Background: The cytokines tumour necrosis factor (TNFα) and interleukin (IL)10 have been implicated in the pathogenesis of Crohn’s disease (CD), with increased concentrations reported in patients with active disease. However, limited data exist on their effects on disease phenotype in the same population. Certain single nucleotide polymorphisms (SNPs) within the promoter region of the IL10 (-1082G/A, -592C/A) and TNFα (-308G/A, -857C/T) genes have been associated with altered levels of circulating IL10 and TNFα.

Methods: We conducted an Australian based case-control study (304 CD patients; 231 healthy controls) of these four SNPs. Further investigation of two SNPs was conducted using a logistic regression analysis.

Results: We identified a possible association of both IL10 SNPs and TNFα-857 with CD. Further investigation of a relationship with disease severity showed a significant association of higher producing IL10-1082G and TNFα-857C alleles with stricturing behaviour, which was strongest when these alleles were combined and persisted after multivariate analysis (p = 0.007; odds ratio (OR) 2.37, 95% CI 1.26 to 4.43). In addition, the TNFα-857CC genotype was independently associated with familial CD (p = 0.03; OR 3.12; 95% CI 1.15 to 8.46).

Conclusion: These two SNPs may help to predict disease behaviour in CD patients, which may be clinically useful in shaping treatment of the disease at an earlier stage.

C rohn’s disease (CD) is a complex, chronic inflammatory disorder that can affect any part of the gastrointestinal tract. The aetiology of the disease has not been fully elucidated but is based on the concept of a genetically susceptible host encountering a single or a series of environmental “hits” to manifest the disease phenotype.1 At least one major CD susceptibility gene, CARD15, has recently been identified, and directly implicates the innate immune system in the pathogenesis of this disease.2 Other potential CD susceptibility genes implicating the intestinal epithelium, and located on chromosomes 5 and 10, remain to be confirmed in independent datasets.3,4

A number of cytokines have been invoked in the chronic inflammatory lesions of CD patients, in particular tumour necrosis factor (TNFα) and interleukin (IL)10. Evidence for a central role for TNFα in CD comes from both human work and animal models, including measurement of TNFα in stool and intestinal tissue,3,4 and the development of the TNFαARE and SAMP1/Yit murine models of CD, which overexpress TNFα and develop a chronic, granulomatous ileitis similar to human CD.3 The role of IL10 in CD pathogenesis was initially suggested by development of enterocolitis in IL10 knockout mice, and because of its potentially important role as an immunoregulatory cytokine.5,6 Cytokine (IL10) and anticytokine (TNFα) strategies have also been exploited in CD treatment, with the latter proving successful in the form of anti-TNFα monoclonal antibodies.7 However, IL10 has not demonstrated significant efficacy over placebo in large, randomised studies in CD, and importantly, the inflammatory lesion seen in the IL10 knockout is predominantly colitis, with no ileal involvement seen in longitudinal studies.8,9

In view of their central role in initiation and control of the inflammatory response, and in other pathways including apoptosis, TNFα and IL10 are attractive candidate susceptibility and/or modifier genes for CD. Hence, TNFα and IL10 may be regulated at the transcriptional level, as several single nucleotide polymorphisms (SNPs) in the promoter regions of both genes have been associated with changes in expression levels.10–12 Promoter region SNPs in IL10 (-1082G allele) and TNFα (-308A allele, -857C allele) genes have been associated with significantly higher levels of circulating IL10 and TNFα, respectively.13–16 A second IL10 promoter SNP (-592) has also been implicated in CD; however, its functional relevance remains unclear.17 Several studies have shown significant association between these SNPs and patients with CD,15 16 18 19 but others have been unable to confirm this.20–22

No study to date has analysed both TNFα and IL10 polymorphisms in the same population, despite the polygenic nature of CD and the potentially important interaction between these cytokines within the mucosa. Although these cytokines have previously been regarded as pro-inflammatory (TNFα) and immunoregulatory (IL10), this concept may now be too simplistic.23 In fact, both TNFα and IL10 activate CD8+ T cells, which have been strongly implicated in the pathogenesis of the CD phenotype displayed by the TNFαARE mouse.24 Another potential immunostimulatory role for IL10 has been observed during some of its pivotal trials for CD. Subcutaneous administration of this agent at the higher dose of 20 μg/kg was associated with significant increases in PHA induced γ-interferon production compared with placebo, and an increase in TNFα levels that did not reach significance.22

The purpose of this study was threefold: to look for association between these four promoter polymorphisms of the TNFα and IL10 genes in a well characterised Australian CD patient cohort (table 1) compared with a carefully matched control population; to further investigate those SNPs that proportioned differently in the CD population in a detailed phenotype–genotype analysis; and finally, to determine whether the TNFα and IL10 genes act synergistically in determining CD phenotype.

PATIENTS AND METHODS

Subjects

The 304 CD patients included in this study are a consecutive series of cases prospectively recruited into a longitudinal IBD research protocol from 1994. All patients were recorded on our IBD database together with relevant phenotypic information including disease distribution, behaviour, and duration, surgery, smoking, and histological data such as granulomas.
Clinical records upon which these assessments were made were available for all patients in the study, from diagnosis to current. Presence of ileal disease was based upon direct ileoscopy and histology and/or small bowel contrast examination. Patients joined the programme by giving their informed and written consent for entry of their clinical history on to a dedicated IBD database, and provision of a blood sample for studying the genetics of IBD. In each case CD diagnosis was confirmed using established clinical, radiological, endoscopic, and histological guidelines.25 26 Disease behaviour was based upon the Vienna classification, 46% of patients (n = 123) had ever/never had a stricture (as defined by the Vienna classification) and defined as either narrowing of the intestinal lumen with proximal dilatation on contrast examination and/or clear evidence of small bowel obstruction on any radiological examination.

Analysis of combined IL10-1082 and TNFα-857 genotypes with disease phenotype was conducted by creating a new two level variable where we compared the difference between having more than two associated mutations and two or less. This was achieved by grouping patients carrying at least three associated alleles (that is, IL10-1082G/TNFα-857C alleles associated with higher circulating levels of each cytokine: GG/CC, GG/CT, and GA/CC) and comparing with patients carrying two or less associated alleles (that is, the remaining genotype combinations) as above. This analysis was repeated with stratification of patients for CARD15 genotype (R702W, G908R, Leu1007insC). Statistical analysis

Genotype and allele frequencies for CD cases and healthy controls for the four SNPs were determined. Significant differences between cases and controls were calculated using a χ² test for all SNPs except TNFα-857, where a Fisher’s exact test was used because the number of observed TT genotypes was <5 in the control group. Maximum likelihood estimates of haplotype frequencies were calculated using haplo.stats and regression analysis packages (brlr, haplo.stats) in the R statistical program.28 Genotype–phenotype analysis was only performed for SNPs where p was <0.2 in case–control analysis.

Association of SNP genotypes to risk factors for CD severity was analysed using a univariate logistic regression model to examine the significance of any association between CD phenotype and a given SNP. Analyses comparing means and proportions in each category used a χ² test (or Fisher’s exact test if required). Stratification of CD patients for presence/absence of any CARD15 variant (R702W, G908R, Leu1007insC) was conducted and the univariate analysis repeated. Logistic regression analysis was also conducted to assess the significance of associations, after adjusting for relevant biological variables (age at diagnosis, disease duration, family type, disease site, disease behaviour, and presence/absence of granulomas) that attained a p value <0.2 at the univariate level. Previous studies indicate that these “primary or biological” variables are potentially significant in determining disease evolution and expression,29 and therefore should be considered in a multivariate analysis. Clinical variables including use of immunosuppression and need for surgery represent consequences of disease expression.

Results

Clinical characteristics of patient population

The major clinical characteristics of this CD population are given in table 1. In addition to the disease behaviour based upon the Vienna classification, 46% of patients (n = 123) were reclassified as having an intestinal stricture independent of penetrating disease, and defined as either narrowing of the intestinal lumen with proximal dilatation on contrast examination and/or clear evidence of small bowel obstruction on any radiological examination.

Genotype and allele frequencies of IL10 and TNFα SNPs

Genotype analysis showed a significant difference in frequencies for the IL10-1082 polymorphism between cases and controls (p = 0.03; table 2), but not for the IL10-592 variant (p = 0.28). While the TNFα-308 polymorphism showed no association with disease (p = 0.72), there was evidence of an
association with TNFα-857 according to Fisher’s test (p = 0.14), due to the absence of the TT genotype in the control population (table 2). Allele frequencies for the two TNFα SNPs were similar for both cases and controls, while CD patients had slightly higher frequencies of IL10-592A (p = 0.12) and IL10-1082G (p = 0.12) alleles compared with controls (data not shown).

**Haplotype analysis**
Haplotype analysis of the two IL10 SNPs showed that all four haplotypes were present in the Australian population. This represents the first report of the GA haplotype in any IBD population. This haplotype was observed in very low frequency in both cases and controls (table 3), while the remaining three haplotypes (GC, AC, and AA) were observed at similar frequencies to previous reports. Comparison of case–control haplotype frequencies showed a trend towards a significant difference ($\chi^2 = 6.53; p = 0.09$). Haplotype analysis of the two TNFα SNPs showed that all four haplotypes were present in the Australian population in similar frequencies as previously reported. There were no significant differences between cases and controls (data not shown).

**Genotype–phenotype associations**
To further test associations between IL10-1082, TNFα-857, and CD, we conducted univariate and multivariate analyses to determine whether these two SNPs contribute to a specific CD phenotype. Variables were included in the multivariate analysis if p was ≤ 0.2 at the univariate level. Gene–gene interaction with CARD15 variants was also investigated.

**IL10-1082**
Univariate analysis for IL10-1082 and clinical variables was conducted comparing all three genotypes (table 4). Patients with the GG genotype were more likely to have complex disease behaviour (stricturing and/or penetrating, 77%) compared with those carrying the AA genotype (57%, OR 2.57, 95% CI 1.10 to 6.03). Patients with a GG genotype were more likely to have undergone any CD surgery correcting for familial CD, duration of disease, disease site and phenotype showed that patients carrying the G allele had higher odds of their disease exhibiting either stricturing (ORGA 2.90, 95% CI 1.36 to 6.19; ORGG 2.10, 95% CI 0.65 to 3.31; ORGG 2.90, 95% CI 1.15 to 7.37) behaviour, although this did not reach significance (p = 0.10). Similar analysis of the ever/never stricturing disease variable maintained the association with IL10-1082G (p = 0.02), where patients carrying the G allele had higher odds of ever having stricturing disease (ORGA 1.64, 95% CI 0.57 to 4.74; ORGG 1.59, 95% CI 0.44 to 5.81), although this was not significant (p = 0.64).

**TNFα 857**
Owing to the small proportion of TNFα-857TT genotypes observed in our cohort (n = 5) both univariate and multivariate analyses of TNFα-857 genotype–phenotype associations was carried out using combined data (combined proportions of CT+TT compared with CC). Univariate analysis

### Table 2
Genotype frequencies for CD cases and healthy controls for the two TNFα (-308 and -857) and two IL10 (-592 and -1082) SNPs

<table>
<thead>
<tr>
<th>Genotype frequencies</th>
<th>CD</th>
<th>Controls</th>
<th>OR</th>
<th>95% CI</th>
<th>$\chi^2$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL10-592</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>0.60</td>
<td>0.67</td>
<td></td>
<td></td>
<td>2.52</td>
<td>0.28</td>
</tr>
<tr>
<td>CA</td>
<td>0.36</td>
<td>0.30</td>
<td>1.33</td>
<td>0.90 to 1.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0.04</td>
<td>0.03</td>
<td>1.56</td>
<td>0.56 to 4.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL10-1082</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>0.27</td>
<td>0.27</td>
<td></td>
<td></td>
<td>6.91</td>
<td>0.03</td>
</tr>
<tr>
<td>GA</td>
<td>0.51</td>
<td>0.40</td>
<td>1.25</td>
<td>0.78 to 1.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0.22</td>
<td>0.33</td>
<td>0.67</td>
<td>0.42 to 1.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα-308</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>0.65</td>
<td>0.69</td>
<td></td>
<td></td>
<td>0.67</td>
<td>0.72</td>
</tr>
<tr>
<td>GA</td>
<td>0.30</td>
<td>0.28</td>
<td>1.14</td>
<td>0.82 to 1.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0.05</td>
<td>0.03</td>
<td>1.85</td>
<td>0.80 to 4.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα-857</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>0.84</td>
<td>0.86</td>
<td></td>
<td></td>
<td>3.99</td>
<td>0.14</td>
</tr>
<tr>
<td>GA</td>
<td>0.14</td>
<td>0.14</td>
<td>0.98</td>
<td>0.59 to 1.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>0.02</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Fisher’s exact test. †Genotype-phenotype analysis was only performed for SNPs where p < 0.2 in case-control analysis.

### Table 3
Estimated haplotype frequencies of the combined IL10-592C/A and IL10-1082G/C promoter polymorphisms at position

<table>
<thead>
<tr>
<th>IL10</th>
<th>Frequency</th>
<th>CD [n = 232]</th>
<th>Controls [n = 182]</th>
</tr>
</thead>
<tbody>
<tr>
<td>-592</td>
<td>-1082</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>G</td>
<td>0.490</td>
<td>0.439</td>
</tr>
<tr>
<td>C</td>
<td>A</td>
<td>0.292</td>
<td>0.374</td>
</tr>
<tr>
<td>A</td>
<td>G</td>
<td>0.036</td>
<td>0.031</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>0.182</td>
<td>0.156</td>
</tr>
</tbody>
</table>

$\chi^2 = 6.37; p = 0.09$.
(Table 5) highlighted significant associations between TNF-857CC and stricture/penetrating disease behaviour (69%), compared with the CT/TT genotypes (50%, OR 2.24, 95% CI 1.02 to 5.96, p = 0.05), and with familial CD (CC = 28% v CT/TT = 14%, OR 2.4, 95% CI 0.97 to 5.96, p = 0.05).

These associations were again found in patients with no CARD15 mutation (disease behaviour, OR 2.94, 95% CI 1.23 to 8.46). A test for the presence of effect modification was also conducted; however, this was not significant.

**Combined IL10/TNFα genotypes and disease phenotype**

To examine whether IL10-1082 and TNFα-857 genotypes might work synergistically to influence CD behaviour, we created a new two level variable to compare the difference between having more than two associated mutations and having two or fewer. Patients carrying at least three associated alleles (that is, IL10-1082/TNFα-857 alleles associated with higher circulating levels of each cytokine; GG/CC, GG/CT, and GA/CC) were compared with patients carrying two or fewer associated alleles (the remaining genotype combinations). This analysis showed an even stronger positive association between IL10-1082G and TNFα-857C with strictureting behaviour, which persisted after multivariate analysis (p = 0.007; OR 2.37; 95% CI 1.26 to 4.43). There was also a moderate positive association with familial CD (p = 0.04; OR 2.14; 95% CI 1.04 to 4.42). These findings were unchanged after stratification for CARD15. A test for the presence of effect modification was also conducted; however, this was not significant.

**DISCUSSION**

IL10 and TNFα are regarded as key players in development and control of the inflammatory response, and hence are thought to play an important role in chronic inflammatory and autoimmune disorders, including IBD. In this study, we investigated the role of four potentially functional IL10 and TNFα SNPs in a large, well characterised CD cohort that had previously been genotyped for the major CARD15 SNPs (R702W, G908R, Leu1007InsC).

There was significant evidence that IL10-1082 genotype distributions differed between cases and controls, and there was weak evidence of an association with TNFα-857, whereas IL10-592 and TNFα-308 showed no such association. Haplotype analysis of the two IL10 SNPs did not strengthen
the observed genotype association with IL10–1082. Multiple previous studies have investigated TNFα SNPs in CD, with both positive and negative results.30-32 Many of these have included small cohorts (35–124 patients) or have been highly selected, including those with fistulising disease alone, or patients treated with infliximab for refractory disease.18-20 22 Two larger studies using association and linkage approaches have shown positive association with the TNFα-857 SNP and positive linkage with the TNF-HLA region on chromosome 6p.19-20 SNPs within the IL10 gene have received less attention despite it being an attractive candidate, as it has a known role in generation of regulatory T cells and the phenotype of the IL10 knockout model.9 13 Studies thus far have been limited to a negative linkage study,30 which used 89 CD affected sibling data have suggested an immunostimulatory role for IL10.33 In the diseased ileum, IL10 does not downregulate proinflammatory cytokines and in patients treated with infliximab for refractory disease.18 20 22 The other major finding for the TNFα-857 polymorphism is the significant association between the CC genotype and familial CD; 43% of patients carrying the CC genotype had a positive family history of IBD compared with only 14% of those carrying other genotypes (CT/TT). This result fits well with data from previous studies, which used multiplex IBD families exclusively, or a combination of multiplex families and sporadic cases to show association between TNFα-857C and CD.14 19

Combining the IL10-1082GG and TNFα-857CC genotypes demonstrated a significant synergistic effect, where patients carrying at least three of four alleles associated with increased cytokine production (G and C) showed a stronger association with strictureing disease compared with either polymorphism alone (p = 0.007; OR 2.37; 95% CI 1.26 to 4.43), which was unaffected by CARD15 status. This is the first report of these two cytokines potentially working together to create a specific disease phenotype, and needs to be confirmed in an independent cohort of patients that have been phenotyped in the same manner using the Vienna classification. It is not clear what mechanism may be at play here. However, a common mediator of chronic inflammation may be a CD8+ T cell. This cell population is essential for the development of ileitis in the TNFαARE model of CD, in which animals overexpress TNFα protein up to threefold.7 Similarly, IL10 can stimulate and increase cytolytic activity in CD8+ T cells, and rescue peripheral blood T cell clones from apoptotic cell death.23 These and other potential mediators of the chronic inflammatory process need to be explored in further observational and functional studies.

Our investigation of IL10 and TNFα SNPs in an Australian CD population suggests that specific SNPs in the promoter regions of both the TNFα and IL10 genes may be useful to predict CD behaviour with respect to disease severity or surgical risk. This may be clinically useful in shaping treatment of the disease at an earlier stage. The above results will need to be verified in an independent ethnically similar cohort using the same methods of assessment of disease related variables as in this study. Future investigation of genes in the IL10 downstream pathway may provide further insight into the genetic contribution to CD pathogenesis.

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TNFα and IL10 SNPs act together to predict disease behaviour in Crohn’s disease

E V Fowler, R Eri, G Hume, S Johnstone, N Pandeya, D Lincoln, D Templeton and G L Radford-Smith

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