12], we use the term 'epistasis' here to mean the masking of a functional effect of one allele at one locus by an allele at a separate locus[13]. If the *DR2b-DR2a* combination were very advantageous, it might favour much stronger linkage disequilibrium between these coding HLA-DR alleles than among interspersed or flanking anonymous SNPs. Studies have highlighted such differences between SNPs and HLA alleles[11,14]. Analysis of the available SNP data in the *DR2* region for Nigerian (Yoruba) and Asian samples in the International HapMap project[15] suggests that there are population differences in linkage disequilibrium patterns for SNPs (Supplementary Fig. 1). This observation is in stark contrast to the strong linkage disequilibrium between *DR2b* and *DR2a*[4–10], and strongly suggests the co-selection of epistatic alleles.

In support of these genetic observations, we have obtained independent biological evidence for an epistatic interaction between

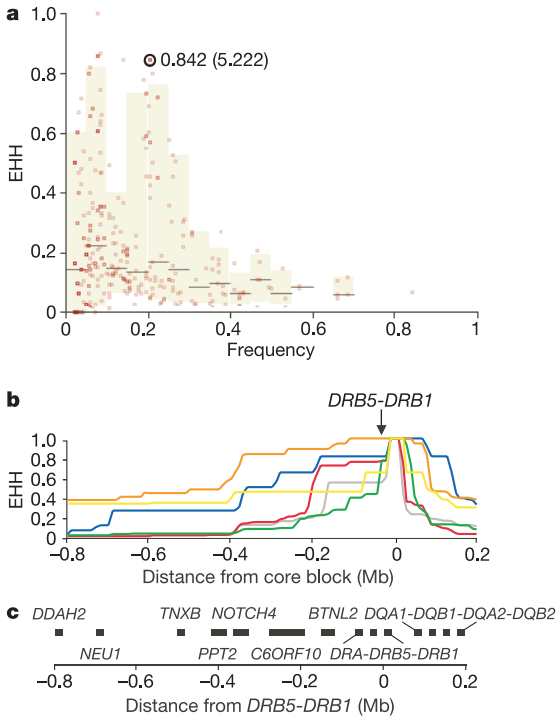

Figure 1 | Analysis of selection around *HLA-DRB1* and *HLA-DRB5* loci using extended haplotype homozygosity. **a**, Distribution of extended haplotype homozygosity (EHH) scores by frequency at 300 kb from the core block. The circled outlier is the HLA-DR2 haplotype with its EHH score and relative EHH score given in parentheses. The shaded bars are 5% and 95% percentiles. **b**, Plot of EHH. The frequencies of the haplotypes are 26% (red), 21% (orange, *DR2* outlier), 18% (green), 16% (grey), 8% (yellow) and 6% (blue). **c**, Approximate gene locations according to the UCSC Genome Bioinformatics website (http://genome.ucsc.edu; March 2006).

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Table 1 | Incidence, severity and mean onset of EAE in humanized mice

Transgene	Disease incidence	Maximum severity score (mean ± s.d.)	Onset in days (mean ± s.d.)
Hy^+-$DR2b^+$	38/44 (86.4%)	7.1 ± 1.0	181.9 ± 66.7
Hy^+-$DR2b^+$-$DR2a^+$	30/55* (54.5%)	6.0 ± 1.7**	221.6 ± 70.1***
Ob^+-$DR2b^+$	9/11 (81.8%)	6.8 ± 1.3	88.1 ± 26.0
Ob^+-$DR2b^+$-$DR2a^+$	7/8 (87.5%)	6.3 ± 1.8	95.8 ± 51.0
Hy^+-$DR2a^+$	0/30	0	-

*$P < 0.001$, **$P < 0.003$ and ***$P < 0.022$ versus Hy^+-$DR2b^+$ transgenic mice.

Table 2 | Frequency of autoreactive T cells in humanized mice

Transgene	Organ	Median frequency of Hy^+CD4^+ or Ob^+CD4^+ T cells (25–75 percentile)
Hy^+-$DR2b^+$	Blood	11.7 (1.18–32.7)
Hy^+-$DR2b^+$-$DR2a^+$	Blood	1.40 (0.06–9.0)*
Hy^+-$DR2b^+$	Thymus	0.17 (0.09–0.45)
Hy^+-$DR2b^+$-$DR2a^+$	Thymus	0.34 (0.12–0.45)
Ob^+-$DR2b^+$-$DR2a^+$	Blood	1.40 (0.70–2.20)
Ob^+-$DR2b^+$	Blood	2.05 (0.60–5.8)

*$P \leq 0.007$ versus Hy^+CD4^+ frequency in blood from Hy^+-$DR2b^+$ transgenic mice.

DR2b and *DR2a*. Our functional studies in humanized mice have dissected the roles of these alleles in predisposition to multiple sclerosis. Previous genetic studies localized the dominant genetic determinant of susceptibility to the region around the *DRB1* and *DRB5* loci on the HLA-DR2 haplotype[1,2], but dissection of the precise DNA variant accounting for this susceptibility has been constrained by the almost complete linkage disequilibrium between the *DR2b* and *DR2a* alleles[16]. Transferring these genes into mice has enabled us to analyse their contributions, either separately or together, to the autoimmune T-cell response and to the resulting multiple-sclerosis-like disease experimental autoimmune encephalomyelitis (EAE).

We used two T-cell antigen receptors (TCRs) derived from individuals affected with multiple sclerosis: Hy.2E11 (Hy) and Ob.1A12 (Ob)[17,18]. Both TCRs recognize the immunodominant epitope in the multiple-sclerosis-relevant autoantigen myelin basic protein, MBP(85–99), when presented by the DR2b molecule (encoded by *DRB1*1501* and the invariant *DRA1*0101* allele; Supplementary Fig. 2 and Supplementary Table 1). The Hy TCR also recognizes several different epitopes presented by the DR2a molecule[19] (encoded by *DRB5*0101* and the invariant *DRA1*0101* allele), a cross-reactivity not seen with the Ob TCR[19]. We generated humanized transgenic mice expressing the *Hy* TCR or *Ob* TCR and *DR2b* or *DR2a* or both of these HLA class II alleles[19,20]. Because they had been crossed to $Rag2^{-/-}$ mice, which do not express endogenous murine TCRs, the only TCR expressed by the mice was the human transgene-encoded TCR.

The Hy^+-$DR2b^+$ mice spontaneously developed very severe EAE (Table 1). Addition of the *DR2a* transgene significantly decreased its incidence, reduced its severity and delayed its onset (Table 1). We also observed complete spontaneous clinical remissions in about 20% of Hy^+-$DR2b^+$-$DR2a^+$ transgenic mice, whereas none of the 38 Hy^+-$DR2b^+$ transgenic mice recovered ($P \leq 0.014$) from EAE. In addition, spontaneous primary progressive EAE was very prevalent in Ob^+-$DR2b^+$ transgenic mice and was not affected by the introduction of *DR2a* (Table 1). These results indicate that the modifying effect of *DR2a* may not simply be due to a nonspecific event, such as DRA chain stealing, but may be due to an epistatic interaction with *DR2b* that depends on a cross-reactive TCR. This conclusion was corroborated by separate experiments showing that neither $CD4^+$ T-cell frequencies nor the severity of EAE in $Hy^+ - DR2b^+$ transgenic mice was affected by the addition of another HLA-DR allele, *DRB1*0401* (data not shown). Taken together, our results imply that, at least in this setting, the *DR2b* allele mediates EAE, whereas *DR2a* functions as a genetic modifier of the resulting disease.

One possible explanation for our observations is that the DR2a molecules induce deletion of Hy^+CD4^+ T cells and thereby modulate the severity of the DR2b-dependent disease. We therefore compared the frequencies of Hy^+CD4^+ T cells in thymi and blood from Hy^+-$DR2b^+$ and Hy^+-$DR2b^+$-$DR2a^+$ transgenic mice on a $Rag2^{-/-}$ background. Whereas we observed no significant difference in their thymi, we found markedly lower frequencies of Hy^+CD4^+ T cells in blood from the Hy^+-$DR2b^+$-$DR2a^+$ mice (Table 2). By contrast, we saw no such differences in the frequencies of Ob^+CD4^+ T cells between Ob^+-$DR2b^+$-$DR2a^+$ and Ob^+-$DR2b^+$ transgenic mice (Table 2). These results suggest that the DR2a molecules do not induce central tolerance in Hy^+-$DR2b^+$-$DR2a^+$ transgenic mice, but instead reduce the number of autoreactive Hy^+CD4^+ T cells in the periphery.

One mechanism of peripheral tolerance is clonal deletion of self-reactive T cells by activation-induced cell death (AICD)[21]. The hallmark of AICD is activation, followed by proliferative expansion and apoptotic cell death of the activated T cells. We tested whether DR2a molecules could induce peripheral deletion by AICD of adoptively transferred Hy^+CD4^+ T cells. In $DR2a^+$ and $DR2b^+$-$DR2a^+$ recipients, the cells underwent multiple divisions within 5 days of transfer (Fig. 2a) and showed a $CD44^+CD62L^-$ phenotype (Supplementary Fig. 3) consistent with activation, whereas almost all cells remained undivided in $DR2b^+$ recipients (Fig. 2a) and predominantly had a $CD44^-CD62L^+$ naive phenotype (Supplementary Fig. 3). Moreover, by day 5, the rate of apoptosis in Hy^+CD4^+ T cells adoptively transferred into $DR2b^+$-$DR2a^+$ and $DR2a^+$ mice was markedly increased as compared with that in $DR2b^+$ mice (Fig. 2b). Hy^+CD4^+ T cells were barely detectable in spleens and blood from the former groups after 15 d from transfer but still persisted in the latter group (Fig. 2c and Supplementary Fig. 4). These results indicate that DR2a molecules induce peripheral deletion of Hy^+CD4^+ T cells by means of AICD. Thus, they show epistasis between the *DR2b* and *DR2a* alleles at a functional level (Supplementary Fig. 5).

One of the hallmarks of the human MHC is the strong linkage disequilibrium that extends across many HLA haplotypes. Although

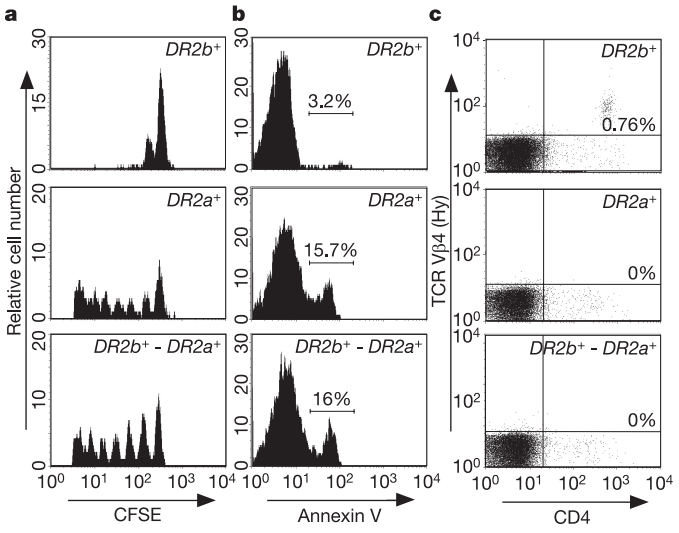

Figure 2 | DR2a induces peripheral deletion of Hy^+CD4^+ T cells by AICD. a, Proliferation of CFSE-labelled Hy^+CD4^+ T cells 5 d after transfer into $DR2b^+$, $DR2a^+$ and $DR2b^+$-$DR2a^+$ transgenic mice. Division of transferred T cells in spleens of the recipient mice was measured by CFSE dilution using flow cytometry. **b**, Apoptosis of Hy^+CD4^+ T cells 5 d after transfer into the indicated recipient mice. Apoptotic Hy^+CD4^+ T cells in spleens of the recipient mice were detected by Annexin-V staining. **c**, Frequency of Hy^+CD4^+ T cells in spleens of $DR2b^+$, $DR2a^+$ and $DR2b^+$-$DR2a^+$ recipient mice 15 d after transfer.

it was originally thought to be unique to the MHC, similar regions of strong linkage disequilibrium are now known to exist elsewhere in the genome[15]. In all cases, the mechanisms for their generation and maintenance are not clear. Several possible explanations exist for long-range linkage disequilibrium such as that apparent on the HLA-DR2 haplotype. The simplest is that strong positive selection for a component of the haplotype has expanded its frequency in recent evolutionary history, creating a founder haplotype that has had inadequate evolutionary time to degrade. This explanation, although potentially important in explaining much linkage disequilibrium in the HLA region, is unlikely to provide the only mechanism for these effects, however, because the *DR2b* and *DR2a* alleles are in strong linkage disequilibrium in all ethnic populations in which they have been studied[4–10]. In addition, this tight linkage disequilibrium is not always paralleled by tight linkage disequilibrium between anonymous SNPs in this region across populations. These data indicate that recent selection of founder haplotypes is unlikely and that the *DRB* alleles on this haplotype probably have repeatedly re-associated through the strength of their functional interaction. Together, therefore, our functional and genetic data support the hypothesis that epistasis is at least partly responsible for this strong linkage disequilibrium.

The epistatic interaction between the *DR2a* and *DR2b* alleles requires a cross-reactive TCR restricted by both corresponding MHC class II molecules. This leads to a mechanism of immune modulation, whereby DR2b molecules mediate a strong immune response, whereas DR2a molecules modify this effect through peripheral T-cell deletion by means of apoptosis. Although the natural antigenic stimulus responsible for the co-selection through epistasis of the two DR alleles is not known, it is likely that in our experimental model a self-antigen produces similar epistatic functional effects. In our model, coexpression of the DR2a and DR2b molecules reduces the magnitude and severity of multiple-sclerosis-like disease and produces remissions that are characteristic of the most common form of multiple sclerosis seen in humans—relapsing-remitting disease[3]. The relevance of our data to the human situation is supported by the observation that intact apoptosis pathways are required for spontaneous clinical remissions in other relapsing remitting EAE models and are crucial for controlling disease severity[22]. Taken together, these observations suggest a situation whereby DR2a operates in concert with DR2b and partially deletes autoreactive T cells to limit autoreactive responses triggered by DR2b, thus creating a less severe and more clinically relevant phenotype (Supplementary Fig. 5).

Although this functional epistasis is occurring in an experimental model, its ability to modulate normal immune responses is apparent. Vigorous host immune responses are ultimately responsible for much of the pathology and tissue damage in various infectious diseases such as viral hepatitis[23] and schistosomiasis[24] in humans and viral lymphocytic choriomeningitis in mouse[25]. Maintaining a balance between controlling the infectious pathogen and causing tissue damage requires a balance in the immune response. The epistasis that we have described provides modulation of the immune response activated by one HLA allele. This epistatic modulation of the severity of such a response could have an important general regulatory role and may therefore be more widespread throughout the MHC and might also contribute to patterns of linkage disequilibrium on other MHC haplotypes.

Separating the effects of individual human alleles at separate loci that show strong linkage disequilibrium can be extremely difficult. The experimental model that we have used for these studies has wide implications for those studying functional contributions of other genetic loci held together in strong linkage disequilibrium. Our humanized murine model has enabled us to characterize the independent and interactive role of two multiple-sclerosis-associated DR alleles *in vivo* and to demonstrate functional effects. By extension, this type of functional approach can provide a powerful mechanism for determining the relevant role of other individual alleles that are indistinguishable in humans because of high or complete linkage disequilibrium.

METHODS

Extended haplotype homozygosity analysis. Extended haplotype homozygosity (EHH)[26] analysis was done with Sweep (http://www.broad.mit.edu/mpg/sweep/) using the Centre d'Etude du Polymorphisme Humain (CEPH) SNP data sets[11] as described in Supplementary Methods.

Integrated haplotype score analysis. We downloaded the genome-wide integrated haplotype scores[27] for the HapMap phase I CEU, CHB + JPT and YRI samples from the Pritchard Lab website (http://pritch.bsd.uchicago.edu/data.html). A window-based ranking process was then followed to obtain a statistic *Q* value for each SNP (see Supplementary Methods).

Mice. Transgenic mice expressing human T-cell receptors (Hy.2E11 and Ob.1A12) and human HLA-DR molecules were generated as described[19,20]. We purchased 129S6/SvEvTac *Rag2*$^{-/-}$ mice from Taconic. Clinical assessment of EAE is described in Supplementary Methods.

Adoptive transfer, apoptosis and *in vivo* proliferation assays. For apoptosis assays, splenocytes from *Hy*$^{+}$ transgenic mice were isolated, washed with PBS and injected intravenously into recipient mice (20×10^6 cells per mouse). To detect apoptotic cells, splenocytes from recipient mice were stained with antibodies against TCR Vβ4 (recognizing the β-chain of Hy TCR) and against CD4, together with Annexin-V and propidium iodide, and analysed by flow cytometry. For *in vivo* proliferation assays, splenocytes from *Hy*$^{+}$ transgenic mice were labelled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) in 0.1% albumin in PBS at 37 °C for 10 min and washed before injection into recipient mice. To measure proliferation of transferred T cells, splenocytes from the recipient mice were stained with antibodies against CD4 and CFSE dilution was analysed by flow cytometry.

Statistical analysis of murine data. Statistical differences in EAE incidence, onset and severity, and in frequencies of Hy^{+}CD4^{+} T cells were determined by a χ^2-test or Fisher's exact test, and Student's *t*-test or the Mann–Whitney rank sum test, respectively. Values of $P \leq 0.05$ were considered statistically significant.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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