Cytokine Polymorphism in Noninfectious Uveitis

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Purpose. Noninfectious uveitis is a sight-threatening immune-mediated intraocular inflammatory disorder. The inheritance of uveitis in multiplex families and its association with known monogenic and polygenic immunologic disorders suggests that common genetic variants underlie susceptibility to uveitis as well as to other immunologic disorders. TNFα and IL10 are strong candidate genes, given the influence of these cytokines on inflammation, immune tolerance, and apoptosis.

Methods. The role of 12 polymorphisms spanning the TNFα and IL10 genomic regions was investigated in 192 uveitis patients and 92 population control subjects from four regional centers in the United Kingdom and Republic of Ireland.

Results. The results demonstrate that uveitis is associated with three haplotype-tagging SNPs (htSNPs) in the IL10 gene: htSNP2 (rs6705650), htSNP5 (rs2222202), and htSNP6 (rs3024490). IL10htSNP2AG/htSNP5TC was the most significantly associated haplotype (P = 0.00085), whereas the ITA+252AA/TNFhtSNP2GC haplotype was protective (P = 0.00051). Furthermore, subgroup analysis showed that the frequency of the TNFd4 allele was higher in patients with nonremitting ocular disease and/or those requiring higher levels of maintenance immunosuppression. Although these associations lost significance after Bonferroni correction, they infer a relationship that may be validated by a larger study.

Conclusions. Since these variants are implicated in the susceptibility and severity of several immunologic disorders, the results support the hypothesis that common genetic determinants influence shared mechanisms of autoimmunity.

Invest Ophthalmol Vis Sci. 2010;51:4133–4142 DOI:10.1167/iovs.09-4583

Uveitis is a sight-threatening intraocular inflammatory disorder with a prevalence exceeding 115 per 100,000.1 Although uveitis itself is localized to the eye, it is a common manifestation of systemic immunologic disease, and is considered part of the immunologic disease continuum.2

The hypothesis that common genetic variants underlie susceptibility to uveitis and other immunologic diseases is strongly supported by several lines of evidence: the inheritance of uveitis in multiplex families,3,4 the association of uveitis with known monogenic (Blau syndrome, neonatal-onset multisystem inflammatory disease)5,6 and polygenic immunologic disorders (Crohn’s disease, sarcoidosis)7,8 and the varying susceptibility to uveitis of different animal species and strains.9 Moreover, quantitative trait loci (QTLs) associated with a rat model of uveitis overlap with loci linked to rheumatoid arthritis (RA), multiple sclerosis (MS), and type 1 diabetes mellitus (T1D) in rats and humans.10 Although there are several different animal models of uveitis, they appear to result in common effector T-cell responses, characterized by the ability to be adoptively transferred with T cells and inhibited by treatment with cyclosporine and other broad-spectrum immunosuppressants.9 Current evidence suggests that both T H1 and T H17 effector cells can independently induce tissue damage in mouse models of uveitis.11 In this respect, the central pathogenic role of T H1 and/or T H17 cells in mediating organ-specific autoimmunity is similar to mouse models of other immunologic diseases, such as experimental autoimmune encephalitis (MS)12 and collagen-induced arthritis (RA).13 Furthermore, both T H1 cell types characteristically produce and promote the production of the proinflammatory cytokine tumor necrosis factor alpha (TNF-α).

The eye is relatively protected from the immune system by the blood–ocular barrier, the immune-inhibitory environment, and active mechanisms of tolerance involving regulatory CD4+ T cells (Tregs).14 Resident retinal cells, such as Müller glia and retinal pigment epithelium (RPE), contribute to the microenvironment through the constitutive expression of cytokines, such as TNFα and the counterregulatory interleukin (IL)-10, and there is evidence that differences between rodent strains in the constitutive or stimulated levels of expression of these cytokines by resident Müller glia, RPE, microglia, and infiltrating T cells, determines their varied susceptibility to the induction of uveitis.15–18 Furthermore, depletion of the CD4+CD25+ Treg population in naïve mice increases their susceptibility to uveitis induction.19 and defective CD4+CD25high Treg cells have been detected in a human form of uveitis.20 Hence, Treg cell function may influence susceptibility to uveitis as well as to other immunologic diseases, like MS, RA, and T1D.21–23 The influence of these cytokines on disease severity is clearly demonstrated by the effects of their neutralization: antagonism of TNF activity with anti-TNF monoclonal antibodies or TNFreceptor1-fusion protein, effectively reduces structural damage to the retina in mice and humans24,25; conversely, neutralization of endogenous IL10 exacerbates inflammation, whereas treatment with IL10 or upregulation of IL10 gene expression corresponds with resolution of disease in mice.26,27 Taken together, this evidence suggests that the consequences of
relative differences in the levels of TNFα and IL10 expression, both constitutively and in response to a pathogenic insult, might influence uveitis susceptibility and severity.

In this study, we sought to systematically investigate the role of polymorphisms in the TNF and IL10 genomic regions that might influence uveitis susceptibility and severity. We selected patients with well characterized uveitides, sharing a common T effector-cell response. Using this approach, our objective was to identify common genetic determinants of the uveitides, which are also likely to influence other immunologic disorders.

METHODS

Subjects

Subjects were recruited from four regional centers in Bristol (Bristol Eye Hospital), Aberdeen (Grampian University Hospitals), Dublin (The Royal Victoria Eye and Ear Hospital), and London (Moordfields Eye Hospital), including 27 patients from a previous study.26,29 Informed consent was obtained from all participants, after explanation of the nature and possible consequences of the study. Subjects were white Caucasians of British or Irish descent for at least two generations. Ethical approval was given by each center and the study adhered to the tenets of the Declaration of Helsinki.

All subjects (198 patients, 92 controls) were given a full ophthalmic examination and categorized into seven diagnostic groups (Table 1). In addition, all patients managed at the four regional centers had undergone routine diagnostic and pretreatment investigations, including a chest radiograph and syphilis serology. Further investigations (e.g., serology for Toxoplasma, Toxocara, Bartonella, Borrelia and Histoplasma capsulatum, for those who had visited endemic areas outside the United Kingdom and Ireland), and/or anterior chamber and vitreous tap, were performed where clinically indicated. Approximately equal numbers of subjects in each uveitic category were selected to mitigate potential stratification bias arising from any one subgroup, since different uveitides have varied prevalence in an unselected population. All patients groups were combined in statistical analyses for increased power. Mean age, age range, and male-female ratios of patients and control subjects were comparable between groups.

For each patient, the clinical course of their ocular inflammatory disease, including visual acuities, disease remissions, and all treatments for eye disease, was documented from disease onset to a census date, common to all patient groups and regional centers and dated after study recruitment was complete. Since a significant proportion of patients were recruited at or soon after their disease onset, with ongoing disease activity (Table 1), we chose to assess clinical course up until a census date that was consistent across all subjects, since both date of recruitment and duration of disease at recruitment varied between patients.

The patients were then assessed on three parameters of disease severity:

1. Ocular remission, defined according to SUN guidelines as inactive disease for at least 3 months after stopping all treatments for eye disease.51
2. Maintenance level of immunosuppression required to control disease activity. Disease control was defined as unchanged or reduced SUN anterior uveitis score53 or uveitis scoring system55 (for posterior segment/intermediate disease) for at least 3 months, with no increase in immunosuppression during this period. We chose as the maintenance level of immunosuppression the most recent combination of medications to consistently sustain disease control (as just defined), since patients often experience multiple episodes of disease control and relapse during their clinical course until an individually tailored level of immunosuppression is achieved. Patients were then grouped according to their level of immunosuppression: Patients

<table>
<thead>
<tr>
<th>Uveitis Classification</th>
<th>Age</th>
<th>Sex</th>
<th>Disease Duration at Recruitment (y)</th>
<th>% in Remission at Recruitment</th>
<th>% Controlled on Maintenance at Recruitment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
<td>Range</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Sarcoïdosis†</td>
<td>30</td>
<td>61</td>
<td>41–86</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>Behçet’s disease‡</td>
<td>31</td>
<td>42</td>
<td>21–67</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>Intermediate uveitis§</td>
<td>32</td>
<td>60</td>
<td>19–91</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>White dots with inflammation¶</td>
<td>35</td>
<td>58</td>
<td>16–90</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>White dots without inflammation¶</td>
<td>31</td>
<td>41</td>
<td>27–65</td>
<td>5</td>
<td>26</td>
</tr>
<tr>
<td>All patients with uveitis</td>
<td>198</td>
<td>50</td>
<td>16–91</td>
<td>72</td>
<td>126</td>
</tr>
<tr>
<td>Healthy controls*</td>
<td>92</td>
<td>50</td>
<td>22–89</td>
<td>27</td>
<td>65</td>
</tr>
</tbody>
</table>

* The number of patients controlled on maintenance therapy divided by the total number of patients on maintenance therapy. Diagnostic criteria for uveitis subgroups:
† Biopsy-proven non-necrotizing granulomatous, or bilateral hilar lymphadenopathy or other characteristic signs on chest X-ray with a raised angiotensin-converting enzyme (ACE) level.
‡ Behçet’s disease International Study Group major criteria.50
§ Bilateral non-necrotizing granulomatous panuveitis following penetrating trauma to one eye with histologic confirmation or clinical confirmation by an experienced ophthalmologist (n = 18 due to incomplete data on 14 SO patients).
¶ Standardization of Uveitis Nomenclature (SUN) Working Group classification.51
† Bilateral non-necrotizing granulomatous panuveitis following penetrating trauma to one eye with histologic confirmation or clinical confirmation by an experienced ophthalmologist (n = 18 due to incomplete data on 14 SO patients).
© No history of autoimmune disorder or inflammatory disorders associated with cytokine gene polymorphism (e.g. T1D, RA, ankylosing spondylitis [AS], systemic lupus erythematosus [SLE], chronic obstructive pulmonary disease, ischemic heart disease, or neoplasia) or eye-specific disorders with a significant genetic predisposition (glaucoma, age-related macular degeneration).

# Table 1. Demographic Details of Study Participants
with remitting disease who did not require maintenance therapy were ranked the lowest, whereas patients who were uncontrolled despite three immunosuppressive treatments or on regular biological therapy (e.g., anti-TNF treatment), were ranked the highest (see Table 8 for a full description of the ranks).

3. Visual outcome: assessed in two ways: first, visual acuity (VA) at the census date, and second, the change in VA from disease onset to the census date (defined as a decrease in Snellen VA of >3 lines, in accordance with SUN guidelines).

### htSNP Selection
Haplotype tagging SNPs (htSNPs) were selected, based on phased population haplotype data from SeattleSNPs (CEPH population; http://www.ncbi.nlm.nih.gov/UniSTS accession numbers were NT_021877 (RefSeq; http://www.ncbi.nlm.nih.gov/UniSTS: provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), AF418271 and AF295024 (SeattleSNPs) for IL10 and TNF genomic regions. Three additional polymorphisms were included because of their known functional influence on transcription: IL10−1082A/G, LTA+252G/A, and TNFΔ38 (Table 2). Sequence accession numbers were NT_0121877 (RefSeq: http://www.ncbi.nlm.nih.gov/RefSeq; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD), AF418271 and AF295024 (SeattleSNPs) for IL10, NT_007592 (RefSeq), and AF241167 and AF241168 (SeattleSNPs) for TNF-A.

### SNP Genotyping
DNA was extracted from peripheral blood mononuclear cells. IL10htSNP1, IL10htSNP2, and IL10htSNP7 were genotyped by restric-

### Table 2. htSNPs Selected in the IL10 and TNF Regions

<table>
<thead>
<tr>
<th>htSNP</th>
<th>Location in Gene</th>
<th>rs Number in dbSNP</th>
<th>Correlated SNPs and Their Location</th>
</tr>
</thead>
</table>
| IL10htSNP1 | −3575 Promoter | rs1800890 | N/A  
| IL10htSNP2 | −2849 Promoter | rs6703630 | N/A  
| IL10htSNP3 | −1082 Promoter | rs1800896 | N/A  
| IL10htSNP4 | −819 Introne 1 | rs1800871 | N/A  
| IL10htSNP5 | +434 Introne 1 | rs2222202 | N/A  
| IL10htSNP6 | +504 Introne 1 | rs3024949 | N/A  
| IL10htSNP7 | +1847 Introne 1 | rs3024949 | N/A  
| LTA+252 | LTA+252 (−3025 bp from TNFA start codon) | rs909253 | N/A |
| TNFahtSNP1 | −308 Promoter | rs1800629 | N/A  
| TNFahtSNP2 | −238 Promoter | rs361525 | N/A  
| TNFahtSNP3 | +488 Introne 1 | rs1800610 | N/A  
| TNFd | TNFd (GA)n (+12785 bp from TNFA start codon) | UniSTS 256848 | N/A  

### Table 3. Associations between Polymorphic Loci and Uveitis

<table>
<thead>
<tr>
<th>Locus</th>
<th>rs Number</th>
<th>Study Cohort</th>
<th>Reference Population*</th>
<th>Comparison of Patients versus Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL10htSNP1 (−3545)</td>
<td>rs1800890</td>
<td>0.48</td>
<td>0.44†</td>
<td>0.745 NS</td>
</tr>
<tr>
<td>IL10htSNP2 (−2849)</td>
<td>rs6703630</td>
<td>0.35</td>
<td>0.31</td>
<td>0.001 0.012</td>
</tr>
<tr>
<td>IL10htSNP3 (−1082)</td>
<td>rs1800896</td>
<td>0.44</td>
<td>0.49†</td>
<td>0.106 NS</td>
</tr>
<tr>
<td>IL10htSNP4 (−819)</td>
<td>rs1800871</td>
<td>0.18</td>
<td>0.23</td>
<td>0.134 NS</td>
</tr>
<tr>
<td>IL10htSNP5 (−434)</td>
<td>rs2222202</td>
<td>0.43</td>
<td>0.46</td>
<td>0.001 0.024</td>
</tr>
<tr>
<td>IL10htSNP6 (−504)</td>
<td>rs3024490</td>
<td>0.19</td>
<td>0.26</td>
<td>0.480 NS</td>
</tr>
<tr>
<td>IL10htSNP7 (+1847)</td>
<td>rs3024493</td>
<td>0.27</td>
<td>0.22</td>
<td>0.008 NS</td>
</tr>
<tr>
<td>LTA+252</td>
<td>rs909253</td>
<td>0.37</td>
<td>0.34†</td>
<td>0.069 NS</td>
</tr>
<tr>
<td>TNFahtSNP1 (−308)</td>
<td>rs1800629</td>
<td>0.21</td>
<td>0.18</td>
<td>0.012 NS</td>
</tr>
<tr>
<td>TNFahtSNP2 (−258)</td>
<td>rs361525</td>
<td>0.08</td>
<td>0.07</td>
<td>0.791 NS</td>
</tr>
<tr>
<td>TNFahtSNP3 (+488)</td>
<td>rs1800610</td>
<td>0.06</td>
<td>0.07</td>
<td>0.212 NS</td>
</tr>
</tbody>
</table>

Two-sided probabilities are calculated by χ² analysis with Bonferroni correction for the number of loci. Significant values are in bold. NS, not significant with a P > 0.05; Puncorr, two-sided uncorrected probability; Pcorr, Bonferroni corrected probability.

* Reference population is the SeattleSNPs CEPH panel, except for IL10htSNP1 to 4, where empirical data from a British control cohort were used.
† Three SNPs were not in HWE, but genotype frequencies did not differ significantly from the reference populations (data not shown).
tion fragment length polymorphism-PCR (RFLP-PCR); IL10htSNP5 and IL10htSNP6 were sequenced together using the same primers; the remaining IL10 and TNF hSNPs were genotyped using sequence specific primers (SSP-PCR) and control primers (recognition of an intronic sequence in HLA-DRB1 40; Supplementary Table S1, http://www.iovs.org/cgi/content/full/51/8/4133/DC1). Primers used for IL10htSNP3, IL10htSNP4, LTA+252, and TNFhtSNP1 genotyping are described elsewhere.41,42

PCR mixtures contained 50 ng genomic DNA, MgCl2 (Supplementary Table S1), 200 µM of each dNTP (Applied Biosystems, Warrington, UK), 1 µM of specific primers with and without control primers (Eurogentec, Romsey, UK), and 0.5 U DNA polymerase (AmpliTaq Gold; Applied Biosystems, Warrington, Cheshire, UK) in 25 µL. Cycling conditions were: 94°C for 5 minutes; 30 cycles of 94°C for 1 minute, Tm°C for 1 minute (Supplementary Table S1), 72°C for 1.5 minutes,

**FIGURE 1.** (A) Haplotype block structure and LD in the region of the LTA, TNF, and LST1 genes on chromosome 6 in the CEU population of the HapMap project.47 Inset: LD between hSNPs of TNFA and LTA in the study population according to Haploview 4.1 49 (B) Haplotype block structure and LD in the IL10 gene on chromosome 1 in the CEU population of the HapMap project.47 Inset: LD between hSNPs of IL10 in the study population using Haploview 4.1.49
and 72°C for 5 minutes on a thermal cycler (Peltier Thermal Cycler, Model 220; MJ Research Systems, Watertown, MA). PCR and restriction digest products were visualized by standard agarose gel electrophoresis.

Quality Control
For a random subset of subjects, the reliability and accuracy of SSP-PCR was checked by DNA sequencing.

TNFα Microsatellite Genotyping
The TNFα microsatellite was genotyped as previously described.15

HLA Typing
HLA class I (A, B, and C) and II (DRB1, DRQA1, and DRQB1) typing was performed by using SSP-PCR at medium resolution,14 to ensure that any associations demonstrated between specific TNF alleles and disease were not secondary to linkage to an associated HLA allele.

Statistical Analyses
Patient and control genotype distributions were analyzed by two-tailed χ² test or Fisher’s exact test (SPSS ver. 14.0; SPSS UK Ltd., Woking, UK, and Epi Info 6.04d; Centers for Disease Control and Prevention, Atlanta, GA). The Bonferroni correction was applied to adjust for number of comparisons (n = total number of loci). The distributions of ordinal phenotypic characteristics were compared by using the Kruskal-Wallis nonparametric test, and the two-tailed χ² test or Fisher’s exact test was used to compare dichotomous groups.

Modeling of haplotypes, genotypes, and allelic associations was performed with the UNPHASED application.15

Hardy-Weinberg Equilibrium
Hardy-Weinberg probabilities for the 11 bi-allelic SNPs were determined for the entire cohort: SNPs at eight loci were in Hardy-Weinberg equilibrium (HWE), whereas SNPs at three loci, IL10htSNP1 (−3545), IL10htSNP3 (−1082), and LTA +252 were not. Although there are no SeattleSNP data available for IL10htSNP1 and IL10htSNP3, genotype frequencies did not differ significantly from those of a British control cohort.13 Similarly, LTA +252 genotype frequencies did not differ significantly from either the SeattleSNP (CEPH) or HapMap (CEU) populations,15 which most closely approximate the British and Irish populations (see Table 3 for comparative minor allele frequencies).

The allele frequencies for the microsatellite marker, TNFα, did not differ significantly from published UK data16 (data not shown).

RESULTS
Prevalence of a self-reported family history of immune-mediated inflammatory disease was 22%; most commonly, RA (20%), undefined “arthritis” (17.5%), uveitis (12.5%), undefined (Type 1 or 2) diabetes mellitus (10%), sarcoidosis (7.5%), MS (7.5%), SLE (5%), Graves’ disease (5%), Behcét’s disease (5%), psoriasis (2.5%), inflammatory bowel disease (2.5%), celiac disease (2.5%), and AS (2.5%). Excluding histories of undefined arthritis or diabetes, the prevalence of a family history of autoimmunity was 16%.

Associations between IL10 and TNF Polymorphisms and Uveitis
Linkage disequilibrium (LD) between the 11 bi-allelic SNPs analyzed in this study is compared with the haplotype block structure of TNF and IL10 in the HapMap CEU population15 in Figure 1.

SNPs at three loci were significantly associated with uveitis: IL10bSNP2, IL10bSNP5, and bSNP6 (Table 3). Further analysis revealed that the alleles IL10bSNP2A, IL10bSNP5T, and bSNP6G were responsible for the association (Table 4). Stratification analyses were performed with UNPHASED, to determine whether a single IL10 locus or combination of IL10 loci contributes most significantly to the association with uveitis. In these analyses, an association with IL10bSNP2 remained significant throughout, in combination with either an association with IL10bSNP5 (P = 0.0072) or with IL10bSNP6 (P = 0.0072), whereas the associations with bSNP5 and bSNP6 (P = 0.0227) or all three SNPs together (P = 0.0081) were less significant.

Further analyses of genotype combinations at these three loci showed that the combination of genotypes, IL10bSNP2AG and IL10bSNP5TC, was the most significantly associated with uveitis (P = 0.00085, odds ratio [OR], 8.13; 95% confidence interval [CI], 2.29–28.82). Two haplotypes with the IL10bSNP2AG/bSNP5TC combination were significantly associated with uveitis in three loci analyses, whereas the IL10bSNP2GG/bSNP5CC/bSNP6GG haplotype was protective, suggesting that IL10bSNP2A and IL10bSNP5T are the risk-conferring alleles (Table 5).

Using UNPHASED to model allelic haplotypes, eight IL10 haplotypes (from seven loci) were predicted with certainty, but none were associated with uveitis.

Although there were no significant associations between the TNF loci and uveitis after Bonferroni correction, we observed phenotypic associations with three alleles: LTA +252G, TNFbSNP1A and TNFbSNP2A (Table 6). These results were suggestive of an underlying relationship with uveitis, involving these three loci, that our study was inadequately powered to detect. Stratification analyses using UNPHASED determined that LTA +252 and TNFbSNP2 genotypes in combination were significantly associated with uveitis (P = 0.0004); whereas associations with LTA +252 and TNFbSNP1 (P = 0.0147) or TNFbSNP1 and TNFbSNP2 (P = 0.0264) or all three SNPs (P = 0.0099) were less significant. The combination of LTA +252A4 and TNFbSNP2G4 genotypes was significantly protective with P = 0.0031.

Ten allelic TNF haplotypes (from five loci) were predicted with certainty using UNPHASED, but none were associated with uveitis. Further stratification analyses to determine whether an HLA class I or II allele was associated with uveitis. Further stratification analyses to determine whether an HLA class I or II allele was associated with uveitis.
http://www.iovs.org/cgi/content/full/51/8/4133/DC1). Nevertheless, we did observe a higher frequency of HLA-B*51 (12.9% patients vs. 3.9% control subjects, \( P_{uncorr} = 0.023; \) OR, 5.4) in patients with Behçet’s disease compared with controls,\(^{50}\) and an association between HLA-DRB1*04 with sympathetic ophthalmia that we have described previously.\(^{29}\)

**Associations between IL10 and TNF Polymorphisms with Severity of Uveitis**

We performed subgroup analyses to determine whether there were additional associations between the 12 loci and three parameters of uveitis severity. We observed a relationship between uveitis severity and the TNF\(_d\) locus that lost significance after Bonferroni correction. TNF\(_d\) genotypes were linked to both the incidence of ocular remission and the maintenance level of immunosuppression required to control ocular disease activity. Analyses using both parameters showed a consistent association between TNF\(_d\)I+ patients and more severe disease, while TNF\(_d\)I− patients were more likely to have remitting disease or require lower levels of immunosuppression (Tables 7, 8).

There were no significant associations with visual outcome by either measure after Bonferroni correction (data not shown).

**DISCUSSION**

The hypothesis that common genetic determinants underlie susceptibility to uveitis and other immunologic diseases is suggested by its inheritance in multiplex families.\(^{3,4}\) In this study, at least 16% of uveitis patients reported a family history of AI or inflammatory disease, a significantly high number compared with estimates of the U.S. population prevalence of autoimmunity of 5% to 8% (based on >80 recognized AI disorders).\(^{31}\) Experimental models and clinical studies suggest that similar deviations in immune-mediated effector pathways and mechanisms of tolerance increase our overall susceptibility to autoimmunity. This effect may involve (1) central mechanisms of tolerance: the clonal deletion of autoreactive T cells and the generation of naturally occurring Ag-specific CD4+CD25+ Tregs, regulated by thymic expression of tissue-specific antigens and autoimmune transcriptional regulator (AIRE); (2) peripheral mechanisms of tolerance: the induction of Ag-specific Tregs from naïve CD4+CD25+ T cells and T effector cell anergy, critically mediated by IL10; and (3) the ability to mount a T\(_{H1}\) and/or T\(_{H17}\) effector cell response, orchestrated largely through TNFα. The constitutive and inducible levels of expression of key cytokines, such as TNFαs and IL10, influence the maturational pathways of specific cell types coordinating the immune response; for example, IL10 promotes the induction of IL10-producing dendritic cells, Tregs, and macrophages which promote tolerance and the resolution of inflammation, whereas TNFα is clearly important during the effector stage of disease through its effects on T effector cell function, proliferation and macrophage activation, when antigen specificity becomes less relevant and inflammation is mediated more by cytokines and bystander recruitment.\(^{52}\) In this study, we have demonstrated significant relationships between uveitis and 3 IL10 loci: bsSNP2 (−2849), bsSNP5, and bsSNP6. Moreover, we have identified phenotypic associations between uveitis and LTA+252 and TNFhtSNP2 (−238), and a relationship between the TNF\(_d\) microsatellite polymorphism and two parameters of uveitis severity: nonremitting ocular disease and maintenance level of immunosuppression. We can speculate that IL10 polymorphisms, associated with an increased susceptibility to uveitis, perturb the mechanisms that normally uphold peripheral tolerance to retinal antigens, whereas genetic variants of TNFα may be more likely to affect uveitis severity through their influence on the effector stage of disease.

The transcriptional regulation of gene expression is now known to depend on conserved noncoding sequences (CNS) far upstream of conventionally annotated promoter regions, within introns and even between genes. These regions are known to harbor regulatory elements, such as enhancers, locus control regions, silencers, insulators, and matrix attachment regions. Nucleosome-free transcriptionally active regions of a gene, identified by hypersensitivity to DNaseI endonuclease activity (hypersensitivity sites or HS), are often found near CNS. Consequently, genetic variants may be associated with a functional effect on transcription either because (1) they are, themselves, positioned within CNS containing regulatory elements and have a direct influence on transcription or (2) they are in LD with other variants that are influencing transcription;

### Table 5. Significant Associations Shown by UNPHASED between IL10bsSNP2, bsSNP5, and bsSNP6

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Patients</th>
<th>Control Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype/Haplotype</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>AG-TC-TG</td>
<td>21</td>
<td>11.1</td>
</tr>
<tr>
<td>AG-TC-GG</td>
<td>37</td>
<td>19.5</td>
</tr>
<tr>
<td>GG-GG-GG</td>
<td>5</td>
<td>2.6</td>
</tr>
</tbody>
</table>

\(n\), number of subjects; %, percentage of subjects; \(\chi^2\), two-sided probability.

### Table 6. Association Between TNF Alleles and Uveitis

<table>
<thead>
<tr>
<th>Allele</th>
<th>SNP rs Number</th>
<th>Patients n</th>
<th>%</th>
<th>Controls n</th>
<th>%</th>
<th>P</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTA+252G</td>
<td>rs909253</td>
<td>137</td>
<td>69.2</td>
<td>47</td>
<td>51.1</td>
<td>0.003</td>
<td>2.2 (1.3-3.6)</td>
</tr>
<tr>
<td>TNFhtSNP1A (−308)</td>
<td>rs1800629</td>
<td>84</td>
<td>42.4</td>
<td>26</td>
<td>28.3</td>
<td>0.021</td>
<td>1.9 (1.1-3.2)</td>
</tr>
<tr>
<td>TNFhtSNP2A (−238)</td>
<td>rs361525</td>
<td>36</td>
<td>18.2</td>
<td>7</td>
<td>8.2</td>
<td>0.018</td>
<td>2.7 (1.2-6.2)</td>
</tr>
</tbody>
</table>

\(n\), number of subjects; %, percentage of subjects; \(P\), two-sided probability.
Table 7. Association Between TNFd Phenotypes and Ocular Remission in Uveitis by $\chi^2$ Analysis

<table>
<thead>
<tr>
<th>TNFd Phenotype</th>
<th>Nonremitting Disease</th>
<th>Remitting Disease</th>
<th>$P_{\text{uncorr}}$</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFd1+</td>
<td>15</td>
<td>32</td>
<td>0.024*</td>
<td>0.4 (0.2–0.9)</td>
</tr>
<tr>
<td>TNFd4+</td>
<td>48</td>
<td>37</td>
<td>0.005*</td>
<td>2.4 (1.3–4.5)</td>
</tr>
</tbody>
</table>

$n$, number of subjects; %, percentage of subjects; $P_{\text{uncorr}}$, two-sided uncorrected probability.

* Not significant after Bonferroni correction.

IL10 is predominantly regulated by transcription, and genetic factors account for 75% of interindividual differences in IL10 production. However, studies that have linked IL10 transcription to SNPs within 1.4 kb of the transcription start site (TSS) report various results. Some variation may be due to differences in experimental conditions and protocol, although more recent evidence suggests that the IL10 promoter extends at least 4 kb of the TSS of the gene. Hence, the variable association of proximal promoter haplotypes, GCC, ACC, and ATA ($−1082, −819, −592$) with more distal functionally important SNPs (and others that remain uncharacterized) may account for these conflicting results. Several studies have found associations between the more distal IL10.R microsatellite and IL10 production. IL10–2849A associates with IL10.R3, linked with low IL10 secretion, while IL10.R2 is linked to high IL10 secretion. Whether these loci or another variant linked to them (inherited in the same haplotype) directly influence gene transcription is currently unknown. Nevertheless, carriers of the IL10–2849A genotype produced significantly less IL10 in response to endotoxin in independent studies. In our study, IL10bSNP2A (−2849A) was associated with uveitis with an OR of 2.2 (95% CI: 1.4–3.7). Hence, individuals who are genetically predisposed to be low IL10 producers, an anti-inflammatory cytokine that promotes tolerance, may be expected to have an increased prevalence of inflammatory disease.

We have also found that IL10bSNP5T and bSNP6G are associated with uveitis with ORs of 2.0 (95% CI: 1.1–3.7) and 3.9 (95% CI: 1.4–10.7), respectively. Most studies have neglected potential functional variants in intronic or 3′UTR noncoding sequences. Hence, the importance of IL10bSNP5 and bSNP6 and the SNPs linked to them has been underinvestigated. Both $T_{\text{IL1}}$ and $T_{\text{IL2}}$ cells display a strong constitutive and inducible H5 site in the 3′ UTR, and $T_{\text{IL1}}$ cells develop H5 sites in introns 3 and 4 on stimulation. The IL10bSNP5 and bSNP6 loci, located in intron 1, are in LD with several other intronic SNPs further 3′ in the gene (Table 2, Fig. 1); in particular, IL10bSNP5 is linked to SNPs in intron 3 (rs1878672) and the 3′ UTR (rs3024496). Although direct evidence is lacking, it remains to be seen whether IL10bSNP5 is the functionally relevant SNP modulating ocular inflammation, or a SNP linked to it. Nevertheless, these data may direct future research to investigate the functional influence of IL10bSNP5 and other noncoding variants in cell types specifically implicated in the pathogenesis of ocular inflammation.

The major histocompatibility complex (MHC) on chromosome 6, differs from the IL10 genomic region, with some examples of long range LD. Although the blocklike microstructure of this region is similar (Fig. 1), longer range LD arises in a subset of MHC haplotypes because of linkage between segments of strong LD. The TNFA and LTA genes are tandemly arranged in the MHC class III region—only 1.2 kb separates the polyadenylation site of LTA and the TSS of TNFA. Strong LD has been demonstrated between TNF coding and promoter SNPs, as far upstream as the LTA+252 SNP, and 8 kb downstream to the TNFd microsatellite locus, in intron 4 of LSTA. Both loci are linked to extended haplotypes across the human MHC region and to the transcriptional activity of TNFA. Some variability in the results of these studies may be attributable again to differences in experimental method and the chance representation of different haplotypes in this region. Neverthless, the TNFA−3084 polymorphism associates with the LTA+252G polymorphism in several combined HLA-TNF-LT haplotypes, and these haplotypes have been linked to TNF production levels and numerous immunopathologic diseases by several independent studies. Furthermore, the TNFd allele is linked to high TNFα production by leukocytes in vitro, and the TNFd allele shows strong LD with TNF−238A. This may explain why, in this study, carriers of LTA+252G, TNFbtSNP1A (−308A) and TNFbtSNP2A (−238A),
associated with increased TNFa production, have a greater chance of developing uveitis, and TNFα carriers were more frequently patients with nonremitting ocular inflammation or those requiring higher levels of immunosuppression. We did not demonstrate any associations between TNF polymorphisms and visual outcome. While a census date was chosen to make a final assessment of clinical course that was consistent across patient groups, the duration of disease at this date did vary between patients. Furthermore, both measures were likely confounded by ongoing disease activity and/or the incidence of cataract and other complications. Further analysis of visual outcome in uveitis would be desirable, correlated with the development of complications and disease duration. Such an analysis would necessitate a larger prospective study with appropriate statistical power.

Although few studies have directly examined the effects of variants in these genes on transcription factor binding and subsequent TNFA expression, HS sites have been identified in the highly conserved proximal TNFA and LTA promoters in several cell lines, including a constitutively active site in human monocyte and T-cell lines.6–68 Furthermore, specific enhancer complexes have been identified in these regions that depend on transcription factor, cell type, and stimulus.99,70 Other regulatory elements and HS have been reported further upstream of the LTA and TNFA promoters,1,7,72 within TNFA intron 3,73 the TNFA 3′-UTR,74 intergenic regions of the TNF locus,69 and a CpG island in exon 4 of the LTB gene. The latter was detected across a range of cell types and demonstrated histone modifications associated with active transcription.75 Hence, the TNFA—508 and −238 loci coincide with highly CNS close to the TSS of the TNFA gene and a constitutive HS spot in T cells, which could account for their influence on transcription. Associations between the LTA+252 and TNFα polymorphisms with disease may again arise due to their influence on transcription in other cell types that have not yet been investigated, or because they are linked to other functional polymorphisms—for example, variants in the highly conserved LTA promoter or CpG island of LTB, that are likely to have regulatory function.

We did not identify a common HLA haplotype or allele that was associated with uveitis susceptibility or severity overall. Our hypothesis would be that tissue-specific factors, influenced by cognate (self-)antigen presentation to MHC-restricted T cells, govern the sites of inflammation in immunologic diseases. Although few studies have directly examined the effects of these genes on transcription factor binding and subsequent TNFa expression, HS sites have been identified in the highly conserved proximal TNFa and LTA promoters in several cell lines, including a constitutively active site in human monocyte and T-cell lines.6–68 Furthermore, specific enhancer complexes have been identified in these regions that depend on transcription factor, cell type, and stimulus.99,70 Other regulatory elements and HS have been reported further upstream of the LTA and TNFA promoters,1,7,72 within TNFA intron 3,73 the TNFA 3′-UTR,74 intergenic regions of the TNF locus,69 and a CpG island in exon 4 of the LTB gene. The latter was detected across a range of cell types and demonstrated histone modifications associated with active transcription.75 Hence, the TNFA−508 and −238 loci coincide with highly CNS close to the TSS of the TNFA gene and a constitutive HS spot in T cells, which could account for their influence on transcription. Associations between the LTA+252 and TNFα polymorphisms with disease may again arise due to their influence on transcription in other cell types that have not yet been investigated, or because they are linked to other functional polymorphisms—for example, variants in the highly conserved LTA promoter or CpG island of LTB, that are likely to have regulatory function.

We did not identify a common HLA haplotype or allele that was associated with uveitis susceptibility or severity overall. Our hypothesis would be that tissue-specific factors, influenced by cognate (self-)antigen presentation to MHC-restricted T cells, govern the sites of inflammation in immunologic disease, whereas factors affecting the shared mechanisms described above will affect the risk and severity of autoimmunity as a whole. We have found that polymorphisms, IL10−2849, LTA+252, TNF−238 and TNFα were linked to uveitis, loci that have been implicated in many other immune-mediated inflammatory disorders.55 Furthermore, these loci and the two additional SNPs associated with uveitis in this study, IL10bSNP5 and h6SNP6, are either located within CNS with functional relevance, or they are linked to regions identified by chromatin assays of inflammatory cell types that are. Hence, the results of transcriptional assays should be interpreted contextually, taking into account cell-type and stimulus and the relationship between loci in the same haplotype or chromosome. Taken together, our results lend further weight to the hypothesis that common genetic determinants underlie the risk of autoimmunity.

Acknowledgments

The authors thank the Immunology and Immunogenetics Department at Southmead Hospital, North Bristol Health Care Trust for HLA typing and all patients and control subjects who participated.

References

Cytokine Polymorphism in Noninfectious Uveitis


