EXTENDED REPORT

MHC class II, tumour necrosis factor α, and lymphotoxin α gene haplotype associations with serological subsets of systemic lupus erythematosus

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Objective: To conduct a case–control study to investigate whether there are independent tumour necrosis factor α (TNFα) or lymphotoxin α (LTα) haplotype associations with SLE or with any of the major serological subsets of SLE.

Methods: 157 patients with SLE were genotyped for HLA-DRB1, HLA-DQB1, TNFα, and LTα alleles by polymerase chain reaction and compared with 245 normal white controls. For TNFα, six single nucleotide polymorphisms (SNPs) at positions −1031, −863, −857, −308, −238, and +488 and for LTα three SNPs at positions +720, +365, and +249 were studied to assign six TNFα haplotypes (TNF1-6) and four LTα haplotypes (LTA1-4). All SLE patients had full serological profiles on serial samples.

Results: The most significant association with SLE overall was with HLA-DR3 ($p<0.001$; odds ratio (OR) = 2.5 (95% confidence interval, 1.6 to 3.8)) and the extended haplotype HLA-DQB1*0201;DRB1*0301;TNF2;LTA2 ($p<0.001$; OR = 2.3 (1.4 to 3.7)). Associations were strongest in the anti-La positive group (13%) of SLE patients (HLA-DR3, OR = 71 (9 to 539); HLA-DQB1*0201, OR = 35 (5 to 267); TNF2, OR = 10 (2.8 to 36), and LTA2, OR = 4.9 (1.1 to 21)). There was an increase in the HLA-DR2 associated extended haplotype (HLA-DQB1*0602;DRB1*1501;TNF1;LTA1) in patients with anti-Ro in the absence of anti-La ($p<0.005$; OR = 3.9 (1.5 to 10)). The HLA-DR7 extended haplotype (HLA-DQB1*0303; DRB1*0701/2; TNF5;LTA3) was decreased in SLE overall ($p<0.02$; OR = 0.2 (0.05 to 0.8)).

Conclusions: The strongest association in this predominantly white population with SLE was between HLA-DR3 and anti-La, which seemed to account for any associations with TNFα alleles on an extended DR3 haplotype.

Systemic lupus erythematosus (SLE) is a chronic multisystem inflammatory disorder predominantly affecting females, characterised by vascular damage and immune deposition in affected organs and the presence of certain sets of circulating autoantibodies. The aetiology is unknown but there is likely to be a complex interaction of hormonal, genetic, and environmental factors operating in a stochastic fashion in the affected individual. The genetic contribution to SLE is well supported by findings of higher concordance in monozygotic versus dizygotic twins, a 20-fold increased risk in siblings, and linkage analysis and case–control studies that have identified various genetic susceptibility loci (see Tsao[1] for a review).

Several studies have shown associations between the major histocompatibility complex (MHC) class II region and SLE, and the most frequently reported association in white populations is with HLA-DR3.[2–4] HLA-DR3 forms part of an ''autoimmune'' haplotype (HLA A1, B8, DR3, DQ2) which in white populations is in linkage disequilibrium with certain tumour necrosis factor α (TNFα) and complement (''C4 null'') alleles located at the nearby MHC class III region. It is uncertain what genes on the extended haplotype may be responsible for disease susceptibility. The associations of MHC class II genes appear to be stronger with autoantibody defined subgroups of patients,[5] which would be consistent with the view that certain class II molecules provide a more favourable mechanism for autoantigen driven, T cell assisted autoantibody generation. Accordingly, MHC class II alleles may be important susceptibility factors for autoantibody generation, whereas other genes on the extended haplotype may influence susceptibility through different mechanisms.

The role of TNFα in SLE is controversial. The administration of exogenous TNFα to NZB/NZW F1 mice delays onset and progression of SLE-like disease,[6] implying a protective role. HLA-DR2 is associated with low levels of TNFα and susceptibility to nephritis.[7] Also, lupus-like syndromes have been reported in patients receiving anti-TNFα treatment.[8] Alternatively, increased levels of TNFα are increased in human SLE and appear to be correlated with disease activity.[9] Therefore, there has been interest in studying biallelic polymorphisms in the promoter region of TNFα that may influence TNFα levels and susceptibility to SLE. In particular, the uncommon variant TNF-308A allele is in close linkage disequilibrium with HLA-DR3[10] and has been found to confer an independent susceptibility to SLE in some studies[11 12] but not all.[13 14] We have therefore studied the frequency of promoter polymorphisms in TNFα and the closely related lymphotoxin α (LTα) gene,[15] which may confer susceptibility in our own SLE population. We also undertook a thorough examination of autoantibody specificities to determine whether autoantibody defined subsets of disease reveal stronger genetic associations.

METHODS

Study population

We studied 157 patients with SLE (19 male and 138 female) attending the Royal National Hospital for Rheumatic

Abbreviations: aCL, anticardiolipin antibodies; ANA, antinuclear antibodies; LTα, lymphotoxin α; MHC, major histocompatibility complex; SLE, systemic lupus erythematosus; SSP, sequence specific primer; TNFα, tumour necrosis factor α

Table 1 Primers used to amplify TNFα SNPs at positions −1031, −836, and −857 and the eight possible haplotypes

Primer mix	5′ Primer	Sequence	3′ Primer	Sequence	Amplified haplotype −1031, −863, −857
1	1971	AAAGGAGAAGCTGAGAAGAT	1	TGGCCCTGTCTTCGTTTAAGT	TAC
2	1971	AAAGGAGAAGCTGAGAAGAT	2	ATGGCCCTGTCTTCATTAAGT	TAT
3	1971	AAAGGAGAAGCTGAGAAGAT	3	GGCCCTGTCTTCGTTAAGG	TCC
4	1971	AAAGGAGAAGCTGAGAAGAT	4	TGGCCCTGTCTTCATTAAGG	TCT
5	1972	AAAGGAGAAGCTGAGAAGAC	1	TGGCCCTGTCTTCGTTTAAGT	CAC
6	1972	AAAGGAGAAGCTGAGAAGAC	2	ATGGCCCTGTCTTCATTAAGT	CAT
7	1972	AAAGGAGAAGCTGAGAAGAC	3	GGCCCTGTCTTCGTTAAGG	CCC
8	1972	AAAGGAGAAGCTGAGAAGAC	4	TGGCCCTGTCTTCATTAAGG	CCT

SNP, single nucleotide polymorphism; TNFα, tumour necrosis factor α.

Diseases, Bath, UK. All fulfilled revised American College of Rheumatology (ACR) criteria for SLE,[16] with the least number fulfilling the individual criteria as follows: malar rash, 53; discoid rash, 10; photosensitivity, 50; oral ulcers, 35; arthritis, 70; serositis, 34; renal disorder, 16; neurological disorder, 14; haematological disorder, 85; immunological disorder, 120; and antinuclear antibody, 150. All SLE patients apart from six were white. The six non-white patients were of Afro-Caribbean (4) and Chinese (2) descent. The control population consisted of 245 healthy blood donors from the same geographical area (west of England).

HLA-DRB1 and HLA-DQB1 genotyping

DNA was prepared from whole blood collected into EDTA using a standard salting out procedure. HLA-DRB1 and HLA-DQB1 alleles were identified using a polymerase chain reaction (PCR) based method with sequence specific primers (SSP).[17 18] Ten 5′ primers and 16 3′ primers combined in 20 primer mixes were used for identifying HLA-DRB1 alleles. Also, in each PCR reaction a pair of primers specific for non-allelic sequences was included. These primers amplified the third intron of HLA-DRB1 genes and functioned as an internal amplification control. Allele specific amplification of HLA-DRB1*04 alleles was carried out using one 5′ primer and eight 3′ primers combined in eight (5′+3′) primer mixes, with control primers in each reaction.[19] Allele specific amplification of HLA-DRB1*01 alleles was established using four 3′ primers and one 5′ primer combined in four reactions.[20] In all, 26 alleles were tested, grouped under 10 broad specificities: DR1 (0101/2, 0103), DR2 (1501/2, 1601/2), DR3 (0301, 0302), DR4 (0401, 0402, 0403, 0404, 0405, 0406, 0407, 0408), DR5 (1101–1104, 1201/2), DR6 (1301, 1302, 1303/4, 1305, 1401/4/5, 1402/3), DR7 (0701/2), DR8 (0801–0805), DR9 (0901), and DR10 (1001).

Eleven 5′ primers and 11 3′ primers combined in 14 primer mixes were used for the identification of HLA-DQB1 alleles.[18] In all, 13 alleles were tested: 0201, 0301/4, 0302, 0303, 0401, 0402, 0501, 0502, 0503, 0601, 0602, 0603, and 0604.

TNFα and LTα genotyping

TNFα polymorphisms at positions −308, −238, and 448 and LTα polymorphisms at positions 720, 365, and 249 were determined using the primers at the concentrations described by Fanning *et al*,[21] with modifications to primers 708 (to ATAGGTTTTGAGGGGCATGG) and 709 (to ATAGGTTTTG AGGGGCATGA). Both primers were used at concentration of 0.56 μM.

TNFα polymorphisms at positions −1031, −863, and −857 were determined using primers generated from GenBank: Z15026, which were combined to amplify eight possible haplotypes (table 1).

The primers were used at a working concentration of 1.1 μM. Control primers C63 and C64[21] were used in each reaction at a working concentration of 0.1 μM. No more than six haplotypes were deduced from the combination of the six TNFα SNPs and are listed in table 2.

PCR preparation

PCR reactions were carried out in a final volume of 10 μl. Each of the 32 reactions consisted of 5 μl of primer mix and 5 μl of PCR reaction mix. Concentrations of components in the reaction mix were: 0.4 mM of each dNTP, reaction buffer (150 mM Tris HCl, pH 8.8; 40 mM $(NH_4)_2SO_4$), 0.02% Tween 20), 3.0 mM MgCl, 0.05 U/ml Taq polymerase (ABgene), sucrose cresol red (0.6 M sucrose, 0.3 mM cresol red), 0.02–0.1 mg/ml DNA.

PCR cycling conditions

PCR amplifications were carried out in MJ Research PTC Pelter Thermal Cycler. For HLA-DRB1 and HLA-DQB1 alleles the following conditions were used: two minutes at 96℃; nine cycles of 25 seconds at 96℃; 50 seconds at 65℃; 30 seconds at 75℃; 19 cycles of 10 seconds at 96℃; 50 seconds at 61℃; 30 seconds at 72℃; 10 minutes at 72℃. For TNFα and LTα alleles the following conditions were used: one minute at 96℃; four cycles of 20 seconds at 96℃; 45 seconds at 70℃; 25 seconds at 72℃; 20 cycles of 25 seconds at 96℃; 50 seconds at 65℃; 30 seconds at 72℃; three cycles of 96℃; 60 seconds at 55℃; 120 seconds at 72℃.

Autoantibody measurement

Antinuclear antibodies (ANA) were measured by indirect immunofluorescence on Hep-2 cells. Antibodies to extractable nuclear antigens (including U1RNP, Sm, Ro/SS-A, and

Table 2 TNFα promoter haplotypes

Haplotype	TNF −1031	TNF −863	TNF −857	TNF −308	TNF −238	TNF +488
TNF1	T	C	C	G	G	G
TNF2	T	C	C	A	G	G
TNF3	C	A	C	G	G	G
TNF4	T	C	T	G	G	A
TNF5	C	C	C	G	A	G
TNF6	C	C	C	G	G	G

TNFα, tumour necrosis factor α.

Table 3 MHC class II associations with SLE and serological subsets of disease

Allele	Controls (n = 244)	SLE (n = 157)	Ro (n = 41)	La (n = 20)	U1RNP (n = 59)	Sm (n = 17)	aCL (>low) (n = 16)	DNA (n = 115)
DR3	52 (21%)	63 (40%) [2.5]	25 (61%) [5.8]	19 (95%) [71]	17 (29%)	8 (48%) [3.2]	5 (31%)	42 (37%) [1.8]
DR6	54 (22%)	23 (15%)	24 (10%)	3 (1.5%)	6 (10%) [0.4]	2 (12%)	3 (19%)	17 (15%)
DR7	62 (25%)	25 (16%) [0.6]	4 (10%) [0.3]	1 (5%)	9 (15%)	3 (18%)	6 (15%)	16 (14%) [0.5]
DQ 0201	86 (35%)	82 (60%) [2]	26 (63%) [3.2]	19 (95%) [35]	25 (42%)	9 (53%)	8 (47%)	58 (50%) [1.8]
DQ 0602	58 (24%)	50 (32%)	15 (37%)	6 (30%)	17 (28%)	8 (47%) [2.9]	5 (30%)	41 (36%)
DQ 0603	24 (10%)	6 (4%) [0.4]	1 (2%)	1 (5%)	3 (5%)	1 (6%)	1 (6%)	4 (3%) [0.4]
DQ 0604	25 (10%)	11 (7%)	1 (2%)	1 (5%)	0 (0%) RR = 0.9	0 (0%)	1 (6%)	8 (7%)

Odds ratios in square brackets.
SLE, systemic lupus erythematosus.

La/SS-B,) were measured by Ouchterlony double diffusion. Anti-double stranded DNA antibodies were measured by enzyme linked immunosorbent assay (ELISA) (Cambridge Life Sciences, Ely, UK) and anticardiolipin antibodies (aCL) by ELISA (Cambridge Life Sciences).

Serial serum samples were available on patients over a span of up to 10 years (median three samples; range 1 to 11 samples). Unequivocal evidence of autoantibody presence on at least one occasion was required for autoantibody assignment. In addition all patients with anti-U1RNP, anti-Sm, or anti-La antibodies had autoantibodies confirmed by western blotting on at least one sample.[22] For patients with aCL, three groups were analysed versus controls; the first group had raised IgG aCL (>15 GPL) on at least one occasion, the second group had raised IgM aCL on at least one occasion, and the third group had either IgG or IgM aCL raised to at least moderate levels (>25 GPL or >25 MPL) on at least two occasions.

Statistical analysis

All clinical, serological, and genetic data were maintained on an Access database. Data were transferred to SSPS for statistical analysis. Genotype frequencies were tested for Hardy–Weinberg equilibrium. TNFα and LTα haplotypes were assigned as published (TNFα haplotypes are shown in table 2). Phenotype (allele carriage) frequencies were compared between the disease and control groups using χ^2 analysis with Bonferroni correction for the number of alleles observed when appropriate (p values given are uncorrected unless stated). Odds ratios (OR) with confidence intervals (CI) are also determined, or relative risk (RR) when a frequency was zero. Allele frequency and gene frequency were also derived and are stated where relevant for comparison with other studies. Haplotype frequencies were determined using the software programme Arlequin (www.lgb.unige.ch/arlequin). Estimations of the linkage disequilibrium between alleles were calculated as D values where D = 0 represented no linkage and D = 1 represented strong linkage disequilibrium. To assess allele or haplotype independence, cross tabulation with stratification analysis (Mantel–Haenszel procedure) was used.

Autoantibody defined groups were compared to controls in a similar manner.

Ethical approval for the study was given by the Bath regional ethics committee and informed written consent was obtained.

RESULTS

MHC class II associations with SLE

Associations between SLE and MHC class II alleles are shown in table 3 (only significant associations are tabulated). As expected, the strongest positive association was between SLE and HLA-DR3 ($p<0.001$; OR = 2.5 (95% CI, 1.6 to 3.8)) (table 3).

The DR3 related extended haplotype (HLADQB1*0201; DRB1*0301; TNF2; LTA2) was also increased ($p<0.001$; OR = 2.3 (95% CI, 1.4 to 3.7)). Homozygosity for HLA-DR3 was not significantly increased in SLE (8/157 *v* 7/244). There was a significant increase in HLA-DQB1*0201 ($p<0.001$; OR = 2 (1.3 to 3.0)), which was in known strong linkage with HLA-DR3 (D = 0.93 for SLE and D = 0.98 for controls); however, HLA-DQB1*0201 was not associated with SLE in the absence of HLA-DR3.

HLA-DR*0701/2 conferred a protective effect for SLE ($p<0.05$; OR = 0.6 (0.3 to 0.9)), although this was not significant after correction. The reduction in HLA-DR*0701/2 was caused by a reduction in the DR7 extended haplotype (HLA-DQB1*0303; DRB1*0701/2;TNF5;LTA3) ($p<0.02$; OR = 0.2 (0.04 to 0.8)).

Table 4 TNF-308 and TNF-238 promoter polymorphism phenotypes and TNFα promoter haplotypes in SLE and serological subsets of disease

TNFα promoter phenotype	Controls (n = 234)	SLE (n = 147)	Ro (n = 38)	La (n = 19)	U1RNP (n = 55)	Sm (n = 18)	aCL (>low) (n = 15)	DNA (n = 107)
−308G	215 (93%)	135 (92%) [0.3]	32 (84%) [0.1]	14 (74%) [0.1]	52 (95%)	17 (95%)	15 (100%)	100 (93%)
−308A	85 (36%)	65 (44%)	21 (55%) [2.2]	16 (84%) [9.4]	21 (38%)	7 (39%)	4 (27%)	38 (36%)
−238G	233 (100%)	143 (97%)	37 (97%)	18 (95%)	55 (100%)	18 (100%)	15 (15%)	103 (96%)
−238A	29 (12%)	9 (6%)	3 (8%)	2 (11%)	1 (2%) [0.1]	0 (0%)	0 (0%)	9 (8%)
TNFα promoter haplotype	**Controls (n = 224)**	**SLE (n = 142)**	**Ro (n = 36)**	**La (n = 19)**	**U1RNP (n = 52)**	**Sm (n = 17)**	**aCL (>low) (n = 14)**	**DNA (n = 103)**
TNF1	175 (79%)	101 (71%)	25 (69%)	11 (58%)	37 (71%)	15 (88%)	10 (71%)	76 (74%)
TNF2	78 (35%)	60 (42%)	19 (53%) [2.1]	16 (84%) [11]	19 (37%)	6 (35%)	4 (29%)	34 (33%)
TNF3	72 (32%)	45 (32%)	7 (19%)	2 (11%)	18 (35%)	1 (6%)	5 (36%)	32 (31%)
TNF4	27 (12%)	11 (8%)	4 (11%)	1 (5%)	3 (6%)	2 (12%)	1 (75)	9 (9%)
TNF5	27 (12%)	8 (6%) [0.5]	2 (6%)	2 (11%)	1 (2%)	0 (0%)	0 (0%)	8 (8%)
TNF6	1 (0%)	1 (1%)	0 (0%)	0 (0%)	1 (2%)	0 (0%)	0 (0%)	0 (0%)

Odds ratios in square brackets.
TNFα, tumour necrosis factor α; SLE, systemic lupus erythematosus.

There was a decrease in HLA-DQB1*0603 ($p<0.05$; OR = 0.4 (0.1 to 0.9)) accompanied by a non-significant reduction in the linked allele HLA-DRB1*1301 (data not shown)

TNFα and LTα allele and haplotype associations with SLE

All TNFα and LTα polymorphisms tested were in Hardy–Weinberg equilibrium. There was an increase in the TNF2 haplotype (containing TNF-308A) although this did not reach significance (table 4).

As expected, TNF2 was in strong linkage with HLA-DR3 (D = 0.69 for SLE and D = 0.69 for controls). There was also an increase in TNF-308A allele frequency (0.26 *v* 0.19 in controls) and in TNF-308A gene frequency (0.14 *v* 0.10 in controls).

The only significant TNFα haplotype association with SLE was a reduction in TNF5, which contains the TNF-238A allele ($p<0.05$; OR = 0.5 (95% CI, 0.2 to 1.0)). Of interest, the TNF5 haplotype was in stronger linkage with HLA*0701/2 in controls (D = 0.53) than in SLE (D = 0.14); this may be explained by the fact that we did not test for DR7 subtypes. Also, there was a reduction of one DR7 extended haplotype in SLE (DQB1*0303;DRB1*0701/2;TNF5;LTA3) (see above), whereas another DR7 extended haplotype (DQB1*0201;DRB1*0701/2; TNF1; LTA1) was very similar in frequency between SLE and controls.

Four LTα haplotypes were identified from the three SNPs studied, as previously described (LTA1, 720C, 365C, 249A; LTA2, 720A, 365G, 249G; LTA3, 720C, 365G, 249A; and LTA4, 720A, 365C, 249A).[21] There were no associations between SLE and Ltα haplotypes, apart from a weak reduction of the LTA3 in SLE ($p<0.05$; OR = 0.6 (0.4 to 1.0)). As noted above, LTA3 forms part of an extended DR7 haplotype which was reduced in SLE.

MHC class II associations with serological subsets of SLE

The significant associations of autoantibody defined groups of patients with SLE and HLA-DRB1 and TNFα promoter phenotypes and haplotypes are shown in table 3. There was a highly significant association between anti-La and HLA-DR3 ($p<0.001$; OR = 71 (9 to 539)). One patient with SLE and anti-La antibodies was HLA-DR3 negative (HLA-DR5,8). Her autoantibody profile consistently demonstrated the presence of anti-U1RNP and anti-Sm antibodies (six serial samples) and on four occasions anti-La antibodies were also present (verified by immunoblotting on one sample tested).

There were a few weak associations with other serological subsets of SLE that became non-significant after correction. HLA-DR3 was increased in patients with anti-Sm antibodies ($p<0.02$; OR = 3.2 (1.2 to 8.9)). There was a decrease in HLA-DR6 in patients with anti-U1RNP antibodies ($p<0.05$; OR = 0.4 (0.2 to 1.0)) which was accounted for by a decrease in the HLA-DRB1*1302 allele (2% *v* 10%; $p<0.05$). The HLA-DQB1 0604 allele was in linkage with DRB1*1302 (D = 1.0) and was also reduced in patients with anti-U1RNP ($p<0.01$; RR = 0.9 (0.86 to 0.94)).

We determined whether there were any MHC class II associations with SLE or other serological subsets that were independent of the association between anti-La and HLA-DR3. In the anti-La negative SLE patients, there was a weak association with HLA-DR3 ($p<0.05$; OR = 1.7 (1.1 to 2.8)) and similarly with HLA-DQB1*0201 ($p<0.05$; OR = 1.5 (1.0 to 2.4)). There was still a protective effect associated with the HLA-DR*0701/2;DQB1*0303 extended haplotype ($p<0.05$; OR = 0.2 (0.05 to 0.9)), and the weak negative association with HLA-DQB1*0603 remained unchanged ($p<0.05$; OR = 0.3 (0.1 to 0.9)). Anti-Sm remained associated with

HLA-DR3 ($p<0.05$; OR = 2.9 (1.0 to 8.1)) and anti-U1RNP negatively associated with HLA-DRB1*1302 ($p<0.05$; OR = 0.2 (0 to 1.2)) and HLA-DQB1*0604 ($p<0.02$; RR = 0.9 (0.86 to 0.94)).

There were 23 patients with anti-Ro antibodies who were anti-La negative. In this group of patients there was an association with the HLA-DR2 extended haplotype (HLA DQB1*0201;DRB1*1501;TNF1;LTA1) ($p<0.005$; OR = 3.9 (1.5 to 10.4)), although there was no significant association with either HLA-DRB1*1501 or DQB1*0201 when analysed separately.

TNFα and LTα associations with serological subsets of SLE

There was a strong association with anti-La positive SLE and the TNF2 haplotype ($p<0.001$; OR = 10 (95% CI, 2.8 to 36)) and carriage of the TNF-308A allele ($p<0.001$; OR = 9.4 (2.7 to 33)). The TNF2 haplotype containing the TNF-308A allele is strongly linked to HLA-DR3 (see above). Associations between anti-Ro and TNF2 were weaker (table 4) and not present in the absence of anti-La. TNF5 that is contained on one of the HLA-DR7 extended haplotypes was reduced in all serological subsets, although this only reached significance for the TNF-238A allele itself and patients with anti-U1RNP ($p<0.05$; OR = 0.1 (0 to 1.0)).

There was a significant association between anti-La positive SLE and LTA2 ($p<0.05$; OR = 4.9 (1.1 to 21)) (and similarly significant associations between anti-La and the individual alleles comprising LTA2). LTA2 is in linkage with HLA-DR3 (D = 0.74 for SLE and D = 0.69 for controls) There was a significant reduction in LTA3 in anti-Sm positive SLE (18% *v* 53%; $p<0.01$; OR = 0.2 (0.1 to 0.7)) which accounted for the previously noted reduction of LTA3 in the total SLE group.

We investigated whether there were any association between anti-La and TNFα or LTα haplotypes that were independent of those with HLA-DR3. There was no independent association of TNF2 with anti-La stratified for HLA-DR3 (Mantel–Haenszel common odds ratio = 1.4 (0.3 to 6.6)) whereas anti-La was associated with HLA-DR3 independently from TNF2 (common odds ratio = 160 (3.5 to 7340)). Similarly, there was no association of anti-La with LTA2 independent from HLA-DR3 (common odds ratios = 0.9 (0.1 to 5.3) *v* 259 (7 to 9524), respectively).

Finally, we investigated whether there were any TNFα or LTα haplotype associations with the anti-Ro (in absence of anti-La) that were independent of other associations on the HLA-DR2 extended haplotype (HLADQB1*0201;DRB1*1501; TNF1;LTA1). The strongest association on this extended haplotype was with LTA1 ($p<0.02$; OR = 3.7 (1.2 to 11)) and there were no significant associations with other individual haplotypes. Within the LTA1 haplotype, the association of anti-Ro (without anti-La) was with the LTα 365C allele ($p<0.02$; OR = 3.7 (1.2 to 11)).

DISCUSSION

The HLA region is located on the short arm of chromosome 6 (6p21.3) and is a likely site for encoding susceptibility genes in SLE. The association of the MHC class II haplotype containing HLA-DRB1*0301 and HLA-DQ*0201 alleles is well established from case–control studies, especially in white populations.[2–4] In other ethnic groups such as Afro-American,[2] African blacks,[23] Taiwanese,[24] and Korean[25] SLE populations there is a stronger association with HLA-DR2 containing haplotypes. More recent results from genome scans involving linkage analysis of lupus families have confirmed at least six susceptibility loci including 6p21.11–21 which is in close proximity to the MHC region.[1] An important association between SLE and HLA-DR3 has been confirmed in our white population, with an odds ratio of 2.5—in keeping with the level of risk reported from other similar studies. HLA-DR7 may have a protective effect which has been reported previously.[3]

The MHC associations with SLE may be more strongly linked to autoantibody generation than to the disease itself.[5] SLE is a heterogeneous condition in which certain autoantibody specificities are associated with more homogeneous patterns of disease. Also, an association between the MHC class II region and specific autoantibody generation is consistent with the concept of an antigen driven process involving T-helper cell recognition.[26 27] All but one of 20 SLE patients with anti-La antibodies in our current study were HLA-DR3 positive (odds ratio 71), suggesting that there may be a strict MHC class II requirement for processing of La peptides, or at least the presence of a HLA-DR3 containing haplotype greatly facilitates anti-La autoantibody generation. The association of HLA-DR3 and anti-La is well supported from previous studies of patients with SLE or Sjögren's syndrome,[27–30] apart from in a Japanese population where HLA-DR3 is rare.[31]

There were no other strong associations between MHC class II genes and autoantibody subgroups, although there was a modest association of anti-Ro in the absence of anti-La and an extended haplotype of HLA-DR2. An association between anti-Ro antibodies alone and HLA-DR2 has been reported previously.[32 33] Of interest, the primary association with anti-Ro (without anti-La) in SLE appeared to be with an LTα haplotype (LTA2) rather than with HLA-DR2 itself, which is somewhat contrary to findings from a large linkage analysis of SLE families.[34] In the latter study, the DRB1*1501 haplotype associated with SLE was narrowed to a 500 kb region that would exclude LTα. A role for transgene complementation at the HLA-DQ locus has been suggested for anti-Ro autoantibodies,[35–37] and we have not typed for HLA-DQA alleles in the current study, nor have we differentiated between anti-52 kDa Ro and anti-60 kDa Ro, which may reveal stronger genetic associations. However, further studies examining possible associations of anti-Ro antibody isotypes and LTα would be of interest.

We did not find the association between anti-U1RNP and HLA-DR4 which has been reported in some studies[37 38] but not others.[39] Anticardiolipin antibodies have been associated with both HLA-DR4[40] and HLA-DR7.[40 41] In an earlier cohort from our centre we have found an association between aCL and HLA-DR4 in SLE,[42] but in the current larger cohort we found no association between aCL and HLA-DR subtypes.

There is some evidence that certain alleles on the TNFα gene exert a susceptibility effect for SLE. The less common TNF-308A allele, which is in strong linkage disequilibrium with HLA-DR3 in white populations,[10] was increased in a European white population with SLE independently from HLA-DR3.[12] There were similar findings in an African-American population,[11] where it was notable that the TNF-308A gene frequency in controls was less than in other studies. In contrast, there was no evidence for an association of SLE with TNFα alleles in a study of black South Africans,[13] or Chinese.[14] Our findings are compatible with the primary association in SLE being with HLA-DR3 rather than with TNFα alleles, and especially in the anti-La subgroup of patients. Differences in studies may relate to ethnic variation or possibly to methodology. The distribution of TNFα haplotype frequency in our local control population is in keeping with other studies using similar methods,[43] and all genotyping results were in Hardy–Weinberg equilibrium. We have confidence in the accuracy of our serological groups, as stringent methods were used for defining autoantibody specificities, and multiple samples were tested from each patient.

Linkage studies have not provided evidence for a primary role of TNFα genes in SLE. In a study of white SLE families using transmission disequilibrium testing there was no evidence for excess transmission of either HLA-DR3 or for haplotypes encoding TNF-308A, but there was excess transmission of HLA*1501 (DR2).[44] In a large linkage analysis study of SLE families using a dense map of microsatellite markers across the MHC class II region, three disease haplotypes were found.[34] In two of the haplotypes (DRB1*1501/DQB1*0602 and DRB1*0801/DQB1*0402) the susceptibility region excluded the TNFα gene. Because of extensive linkage disequilibrium, an effect from the TNFα gene was not excluded in the third haplotype bearing DRB1*0301/DQB1*0201. However, the investigators concluded that the major susceptibility genes were likely to be within the MHC class II region.

Conclusions

Our findings point to a strong association between HLA-DR3 and anti-La positive SLE with no evidence for a separate contribution from the TNFα gene. Genetic studies of SLE involving the MHC class II region will benefit by stratifying for this effect. A possible association between anti-Ro antibodies and polymorphisms within the LTa gene warrants further investigation.

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REFERENCES

1 **Tsao BP**. The genetics of human systemic lupus erythematosus. *Trends Immunol* 2003;**24**:595–602.
2 **Reveille JD**, Moulds JM, Ahn C, Friedman AW, Baethge B, Roseman J, *et al*. Systemic lupus erythematosus in three ethnic groups. I. The effects of HLA Class II, C4, and CR! alleles, socioeconomic factors, and ethnicity at disease onset. *Arthritis Rheum* 1998;**41**:1161–72.
3 **Gladman DD**, Urowitz MB, Darlington GA. Disease expression and class II HLA antigens in systemic lupus erythematosus. *Lupus* 1999;**8**:466–70.
4 **Hartung K**, Baur MP, Coldewey R, Fricke M, Kalden JR, Lakomek HJ, *et al*. Major histocompatibility complex haplotypes and complement C4 alleles in systemic lupus erythematosus. Results of a multicenter study. *J Clin Invest* 1992;**90**:1346–51.
5 **Arnett FC**. Genetic studies of human lupus in families. *Int Rev Immunol* 2000;**19**:297–317.
6 **Gordon C**, Ranges GE, Greenspan JS, Wofsy D. Chronic therapy with recombinant tumor necrosis factor-alpha in autoimmune NZB/NZW F1 mice. *Clin Immunol Immunopathol* 1989;**52**:421–34.
7 **Jacob CO**, Fronek Z, Lewis GD, Koo M, Hansen JA, McDevitt HO. Heritable major histocompatibility complex class II-associated differences in production of tumor necrosis factor-α: Relevance to genetic predisposition to systemic lupus erythematosus. *Proc Natl Acad Sci USA* 1990;**87**:1233–7.
8 **Shakoor N**, Michalska M, Harris CA, Block JA. Drug-induced systemic lupus erythematosus associated with etanercept therapy. *Lancet* 2002;**359**:579–80.
9 **Studnicka-Benke A**, Steiner G, Petera P, Smolen JS. Tumour necrosis factor alpha and its soluble receptors parallel clinical disease and autoimmune activity in systemic lupus erythematosus. *Br J Rheumatol* 1996;**35**:1067–74.
10 **Wilson AG**, De Vries N, Pociot F, Di Giovine FS, van der Putte LBA, Duff GW. An allelic polymorphism within the human tumour necrosis factor α promoter region is strongly associated with HLA A1, B8 and DR3 alleles. *J Exp Med* 1993;**177**:557–60.
11 **Sullivan KE**, Wooten C, Schmeckpeper BJ, Goldman D, Petri MA. A promoter polymorphism of tumor necrosis factor a associated with systemic lupus erythematosus in African-Americans. *Arthritis Rheum* 1997;**40**:2207–11.
12 **Rood MJ**, van Krugten MV, Zanelli E, van der Linden MW, Keijsers V, Schreuder GM, *et al*. TNF-308A and HLA-DR3 alleles contribute independently to susceptibility to systemic lupus erythematosus. *Arthritis Rheum* 2000;**43**:129–34.
13 **Rudwaleit M**, Tikly M, Khamashta M, Gibson K, Klinke J, Hughes G, *et al*. Interethnic differences in the association of tumor necrosis factor promoter polymorphisms with systemic lupus erythematosus. *J Rheumatol* 1996;**23**:1725–8.
14 **Chen CJ**, Yen JH, Tsai WC, Wu CS, Chiang W, Tsai JJ, *et al*. The TNF2 allele does not contribute towards susceptibility to systemic lupus erythematosus. *Immunol Lett* 1997;**55**:1–3.
15 **Wilson AG**, Di Giovine FS, Duff GW. Genetics of tumour necrosis factor-α in autoimmune, infectious and neoplastic diseases. *J Inflamm* 1995;**45**:1–12.
16 **Tan EM**, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, *et al*. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982;**25**:1271–7.
17 **Olerup O**, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: An alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 1992;**39**:225–35.
18 **Olerup O**, Aldener A, Fogdell A. HLA-DQB1 and -DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. *Tissue Antigens* 1993;**41**:119–34.
19 **Zetterquist H**, Olerup O. Identification of the HLA-DRB1*04, -DRB1*07, and -DRB1*09 alleles by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. *Human Immunol* 1992;**34**:64–74.
20 **Olerup O**, Zetterquist H. HLA-DRB1*01 subtyping by allele-specific PCR amplification: a sensitive, specific and rapid technique. *Tissue Antigens* 1991;**37**:197–204.
21 **Fanning GC**, Bunce M, Black CM, Welsh KI. Polymerase chain reaction haplotyping using 3′mismatches in the forward and reverse primers: application to the biallelic polymorphisms of tumour necrosis factor and lymphotoxin α. *Tissue Antigens* 2000;**50**:23–31.
22 **McHugh NJ**, James I, Maddison P. Clinical significance of antibodies to a 68 kDa U1RNP polypeptide in connective tissue disease. *J Rheumatol* 1990;**17**:1320–8.
23 **Rudwaleit M**, Tikly M, Gibson K, Pile K, Wordsworth P. HLA class II antigens associated with systemic lupus erythematosus in black South Africans. *Ann Rheum Dis* 1995;**54**:678–80.
24 **Lu LY**, Ding WZ, Fici D, Deulofeut R, Cheng HH, Cheu CC, *et al*. Molecular analysis of major histocompatibility complex allelic associations with systemic lupus erythematosus in Taiwan. *Arthritis Rheum* 1997;**40**:1138–45.
25 **Lee HS**, Chung YH, Kim TG, Kim TH, Jun JB, Jung S, *et al*. Independent association of HLA-DR and FCgamma receptor polymorphisms in Korean patients with systemic lupus erythematosus. *Rheumatology (Oxford)* 2003;**42**:1501–7.
26 **McCluskey J**, Farris AD, Keech CL, Purcell AW, Rischmueller M, Kinoshita G, *et al*. Determinant spreading: lessons from animal models and human disease. *Immunol Rev* 1998;**164**:209–29.
27 **Rischmueller M**, Lester S, Chen Z, Champion G, Van Den BR, Beer R, *et al*. HLA class II phenotype controls diversification of the autoantibody response in primary Sjogren's syndrome (pSS). *Clin Exp Immunol* 1998;**111**:365–71.
28 **Skarsvag S**, Hansen KE, Moen T, Eggen BM. Distributions of HLA class II alleles in autoantibody subsets among Norwegian patients with systemic lupus erythematosus. *Scand J Immunol* 1995;**42**:564–71.
29 **Wilson AG**, Gordon C, Di Giovine FS, De Vries N, Van de Putte LB, Emery P, *et al*. A genetic association between systemic lupus erythematosus and tumor necrosis factor alpha. *Eur J Immunol* 1994;**24**:191–5.
30 **Hartung K**, Ehrfeld H, Lakomek HJ, Coldewey R, Lang B, Krapf F, *et al*. The genetic basis of Ro and La antibody formation in systemic lupus erythematosus. Results of a multicenter study. The SLE Study Group. *Rheumatol Int* 1992;**11**:243–9.
31 **Miyagawa S**, Shinohara K, Nakajima M, Kidoguchi K-I, Fujita T, Fukumoto T, *et al*. Polymorphism of HLA class II genes and autoimmune responses to Ro\SS-A - La\SS-B among Japanese subjects. *Arthritis Rheum* 1998;**41**:927–34.
32 **Arnett FA**, Hamilton RG, Reveille JD, Bias WB, Harley JB, Reichlin M. Genetic studies of Ro (SS-A) and La (SS-B) autoantibodies in families with systemic lupus erythematosus and primary Sjogren's syndrome. *Arthritis Rheum* 1989;**32**:413–19.
33 **Hamilton RG**, Harley JB, Bias WB, Roebber M, Reichlin M, Hochberg MC, *et al*. Two Ro (SS-A) autoantibody responses in systemic lupus erythematosus. Correlation of HLA-DR/DQ specificities with quantitative expression of Ro (SS-A) autoantibody. *Arthritis Rheum* 1988;**31**:496–505.
34 **Graham RR**, Ortmann WA, Langefeld CD, Jawaheer D, Selby SA, Rodine PR, *et al*. Visualizing human leukocyte antigen class II risk haplotypes in human systemic lupus erythematosus. *Am J Hum Genet* 2002;**71**:543–53.
35 **Fujisaku A**, Frank MB, Neas B, Reichlin M, Harley JB. HLA-DQ gene complementation and other histocompatibility relationships in man with the anti-Ro/SSA autoantibody response of systemic lupus erythematosus. *J Clin Invest* 1990;**86**:606–11.
36 **Reveille JD**, Macleod MJ, Whittington K, Arnett FC. Specific amino acid residues in the second hypervariable region of HLA-DQA1 and DQB1 chain genes promote the Ro (SS-A)/La (SS-B) autoantibody responses. *J Immunol* 1991;**146**:3871–6.
37 **Harley JB**, Sestak AL, Willis LG, Fu SM, Hansen JA, Reichlin M. A model for disease heterogeneity in systemic lupus erythematosus. Relationships between histocompatibility antigens, autoantibodies, and lymphopenia or renal disease. *Arthritis Rheum* 1989;**32**:826–36.
38 **Smolen JS**, Klippel JH, Penner E, Reichlin M, Steinberg AD, Chused TM, *et al*. HLA-DR antigens in systemic lupus erythematosus: association with specificity of autoantibody responses to nuclear antigens. *Ann Rheum Dis* 1987;**46**:457–62.
39 **Yao Z**, Seelig HP, Ehrfeld H, Renz M, Hartung K, Deicher H, *et al*. HLA class II genes and antibodies against recombinant U1-nRNP proteins in patients with

systemic lupus erythematosus. SLE Study Group. *Rheumatol Int* 1994;**14**:63–9.
40 **Galeazzi M**, Sebastiani GD, Tincani A, Piette JC, Allegri F, Morozzi G, *et al*. HLA class II alleles associations of anticardiolipin and anti-beta2GPI antibodies in a large series of European patients with systemic lupus erythematosus. *Lupus* 2000;**9**:47–55.
41 **Granados J**, Vargas-Alarcon G, Drenkard C, Andrade F, Melin-Aldana H, Alcocer-Varela J, *et al*. Relationship of anticardiolipin antibodies and antiphospholipid syndrome to HLA-DR7 in Mexican patients with systemic lupus erythematosus (SLE). *Lupus* 1997;**6**:57–62.
42 **McHugh NJ**, Maddison PJ. HLA-DR antigens and antibodies to cardiolipin in systemic lupus erythematosus. *Arthritis Rheum* 1989;**32**:1623–4.
43 **Grutters JC**, Sato H, Pantelidis P, Lagan AL, McGrath DS, Lammers JW, *et al*. Increased frequency of the uncommon tumor necrosis factor −857T allele in British and Dutch patients with sarcoidosis. *Am J Respir Crit Care Med* 2002;**165**:1119–24.
44 **Tsuchiya N**, Kawasaki A, Tsao BP, Komata T, Grossman JM, Tokunaga K. Analysis of the association of HLA-DRB1, TNFalpha promoter and TNFR2 (TNFRSF1B) polymorphisms with SLE using transmission disequilibrium test. *Genes Immun* 2001;**2**:317–22.