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Inflammatory bowel disease is associated with a *TNF* polymorphism that affects an interaction between the OCT1 and NF-κB transcription factors

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Tumour necrosis factor-α (*TNF*) expression is increased in inflammatory bowel disease (IBD), and *TNF* maps to the *IBD3* susceptibility locus. Transmission disequilibrium and case–control analyses, in two independent Caucasian cohorts, showed a novel association of the TNF_{-857C} promoter polymorphism with IBD (overall $P = 0.001$ in 587 IBD families). Further genetic associations of TNF_{-857C} with IBD sub-phenotypes were seen for ulcerative colitis and for Crohn's disease, but only in patients not carrying common NOD2 mutations. The genetic data suggest a recessive model of inheritance, and we observed *ex vivo* lipopolysaccharide-stimulated whole-blood TNF production to be higher in healthy TNF_{-857C} homozygotes. We show the transcription factor OCT1 binds TNF_{-857T} but not TNF_{-857C}, and interacts *in vitro* and *in vivo* with the pro-inflammatory NF-κB transcription factor p65 subunit at an adjacent binding site. Detailed functional analyses of these interactions in gut macrophages, in addition to further genetic mapping of this gene-dense region, will be critical to understand the significance of the observed association of TNF_{-857C} with IBD.

INTRODUCTION

Crohn's disease (CD, OMIM 266600) and ulcerative colitis (UC, OMIM 191390), the inflammatory bowel diseases (IBD), are common chronic inflammatory diseases of the gastrointestinal tract, with an overall prevalence of up to 4 per 1000 in the UK population (1). Considerable epidemiological evidence from family and twin studies suggests a genetic as well as an environmental component to disease susceptibility. A region on chromosome 6p21, IBD3, has been identified as an IBD-susceptibility locus in four independent linkage studies (2–5). IBD3 encompasses the tumour necrosis factor α (TNF) gene, a strong positional and functional candidate gene for IBD.

TNF levels are elevated in the serum, mucosa and stool of IBD patients, and infusion of monoclonal anti-TNF antibody is a highly efficacious IBD therapy (6–9). Increased TNF biosynthesis by deletion of 3′ regulatory elements from the TNF transcript in mice results in a CD-like phenotype (10) and $TNF^{-/-}$ mice show marked reduction in chemically induced intestinal inflammation (11). The transcription factor NF-κB is necessary for TNF activation in monocytes (12–15), the predominant TNF-producing cells in IBD. Increased levels of both TNF production and NF-κB nuclear translocation have been shown in lamina propria mononuclear cells derived from IBD patients (16,17). In humans, transcriptional regulation of TNF is cell- and stimulus-specific (18), and involves a variety of regulatory elements sited in the 5′ promoter region (14,15,19–21), which can affect transcription factor binding (22,23). TNF production is under a strong genetic influence (24). We tested common TNF promoter variants for association with IBD, and studied molecular mechanisms underlying the disease-associated $TNF_{-857C/T}$ polymorphism.

RESULTS

The TNF_{-857C} promoter variant shows association with IBD in two independent cohorts

The TNF_{-857C} allele showed significant association with IBD and ulcerative colitis in an initial TDT analysis (set A, Table 1). Although the CD-phenotype overall did not show association, when we analysed CD-affected offspring without mutations in NOD2, significant association with TNF_{-857C} was observed.

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Table 1. Family-based association analysis of TNF promoter polymorphisms in set A families[a]

Phenotype	Trios	$TNF_{-1031T/C}$	$TNF_{-863C/A}$	$TNF_{-857C/T}$	$TNF_{-308G/A}$
		P	P	P	P
IBD	556	0.88	0.90	0.004[c]	0.18
UC	252	0.35	0.92	0.036[c]	0.11
CD	294	0.25	1.0	0.15	0.83
CD/NOD2 – [b]	194	0.44	0.92	0.045[c]	0.46
CD/NOD2 +	100	0.42	0.76	1.0	0.63

[a]Empirical P-values calculated from 1000 simulations.
[b]CD/NOD2-, Crohn's disease offspring not carrying a NOD2 Arg702Trp, Gly908Arg or Leu1007fsinsC mutation.
[c]Positive associations observed with the major allele TNF_{-857C}.

None of the other three common TNF promoter variants tested showed association with any of the phenotypes analysed. It is necessary to account for the multiple statistical comparisons performed in this analysis of several genetic variants and phenotypes. However, a standard Bonferroni correction is likely to be highly conservative, owing to the presence of linkage disequilibrium between genetic variants and because the phenotypes tested are not independent of each other. Alternatively, the hypothesis of association could be confirmed in a further set of samples. We therefore tested the TNF_{-857C} variant in a second independent IBD cohort (set B) and a cohort of healthy controls. Case–control analysis (Table 2) replicated the association of IBD with the TNF_{-857C} allele.

At the haplotype level, five common (>1% founder frequency) TNF single-nucleotide polymorphism (SNP) haplotypes were observed, with $TNF_{-1031T/-863C/-857C/-308G}$ being the most frequent (founder frequency 54%). The minor TNF_{-308A} and TNF_{-857T} alleles were found on separate unique haplotypes: $TNF_{-1031T/-863C/-857C/-\underline{308A}}$ and $TNF_{-1031T/-863C/-\underline{857T}/-308G}$ (20% and 6% respectively), whereas the minor TNF_{-1031C} allele was observed on two haplotypes: $TNF_{-\underline{1031C/-863C}/-857C/-308G}$ (6%) or $TNF_{-\underline{1031C/-863A}/-857C/-308G}$ (14%). Distortion towards non-transmission was observed in the IBD and UC phenotypes for the single haplotype containing the TNF_{-857T} variant (Table 3). However, of the four separate haplotypes containing TNF_{-857C}, only $TNF_{-\underline{1031C/-863C}/-857C/-308G}$ and CD showed significant association (not significant after NOD2 stratification), suggesting the primary effect to be at the single marker level.

Family-based association analysis using dominant and recessive models (data not shown) and case–control analysis of the entire family cohort (Table 2) demonstrated recessive inheritance of TNF_{-857C} to be the best-fit genetic model in all associated phenotypes. Relative risks in TNF_{-857C} homozygotes for IBD, CD (not carrying NOD2 mutation) and UC were 2.1 [95% confidence interval (CI) = 1.3–3.1], 2.4 (95% CI = 1.4–4.2) and 2.4 (95% CI = 1.4–4.0) respectively.

We previously reported linkage to the IBD3 locus in a subset of the families studied here, and repeated these analyses using the same microsatellite genotypes and methods with stratification for TNF_{-857C} (5). Mean allele sharing in the peak region of linkage (D6S265–291) was 57.5% amongst all IBD sibling pairs, and 62.2% amongst IBD sibling pairs homozygous for TNF_{-857C}, suggesting that TNF_{-857C} contributes to the positive linkage previously observed.

Higher stimulated TNF production in TNF_{-857C} homozygotes

To test whether the TNF_{-857C} allele (or other genetic variants in linkage disequilibrium with TNF_{-857C}) might have a functional effect on TNF production, we stimulated whole blood from healthy controls with lipopolysaccharide (LPS) ex vivo. TNF production was significantly higher in individuals homozygous for TNF_{-857C} ($n = 35$, mean 65.5 ± 5.7 pg/ml) than in TNF_{-857T} allele carriers ($n = 11$, 46.1 ± 6.5) at 2 hours post LPS stimulation ($P = 0.03$).

Table 2. $TNF_{-857C/T}$ association analysis in healthy controls and unrelated affected cases

Cohort	Phenotype	No. of affected individuals	CC[a]	TC[a]	TT[a]	T allele frequency (%)	T carriage frequency (%)	P-value[b]
Healthy controls		278	231	46	1	8.6	16.9	
Set B[a]	IBD	130	119.3	10.2	0.5	4.3	8.2	0.029
	UC	73	67.8	4.7	0.5	3.9	7.1	0.080
	CD/NOD2 –	60	53.8	6.2	0	5.1	10.3	0.26
Combined (sets A + B)	IBD	587	533.8	51.7	1	4.7	9.1	0.0014
	UC	304	280.1	23.4	0.5	4.0	7.9	0.0010
	CD/NOD2 –	241	222.0	19.0	0	3.9	7.9	0.0022

[a]Genotypes from families averaged over 1000 samples of random unrelated affected individuals.
[b]Versus healthy controls.

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Table 3. Haplotype TDT analysis of TNF promoter polymorphisms in set A families[a]

Haplotype	Frequency (%)	IBD T/U	P	CD T/U	P	UC T/U	P
$TNF_{-1031T/-863C/-857C/-308G}$	53.7	225/220	0.83	108/121	0.42	111/94	0.27
$TNF_{-1031T/-863C/-857C/-\underline{308A}}$	19.9	149/128	0.26	75/71	0.76	70/55	0.24
$TNF_{-1031T/-863C/-\underline{857T}/-308G}$	6.0	40/68	0.01	23/31[b]	0.31	17/35	0.02
$TNF_{-\underline{1031C}/-\underline{863C}/-857C/-308G}$	6.1	56/41	0.14	37/19[b]	0.02	19/22	0.65
$TNF_{-\underline{1031C}/-\underline{863A}/-857C/-308G}$	14.0	102/109	0.65	57/55	0.86	44/52	0.45

[a]Haplotype frequency shown in parents from all IBD families; T/U, transmitted/untransmitted alleles from heterozygous parents to affected offspring.
[b]NOD2 stratification omitted for clarity. For $TNF_{-1031T/-863C/-\underline{857T}/-308G}$, CD NOD2 −, T/U 11/20, P = 0.12; CD NOD2 +, T/U 12/11, P = 0.85. For $TNF_{-\underline{1031C}/-\underline{863C}/-857C/-308G}$, CD NOD2 −, T/U 26/15, P = 0.093; CD NOD2 +, T/U 11/4, P = 0.071.

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OCT1 specifically binds TNF_{-857T} but not TNF_{-857C}

A fragment spanning bp −879 to −845 of the TNF promoter and containing either the TNF_{-857C} (Fig. 1A, lanes 3 and 4) or the TNF_{-857T} (lanes 5 and 6) variant was incubated with nuclear extracts derived from the human monocyte cell line MonoMac6, and analysed in an electromobility shift assay (EMSA). There was no major constitutive DNA–protein interaction with the TNF_{-857C} allele (lane 3), but the TNF_{-857C} allele formed two inducible complexes with the nuclear extracts derived from the LPS-activated cells (strong bands, lane 4). These complexes were also formed with the shorter DNA fragment (bp −879 to −858) that did not extend to the $TNF_{-857C/T}$ polymorphic site (lane 2) and were identical to previously identified NF-κB p50–p65 and p50–p50 complexes (22). In contrast, the TNF_{-857T} allele formed an additional high-molecular-weight constitutive complex (asterisked bands lanes 5 and 6). This complex was also observed with a shorter DNA fragment (−864 to −845 bp) that did not extend to the NF-κB-binding site (lanes 7 and 8). Thus, strong binding of a high-molecular-weight complex occurred with the TNF_{-857T} allele (lanes 5–8) but not the TNF_{-857C} allele (lanes 3, 4, 9 and 10).

The TNF_{-857T} variant (A<u>T</u>GAAGAC) might represent a potential binding site for the OCT1 transcription factor, since five out of eight nucleotides fit the consensus OCT1-binding sequence (ATGCAAAT) (25). An excess of unlabelled oligoduplex corresponding to the OCT1 consensus sequence or anti-OCT1 antibody was used in an EMSA with the −879/−845 promoter fragment. A 10× excess of OCT1 consensus sequence in the binding reaction completely abolished the formation of the high-molecular-weight complex (Fig. 1B, lane 5), as did the presence of anti-OCT1 antibody (lane 9). In contrast, the excess of a duplex corresponding to the NF-κB site had no effect on the high-molecular-weight complex (lanes 3, 4, 7 and 8), but diminished the NF-κB-specific complexes at 10× (lane 3) and abolished them at 100× (lane 4). Antibody against NF-κB p50 or p65 had no effect on the high-molecular-weight complex, but resulted in the clearance of NF-κB complexes (lanes 7 and 8). Taken together, these findings indicate that the TNF_{-857T} polymorphism permits the binding of OCT1 immediately adjacent to a binding site for NF-κB (see Fig. 3 below).

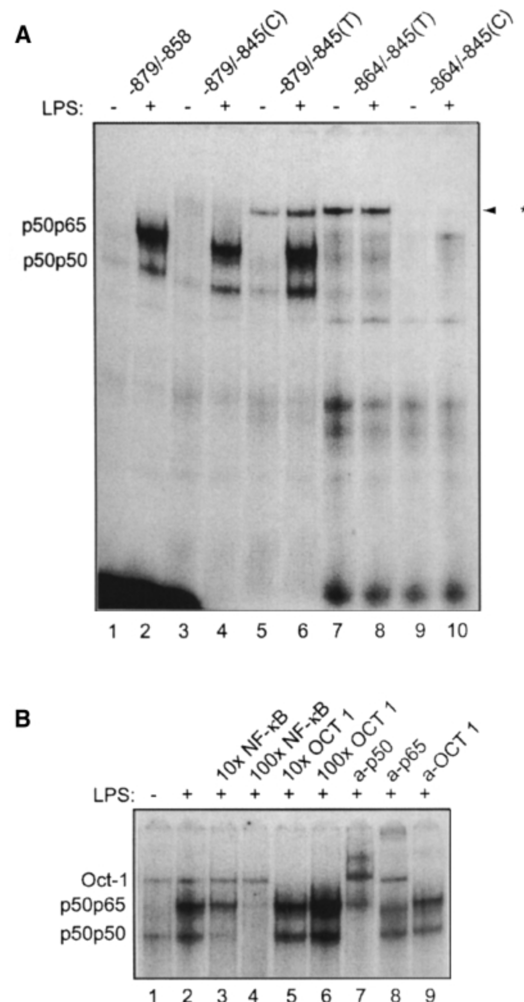

Figure 1. Nuclear factors binding to TNF_{-857C} and TNF_{-857T} variants. (A) Nuclear extracts from MonoMac6 cells prior to LPS stimulation or after they have been stimulated for 1 hour were used in EMSA with radiolabelled probes corresponding to −879/858 nucleotides of the TNF promoter (lanes 1 and 2); −879/−845 nucleotides (lanes 3 and 4 for the TNF_{-857C} allele and lanes 5 and 6 for the TNF_{-857T} allele); −864/−845 nucleotides (lanes 7 and 8 for the TNF_{-857T} allele and lanes 9 and 10 for the TNF_{-857C} allele). An additional high-molecular-weight band is indicated by an asterisk (*). (B) EMSA competition and supershift experiment with MonoMac6 nuclear extracts and −879/−845(T) radiolabelled probe with 10× and 100× excess of unlabelled NF-κB consensus site (lanes 3 and 4) or OCT1 consensus site (lanes 5 and 6); or antibody against NF-κB p50 (lane 7), p65 (lane 8) and OCT1 (lane 9).

OCT1 interacts with NF-κB in vitro

We show that a 34 bp DNA sequence in the distal TNF promoter could bind both OCT1 and NF-κB, raising the

question of whether OCT1 might functionally interact with NF-κB, as has been described for OCT1 and various other transcription factors (26–29). We generated fusion proteins of glutathione S-transferase (GST) with either NF-κB p50 (p50–GST) or the Rel homology domain (RHD) of NF-κB p65 (p65Δ–GST). The fusion proteins bound to agarose beads were then used to examine whether these specific NF-κB elements could interact with OCT1, in an in vitro pull-down assay. In vitro translated labelled OCT1 protein interacted with both p50–GST and p65Δ–GST, but not with GST alone (Fig. 2A). Neither of the GST-tagged proteins retained a significant amount of in vitro translated control TRAF protein (data not shown).

The OCT1 POU_H domain binds NF-κB

The OCT1 DNA-binding domain, the POU (for Pit, Oct, UNC), has been shown by three-dimensional structural analysis to consist of a bipartite DNA-binding domain containing POU-specific (POU_S) and POU-homeodomain-like (POU_H) domains tethered together by a hypervariable linker (30). Three C-terminal deletion mutants of OCT1 were translated in vitro and used in the pull-down assay with either matrix-bound p50–GST or p65Δ–GST (Fig. 2B). The most distal C-terminal domain deletion mutant had no effect on the interaction (lanes 10 and 14). However, the presence of an intact POU_H domain was essential for the interaction with both p50 and p65 subunits of NF-κB (as observed in lanes 11 and 15, in which the POU_H domain is absent, and lanes 12 and 16, in which both POU_H and POU_S domains are absent). The DNA-binding affinity of a truncated OCT1 protein lacking the POU_H domain was only slightly decreased compared with the full-length protein (data not shown). Experiments using OCT1 protein overexpressed in COS cells confirmed differential binding of OCT1 to TNF_{-857T} but not TNF_{-857C} (data not shown), as observed in the EMSA assays.

A reverse pull-down experiment was performed with a matrix-bound fusion protein of GST and the OCT1 POU domain consisting of both POU_S and POU_H. In vitro translated p65Δ interacted with POU–GST but not with GST alone (data not shown).

NF-κB p65 and OCT1 interact in vivo

The observation that OCT1 can bind to NF-κB in vitro does not prove that this interaction can take place in the intracellular environment of mammalian cells. To explore this question, COS-7 cells were co-transfected with NF-κB p65- and OCT1-expressing plasmids and all newly synthesized proteins were labelled with [^{35}S]methionine. The OCT1 construct was His-tagged. Total proteins were extracted and OCT1-containing complexes were precipitated with anti-His antibody attached to agarose beads. The immunoprecipitated complex was then electrophoresed under reducing conditions (SDS–PAGE), showing a radiolabelled band of approximately 90 kDa corresponding to OCT1 (Fig. 2C, left). When the electrophoresed product was examined by western analysis using anti-p65 antibody, a band of the expected size for NF-κB p65 was observed, demonstrating a co-immunoprecipitation of p65 with OCT1 (Fig. 2C, middle and right). Taken together, these findings indicate that NF-κB p65 can physically interact with OCT1 through its POU domain in vitro and in vivo.

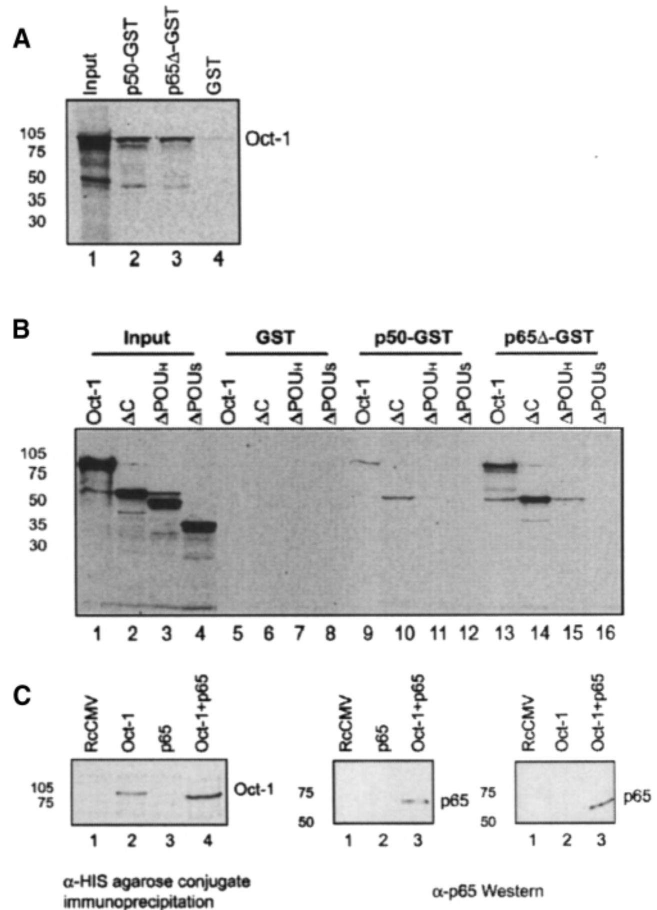

Figure 2. OCT1 interacts with NF-κB in vitro and in vivo. (A) ^{35}S-labelled in vitro translated OCT1 was incubated with either GST alone (lane 4) or with p50– or p65Δ–GST fusion proteins (lanes 2 and 3) bound to glutathione-agarose beads. Twenty-five percent of the ^{35}S-labelled OCT1 added to the reactions is shown as Input (lane 1). (B) ^{35}S-labelled in vitro translated full-length and truncated OCT1 protein was incubated with matrix-bound p50–GST (lanes 9–12) or p65Δ–GST (lanes 13–16) fusion proteins or GST alone (lanes 5–8). Input (lanes 1–4) indicates 25% of labelled proteins used in reactions. (C) COS-7 cells were transfected either with an empty RcCMV expression vector (lane 1), CMV–OCT1–His (lane 2 left and right panel), CMV–p65 (lane 3 left panel and lane 2 middle panel), or the combination of CMV–OCT1–His and CMV-p65 (lane 4 left panel and lane 3 middle and right panels). Newly synthesized proteins were radiolabelled with [^{35}S]methionine. OCT1-containing complexes were immunoprecipitated using anti-His antibody attached to the agarose beads, run on 10% SDS–PAGE and subjected to autoradiography (left panel). The co-immunoprecipitation of p65 with OCT1 was detected by western blotting using anti-p65 antibody (middle and right panels).

Reporter gene analysis in COS-7 cells

To further explore the in vivo interaction between NF-κB and OCT1, we performed transient co-transfection experiments in COS-7 cells. We generated a luciferase reporter gene construct of the distal segment of the TNF promoter (between −922 and −803 bp) linked to the TNF minimal promoter (−83). This construct was transfected into COS-7 cells with OCT1, and

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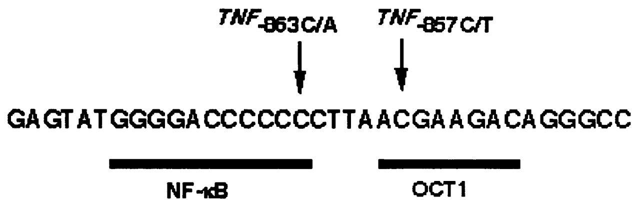

Figure 3. EMSA probe corresponding to −879 to −845 bp of the TNF promoter. Arrows mark SNP locations, and bars mark putative NF-κB and OCT1 consensus binding sites.

NF-κB p65- and p50-expressing plasmids. No significant difference in promoter activity was observed between the TNF_{-857T} and TNF_{-857C} alleles in triplicate experiments (data not shown).

DISCUSSION

Recently mutations in a monocyte-expressed gene, NOD2, have been shown to be associated with CD (31,32), and our data suggested that the population-attributable risk due to common NOD2 mutations in CD was approximately 20% (manuscript submitted). A large body of data implicates TNF in the pathogenesis of both CD and UC, and we postulated that genetic variation in TNF expression might also play a role in IBD susceptibility. Although associations with TNF promoter polymorphisms have been reported for other diseases, there have been no published papers examining the role of the $TNF_{-1031T/C}$, $TNF_{-863C/A}$ or $TNF_{-857C/T}$ variants in Caucasian IBD.

Transmission disequilibrium testing (TDT) demonstrated association of TNF_{-857C} with IBD overall and with UC. Interestingly, when a subset of CD patients carrying disease-associated variants in NOD2 was removed from the analysis, association of TNF_{-857C} with CD was observed. These data suggest TNF_{-857C} and NOD2 variants act independently to confer CD susceptibility (genetic heterogeneity), and further investigation is necessary to assess the significance of this finding. Importantly, we confirmed the association of TNF_{-857C} with IBD in a case–control analysis of a second cohort of IBD families and healthy controls. TDT is more robust than case–control analysis in the presence of population stratification, of particular relevance here due to the observation of different TNF_{-857C} allele frequencies in other ethnic populations (33,34). TDT, however, is less powerful than case–control analysis, because only heterozygous parents are informative (35). The strongest association of TNF_{-857C} with IBD was observed when unrelated cases from both cohorts of families studied were compared with healthy controls.

The positive association of TNF_{-857C} with UC observed here in Caucasians is supported by a small Japanese case–control study (33). The same study reported an association of TNF_{-857T} with Japanese CD, although a significant effect of this allele was not observed in a further report in a larger patient cohort (36). Interestingly, both NOD2 and IBD5 (37) variants are rare in the Japanese population (K. Negoro, personal communication), and the CD phenotype observed in Japanese is somewhat different to Caucasians, being less prevalent, less familial, and of a reversed male/female ratio (38–40). It is therefore possible that susceptibility to CD is inherited by alternative genetic mechanisms in Japanese patients.

When the segment of DNA containing the TNF_{-857} polymorphism was incubated with nuclear extracts from human monocytes, we found evidence of specific binding to the transcription factor OCT1, but only in the presence of the TNF_{-857T} allele. This is consistent with recently published data on OCT1 binding to this part of the TNF promoter region in B cells (41). We have previously identified a complex pattern of NF-κB interactions at the adjacent TNF_{-863} polymorphism (22), which allowed us to map OCT1 binding to the region spanning the TNF_{-857} site, immediately adjacent to the NF-κB binding site (Fig. 3). OCT1 is a broadly expressed transcription factor that is known to acquire cell-specific activating properties through interaction with other transcription factors, but has not previously been reported to interact with NF-κB. We show that OCT1 can interact with the Rel homology domain of NF-κB and that this interaction maps to the POU domain of OCT1, with POU_H being critical for the interaction. Both domains are DNA-binding; therefore the protein–protein interaction could affect the binding of these factors to the TNF promoter.

The association of TNF_{-857C} with IBD in Caucasians is particularly interesting, since our data suggest that TNF production in whole blood is increased in TNF_{-857C} homozygotes. Previous studies have demonstrated both increased TNF production and NF-κB activation in human IBD. We observe (i) that this part of the TNF promoter region contains adjacent binding sites for OCT1 and for NF-κB; (ii) that OCT1 is capable of interaction with NF-κB; (iii) that strong OCT1 binding to this region occurs only in the presence of the TNF_{-857T} allele. It is worth noting that the $TNF_{-376G/A}$ polymorphism, associated with susceptibility to cerebral malaria in Africans (23) [albeit extremely rare in Caucasians (42)], also shows allele-specific binding of OCT1 that appears to alter constitutive TNF expression. These findings, taken in conjunction with the large body of literature on the role of TNF and NF-κB in the pathogenesis of IBD, suggested the following model. When unknown factors in the gut stimulate specific cell types (such as lamina propria monocytes/macrophages), this causes nuclear translocation of NF-κB, resulting in local TNF production, which in turn promotes bowel inflammation. The signalling pathway involves the NF-κB-binding site located at −873 to −863 bp in the TNF promoter region, which is adjacent to an OCT1-binding site at −858 to −851 bp. OCT1 can physically interact with NF-κB and inhibit its transactivating effects. The TNF_{-857C} allele ablates OCT1 binding at this site, and might therefore act to further augment the NF-κB-mediated inflammatory response.

Although the allele-specific effects of $TNF_{-857C/T}$ on whole-blood TNF production support this model, the effect of this variant on primary gut monocytes/macrophages, the critical TNF-producing cells in the gut, remains unknown. These cells have now been implicated in CD pathogenesis by the discovery of disease-associated polymorphisms in the monocyte-restricted protein NOD2. At present, reliable techniques to study allele-specific effects within primary gut tissues are unavailable. We therefore performed reporter gene expression experiments using an artificial system, and did not

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observe any difference between the two alleles. Published studies of the functional effect of the $TNF_{-857C/T}$ polymorphism have all used different methodologies, and no clear consensus has emerged (43–45). Our experiments with COS-7 cells, a simian kidney fibroblast line, represent a highly reductionist system, which we used as a tool to investigate the effects of DNA polymorphisms on the binding of specific transcription factors. TNF regulation is highly cell- and context-specific, and in vitro systems can only provide a very limited insight into how TNF is regulated in vivo in specific tissues (such as the gut) under the influence of biologically relevant stimuli (46), where other transcription factors in a macromolecular system may contribute to the functional effects of this allele. Progress in this field is greatly impeded both by the difficulty of accessing the relevant primary cell type and by the refractoriness of monocytes to plasmid transfection for reporter gene analysis.

Our genetic data clearly identify the TNF_{-857C} allele as a marker of susceptibility to IBD in the UK population, but the association data alone do not help distinguish whether this variant is a true disease allele or a marker allele in linkage disequilibrium with a neighbouring functional polymorphism. Although the association is robust, extensive further studies to map this gene-dense region will be necessary to attempt to identify which of the many HLA gene variants likely to be in linkage disequilibriium with TNF_{-857C} allele might be the causal variant. However, several recent studies suggest that identification of causal mutations may not prove possible with genetic analysis alone, owing to the existence of large haplotypic blocks within the genome (such as the long-range haplotypes found in the HLA) (37,47–49). The higher TNF production in whole blood from TNF_{-857C} homozygotes suggests a biological function, and our observations of allele-specific effects on transcription factor binding suggest that interactions between NF-κB and OCT1 in the TNF promoter occurring in gut tissues may be of potential relevance to the pathogenesis of IBD. Extensive studies to map this gene-dense region in detail, and further analyses of the interaction between the NF-κB and OCT1 transcription factors in gut macrophages, are now necessary to understand the significance of the observed association of TNF_{-857C} with IBD.

MATERIALS AND METHODS

Subjects

Northern European Caucasian families were ascertained from the UK, and the diagnosis of CD or UC confirmed using standard criteria (50). Set A, families with both parents available, comprised 457 families (one or more children affected with IBD) containing 294 CD trios, 252 UC trios and 10 indeterminate colitis trios. A second, independent, set of 130 families (set B) comprised families containing two or more children affected with IBD, without both parents available. 101 families from set A and all of set B were used in our previously reported linkage study of the IBD3 locus (5). Healthy unrelated individuals were recruited from the UK Blood Transfusion Service. Informed consent and full ethical approval were obtained.

Genotyping

We isolated genomic DNA from peripheral blood, and performed PCR using primers as described for the NOD2 (manuscript submitted), $TNF_{-308G/A}$, $TNF_{-857C/T}$ and $TNF_{-863C/A}$ polymorphisms (42). The $TNF_{-1031T/C}$ polymorphism was amplified with the forward primer (5′-CAGGGGAAGCAAAGGAGAAG-3′) and reverse primer (5′-CGACTTTCATAGCCCTGGAC-3′). PCR products were digested overnight with restriction enzymes (for $TNF_{-308G/A}$, NcoI; for $TNF_{-857C/T}$ and $TNF_{-863C/A}$, HypCH4IV; and for $TNF_{-1031T/C}$, BbsI), and separated by agarose gel electrophoresis. Two investigators, unaware of an individual's affection/pedigree status, called genotypes independently, and conflicts were either resolved or the assays were repeated.

Association analysis

Checks were made for misinheritance, and the general score statistic for the TDT calculated using the GASSOC program package (v1.05) for individual polymorphisms (51) or by an unbiased multilocus haplotype method (52). Both programs correct for testing multiple siblings from the same family; thus the P-value obtained for the TDT is a valid test of association in the presence of linkage. Positive associations were further analysed under assumption of dominant or recessive genetic mechanisms (GASSOC GDOM or GREC statistics). To assess gene–gene interaction of TNF and NOD2 variants for the CD phenotype, we further analysed CD patients who were or were not carriers for common NOD2 variants associated with CD (Arg702Trp, Gly908Arg, Leu1007fsinsC). To obtain unrelated case cohorts from the families in sets A and B (unbiased by linkage), a single affected individual per family was selected at random, and genotypes totalled. The average was taken of 1000 such random selections, and allele counts compared with healthy controls using Fisher's exact test.

TNF production by stimulated whole blood

Whole blood from healthy controls was collected into sterile tubes with heparin 20 iu/ml, diluted with an equal volume of RPMI 1640 and incubated with or without 10 ng/ml LPS from Escherichia coli 055 : B5 (Sigma) in 5% carbon dioxide at 37°C. Supernatants were harvested at 2 hours after stimulation and TNF levels were measured by ELISA (R&D Systems) as described previously (53). Sample data was corrected for the unstimulated TNF level, results according to genotype expressed as mean ± SEM, and compared using a two-tailed t-test.

DNA constructs

Human p50- and p65-expressing constructs in an Rc/CMV vector (Invitrogen) were described previously (54). The protein sequences corresponding to amino acids 2–400 of p50 and 2–306 of p65 were recovered by PCR using the appropriate primers and cloned into the bacterial expression vector pGEX-4T-1 (Amersham Pharmacia Biotech) encoding the glutathione S-transferase tag (GST). The (−83)–pGL3 construct [further referred to as (−83)] was generated by PCR amplification using TNF(−83)–BglII primer (5′-aatagatctGGA

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AGTTTTCCGCTGG-3′) and vector-specific primer HindIII (5′-AATGCCAAGCTTG GAAGAG-3′) and (−1173)–pGL3 construct as DNA template, and subsequently cloned into HindIII/BglII sites of a modified pGL3-basic vector. The region between −922 and −803 bp was amplified by PCR using the following primers: forward(F) (KpnI): 5′-aatggtaccCCACAG-CA ATGGGTAGGA-3′; reverse(R) (SacI): 5′-aatagagctcG-GAGGTCC TGGAGGCTC-3′; with TNF promoter wild-type and −857T polymorphic mutant as DNA templates. PCR fragments were cloned into KpnI/SacI sites of the (−83) construct.

The sequence corresponding to amino acids 1–742 of human OCT1 was recovered by PCR using the appropriate primers [OCT1 F(BamHI): 5′-aatggatccATGAACAATCCGT CA-GAAA-3′ and OCT1 R(XhoI): 5′-aatctcgagCTGTGCCTTG-GAGGCG-3′] and cDNA derived from MonoMac6 cells. cDNA was cloned into BamHI/XhoI sites of the eukaryotic expression vector pcDNA3 (Invitrogen). The OCT1 C-terminal deletion constructs were generated by PCR using the same forward primer and three reverse primers and the full-length OCT1 as the DNA template:

OCT1 ΔC (XhoI): 5′-aatctcgagTGGTGGGTTGATTCT-3′ (438 amino acids);

OCT1 ΔPOU_H (XhoI): 5′-aatctcgagTGAGAGGTTCTCTGC-3′ (357 amino acids);

OCT1 ΔPOU_s (XhoI): 5′-aatctcgagGGGAGTATCAATTGG-3′ (276 amino acids);

PCR fragments were cloned into the pcDNA3 expression vector. All constructs were verified by DNA sequencing.

Protein extracts and EMSA

Oligonucleotide probes were radiolabelled with [α-^{32}P]dCTP: −879/−845(C/T) F: 5′-agctGAGTATGGGGACCCCCCCT TAA[C/T]GAAGACAGGGCC-3′; −879/−845(C/T) R: 5′-agctGGCCCTGTCTTC[G/A] TTAAGGGGGGGTCCCCA-TACTC-3′; −864/−845(C/T) F: 5′-agctCCCTTAA[C/T] GAAGACAGGGCC-3′; −864/−845(C/T) R: 5′-agctGGCCCTGTCTTC[G/A]TTAA GGG-3′; −879/−858 (described as κB1 in 22).

MonoMac6 cells ($10–20 \times 10^6$) were stimulated with 100 ng/ml LPS for 1 hour and nuclear extracts were prepared as described previously (55). The binding reaction contained 12 mM HEPES, pH 7.8, 80–100 mM KCl, 1 mM EDTA, 1 mM EGTA, 12% glycerol and 0.5 μg of poly dI–dC (Amersham Pharmacia Biotech). Protein extracts (1–4 μg) were mixed in an 8 μl reaction with 0.2–0.5 ng of labelled probe ($1–5 \times 10^4$ cpm) and incubated at room temperature for 10 minutes. Where indicated, a competitive cold probe corresponding to the consensus binding site for NF-κB (described as κB1 in 22) or OCT-1 (OCT1 F : 5′-agctCCCTTAATGCAAATAGG; OCT1 R: 5′-agctCCTATTTG-CATTAAGGG) or antibody against NF-κB p65 and p50 or against OCT-1 (Santa Cruz Biotechnology) were added prior to the radiolabelled probe. The reaction was analysed by non-denaturing 5% polyacrylamide gel electrophoresis at 4°C.

Protein–protein interactions

Proteins were translated in vitro using the TNT quick-coupled transcription–translation kit (Promega, Southampton, UK) in a 10 μl reaction containing [^{35}S]methionine (Amersham Pharmacia Biotech). Glutathione-bound agarose beads containing normalized amounts of p50, p65Δ or POU domain GST–fusion proteins, expressed in BL21(DE3)LysS cells (Novagen) and purified according to the GST Gene Fusion System manual (Amersham Pharmacia Biotech), were equilibrated in buffer A and subsequently incubated with 3 μl of in vitro translated protein at 4°C for 2 hours as described in (56). Beads were washed with the same buffer, and bound labelled proteins were visualised on 10% SDS–PAGE gel.

For the in vivo immunoprecipitation experiment, COS-7 cells were transfected with CMV–OCT1- and CMV–p65-expressing constructs and labelled with [^{35}S]methionine in methionine-free medium for 6 hours, and total protein extracts were prepared by lysing cells in RIPA buffer ($1 \times$ PBS, 1% NP-40, 0.5% Na-deoxycholate and 0.1% SDS) supplemented with protease inhibitors (Boehringer Mannheim). Twenty microlitres of His–agarose-conjugated antibody (Santa Cruz) were added to 100–500 μg of protein extract and incubated at 4°C overnight. Pellets were collected and washed three times with RIPA buffer and once with $1 \times$ PBS. Bound labelled proteins were visualized on 10% SDS–PAGE gel, and co-precipitated NF-κB p65 protein was visualized by western blotting using Δ-p65 antibody (Santa Cruz) and an enhanced chemoluminescence system (Amersham Pharmacia Biotech).

Cell culture, transfections and luciferase assay

MonoMac6 cells were maintained as described previously (57). COS-7 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.2 mML-glutamine and 0.1% glucose. Transient transfections of luciferase reporter gene- and protein-expressing constructs were performed on COS-7 cells using Fugene 6 non-liposomal reagent (Boehringer Mannheim). After transfection, cells were incubated for 24 hours prior to harvesting. The luciferase assay was performed using a Turner Designs Luminometer Model 20 (Promega).

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