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The role of the Toll receptor pathway in susceptibility to inflammatory bowel diseases

PL De Jager[1,2], D Franchimont[3], A Waliszewska[1,2], A Bitton[4], A Cohen[5], D Langelier[6], J Belaiche[7], S Vermeire[8], L Farwell[2], A Goris[9], C Libioulle[10], N Jani[11], T Dassopoulos[12,13], G Bromfield[14], B Dubois[9], JH Cho[11], SR Brant[12,13], R Duerr[11], H Yang[15], JI Rotter[15], MS Silverberg[16], AH Steinhart[16], MJ Daly[2], DK Podolsky[17], E Louis[7], DA Hafler[1,2] and JD Rioux[1,2,18], Quebec IBD Genetics Consortium and NIDDK IBD Genetics Consortium

[1]Department of Neurology, Center for Neurologic Diseases, Brigham & Women's Hospital and Harvard Medical School, Boston, MA, USA; [2]Broad Institute, Massachusetts Institute of Technology and Harvard University, Cambridge, MA, USA; [3]Division of Gastroenterology, Montreal General Hospital, McGill University, Montreal, Quebec, Canada; [4]Department of Gastroenterology, McGill University Health Centre, McGill University, Montreal, Quebec, Canada; [5]Montreal Jewish General Hospital, Montreal, Quebec, Canada; [6]Centre Hospitalier Universitaire de Sherbrooke - Hopital Fleurimont, Sherbrooke, Quebec, Canada; [7]CHU, Division of Gastroenterology, University of Liege, Liege, Belgium; [8]Division of Gastroenterology, University Hospital Gasthuisberg, Leuven, Belgium; [9]Section of Experimental Neurology, University of Leuven, Leuven, Belgium; [10]Centre for Biomedical Integrative Genoproteomics, University of Liege, Liege, Belgium; [11]Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA; [12]Department of Medicine, School of Medicine, Harvey M. and Lyn P. Meyerhoff Inflammatory Bowel Disease Center, Johns Hopkins University, Baltimore, MD, USA; [13]Department of Epidemiology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA; [14]Department of Medicine, University of Chicago, Chicago, IL, USA; [15]Division of Medical Genetics, Department of Medicine, Steven Spielberg Pediatric Research Center, Inflammatory Bowel Disease Research Center, Cedars-Sinai Medical Center, Los Angeles, CA, USA; [16]Division of Gastroenterology, Mount Sinai Hospital IBD Center, University of Toronto, Toronto, Ontario, Canada; [17]Gastrointestinal Unit, Department of Medicine, Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA and [18]Montreal Heart Institute, University of Montreal, Montreal, Quebec, Canada

Abstract: The intestinal flora has long been thought to play a role either in initiating or in exacerbating the inflammatory bowel diseases (IBD). Host defenses, such as those mediated by the Toll-like receptors (TLR), are critical to the host/pathogen interaction and have been implicated in IBD pathophysiology. To explore the association of genetic variation in TLR pathways with susceptibility to IBD, we performed a replication study and pooled analyses of the putative IBD risk alleles in NFKB1 and TLR4, and we performed a haplotype-based screen for association to IBD in the TLR genes and a selection of their adaptor and signaling molecules. Our genotyping of 1539 cases of IBD and pooled analysis of 4805 cases of IBD validates the published association of a TLR4 allele with risk of IBD (odds ratio (OR): 1.30, 95% confidence interval (CI): 1.15-1.48; P= 0.00017) and Crohn's disease (OR: 1.33, 95% CI: 1.16-1.54; P= 0.000035) but not ulcerative colitis. We also describe novel suggestive evidence that TIRAP (OR: 1.16, 95% CI: 1.04-1.30; P= 0.007) has a modest effect on risk of IBD. Our analysis, therefore, offers additional evidence that the TLR4 pathway - in this case, TLR4 and its signaling molecule TIRAP - plays a role in susceptibility to IBD.

Keywords: Toll-like receptor; inflammatory bowel disease; NFKB1; TLR4; TIRAP

Introduction

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are two chronic inflammatory conditions that predominantly affect the gastrointestinal tract. These classifications are important for the clinical management of the disease. However, in reality, there are certain cases that either overlap both categories or fit in neither, suggesting the existence of a spectrum of diseases collected under the IBD rubric. In addition, epidemiologic studies have revealed that relatives of a patient with UC or CD have a higher risk of developing either form of IBD.[1-3] This suggests that UC and CD share certain genetic susceptibility loci and that the ultimate phenotypic expression of the disease also probably depends on other factors such as genetic modifier loci and environmental influences.

One important environmental contribution comes from the bacteria that reside in the gastrointestinal tract. Endogenous and pathogenic bacteria and mycobacteria have long been suspected of playing a role in IBD, but their involvement in initiating or exacerbating IBD has not been definitively demonstrated in humans.[4] Strong support for this hypothesis, however, comes from murine model systems in which susceptible mice kept in germfree conditions do not develop spontaneous colitis.[5] Furthermore, genetic studies in humans and mice have made a convincing link between the innate immune system and chronic inflammation. Specifically, studies have demonstrated that genetic variants in the host's pattern recognition receptors (PRR) can lead to increased susceptibility to colitis. PRRs are molecules of different families, such as the CARD/NOD molecules and Toll-like receptors (TLR), that recognize microbial products and initiate inflammatory responses as part of the innate

immune response to pathogens. An allele of murine TLR4, *Lpsd,* was first noted to decrease the severity of experimental colitis induced by dextran sulfate sodium (DSS),[6] and more recently, TLR4$^{-/-}$ and MyD88$^{-/-}$ mice have been shown to have more severe DSS-induced colitis than their wild-type littermates.[7-9] In addition, mutations in CARD15/NOD2 have been associated with susceptibility to human CD[10-12] and shown to affect nuclear factor-κB (NF-κB) activation, interleukin (IL)-1β processing, and resistance to intestinal *Listeria monocytogenes* infection in mice.[13-14]

Within the TLR family of PRR, there are 10 different transmembrane receptors (TLR1-10) that are found either on the extracellular surface of cells (TLR1, 2 and 4) or within intracellular compartments such as endosomes (TLR3 and 7-9).[15] An eleventh TLR sequence (TLR11) was described after the initiation of this project and was therefore not assessed in our study; it is still unclear whether TLR11 produces a functional protein in humans.[16] The function of the TLR genes and their associated signaling mechanisms has been characterized in great detail in recent years, particularly in the mammalian bowel. In mammals, the intestinal epithelium is a major barrier to the entry of microbes into the organism. It is therefore not surprising that the TLR molecules and elements of their signaling machinery are expressed not only by resident ileal and colonic macrophages but also by components of the intestinal epithelium.[17-19] Furthermore, these expression patterns are altered in the context of CD or UC and in the presence of an inflammatory mediator.[20-23] This evidence, coupled with the observation that MYD88 and TLR4 alleles affected the severity of experimental colitis in the mouse, led us to initiate an assessment of genetic variation within the TLR pathway for association with human IBD.

A total of 23 genes implicated in TLR signaling pathways at the project's initiation in 2002 were selected for study. These genes include the 10 TLR genes as well as genes whose products interact with the TLR directly at the cell surface (MD-2) or intracellularly (MYD88, TIRAP and TOLLIP), and certain genes (IRAKI, IRAK2, MAP3-K7IP2/TAB2 and TRAF6) whose products link the latter adaptor proteins to signaling cascades that activate the NF-κB pathway (IKKA/CHUK, IKBKB, IKbKG, MAP3K14/NIK and NFKB1).[15] At the study's initiation, two of the selected genes, NFKB1 and TLR4, were reported to have each one allele associated with susceptibility to either CD or UC.[24,25] In the first part of our study, we have therefore performed a replication study of these two alleles (NFKBl^{94delATTG} and TLR4^{299Gly}) and extended the analysis to include all published data on these alleles, as individual replication studies have provided inconsistent results.[26-35] Combining the results in this fashion provides the necessary statistical power to evaluate genetic risk factors of modest effect, such as those observed for multigenic diseases.[36]

In the second part of the study, we screened all 23 genes for association with susceptibility to IBD. Current collections of IBD samples are not large enough to adequately investigate rare alleles that may contribute to risk of IBD. We therefore focused our study on the assessment of common (frequency >0.10) alleles in these genes. The initial screen of the TLR pathway was performed using two sample collections from Canada, and results from this screen were then followed by a powerful replication study in three additional sample collections. This validation effort identified an allele in TIRAP as having evidence for contributing to the risk of developing IBD. In addition, the pooled analyses including published data demonstrate that TLR4 but not NFKB1 has one allele that is associated with susceptibility to IBD and to its CD subset.

Results

Confirming known associations to CD or UC susceptibility

We first set out to confirm the published associations of , susceptibility to CD or UC with NFKB1 and TLR4 alleles. Specifically, we attempted to replicate the putative association of the -94delATTG allele of NFKB1 (NFKB1 ^{94delATTG}) with UC and the 299Gly allele of TLR4 (TLR4^{299G1y}) with CD and UC.[24,25] We therefore genotyped these two putative risk alleles in the nearly 2500 study subjects (Table 1; IBD-1 to -5) for the replication analysis, and then we combined these data with those contained in all published reports of association in IBD, CD or UC using a Mantel-Haenszel pooled analysis approach.

For evaluation of the TLR4^{299Gly} allele, the data available was extensive: 4805 IBD subjects of mostly European origin from 14 different sample collections (Table 2) have been genotyped and showed no significant evidence of genetic heterogeneity (Pearson χ^2 goodness-of-fit test, P = 0.055). While the results from the typing of our own samples do not provide significant evidence of association with CD, UC or IBD (Table 2), it is not inconsistent with such an effect. In fact, the pooled analyses of these results with all of the published replication studies (Table 2), excluding the original study to examine an independent effect, demonstrates that there is a significant association between TLR4^{299Gly} and the risk of IBD (odds ratio (OR) = 1.22, 95% confidence interval (CI): 1.06-1.40; P = 0.002) and confirms the original study. While the original published study estimated a

relatively strong genetic effect (OR = 2.34) for this allele, combining all of the current results with the original results provide a more accurate estimate of genetic effect (Tables 3 and 4), one which is more modest (OR = 1.26, 95% CI: 1.10-1.43) although quite significant (P = 0.00017). This association is also found for the CD subgroup of IBD (OR = 1.33, 95% CI: 1.16-1.54; P = 0.000035), but there is no statistically significant evidence of association for the UC subgroup (P = 0.058). In terms of the putative NFKB1 ^{94delATTG} risk allele, Karban *et al.*[25] first reported an association with risk of developing UC. We therefore tested this specific hypothesis in our entire set of samples but did not find significant evidence of association (OR = 0.99, 95% CI: 0.81-1.2; Supplementary Table 2). However, combining these results with previously published replication studies (Supplementary Table 2) in a pooled analysis provided modest evidence of association between risk of UC and NFKB1 ^{94delATTG} (OR = 1.12, 95% CI: 1.01-1.23, P = 0.012). There was no evidence of association when the allele was analyzed in CD and all IBD samples (data not shown).

Table 1: *Subject samples examined as part of the TLR pathway screen in IBD*

	Numbers of affected subjects			
	IBD	***CD***	***UC***	***Control***
Screening samples				
IBD-1: Canadian trios[a]	160	135	25	N/A[b]
IBD-2: Canadian C/C[c]	114	114	0	68
Total screening	274	249	25	68
Replication samples				
IBD-3: Belgian C/C	608	449	148	479
IBD-4: Belgian trios	104	83	21	N/A
IBD-5: NIDDK trios (Canada and USA)	933	593	304	N/A
Total replication	1645	1125	473	479

Abbreviations: CD, Crohn's disease; IBD, inflammatory bowel diseases; NIDDK, National Institute of Diabetes and Digestive and Kidney; TLR, Toll-like receptors; UC, ulcerative colitis.
[a]Trios, a patient with IBD and both parents.
[b]N/A, not applicable given that these are mother-father-affected child trios.
[c]C/C, case-control.

IBD susceptibility gene discovery in the TLR pathway

In the second part of our study we conducted a screen of the common genetic variation in 23 genes involved in TLR signaling pathways for association to IBD. These genes included the 10 genes encoding the TLRs (TLR1-10), eight of their adaptor proteins, and five molecules involved in the control of the NFKB1 signaling cascade. This study was initiated before the data from the International HapMap project was available, and therefore our study design consisted of (1) a validation step in which we genotyped 379 single nucleotide polymorphisms (SNPs) selected from these 23 genes in 86 trios to determine the linkage disequilibrium (LD) patterns surrounding each of the candidate genes, (2) using the LD information for the selection of 115 SNPs that tag the common variation in these genes, (3) an association screen where the tag SNPs were typed in 274 Canadian IBD samples and their family- and population-based controls samples (Table 1, IBD-1 and -2), and (4) a replication study where we typed the SNPs having suggestive or significant evidence of association in the screen, in 1645 cases and their controls from Belgium, Canada and the United States (Table 1, IBD-3 to -5).

Specifically following the typing of the 115 SNPs in the screening set of samples, we determined that there was no significant genetic heterogeneity between the two sample collections that make up the screening set (data not shown), and therefore the results from these cohorts were combined to provide maximal statistical power. These analyses identified a single non-coding SNP in TIRAP and four haplotypes in IRAK2, TLR3 and TLR4 as having suggestive evidence (P<0.05) of association to IBD (Table 3). These putative associations were then tested in the replication sample set of 1645 cases of IBD and their controls. Again there was no significant evidence of heterogeneity between the different sample collections that constitute the replication cohort, and therefore we performed a combined analysis of cohorts IBD-3 to -5. This analysis revealed that only the TIRAP SNP (rs671492) had an association (P = 0.032) that suggested replication of the initial observation of increased susceptibility to IBD in subjects carrying the 'G' allele. Specifically the minor 'G' allele of rs671492 (rs671492^{G}) has an OR of 1.13 (95% CI: 1.01-1.28; P = 0.032) for susceptibility to IBD in the replication samples; while this statistical evidence is suggestive of replication, it falls above a formal threshold of significance (P<0.01) once we correct for the five hypotheses tested in the replication phase of the study. Nonetheless, these data present an

attractive hypothesis to be pursued further, and, to obtain a more accurate estimate of the magnitude of the effect of rs671492^{G} on risk of IBD, we pooled the screening and replication data and estimated that the rs671492^{G} allele has a true but modest effect on risk to IBD (OR = 1.16, 95% CI: 1.04-1.30). Since the CD and UC subgroups of IBD have distinct genetic associations, we partitioned the pooled stages 1 and 2 data along these phenotypic categories and conducted separate analyses on each phenotypic subgroup. Subjects (1374) with CD (collections IBD-1 to -5) were used in this secondary analysis of the rs671492^{G} allele (OR = 1.10, 95% CI: 0.97-1.24); on the other hand, the smaller subgroup of 452 subjects with UC (collections IBD-3 and -5) revealed an OR of 1.38 (95% CI: 1.12-1.71) for the rs671492^{G} allele. The IBD-1 and -4 collections were not used in the latter analysis given the small number of UC cases that they contain.

Discussion

Previous studies of animal models of IBD as well as the discovery of IBD susceptibility alleles in the human CARD15 gene have indicated that the innate immune response in patients who develop IBD may be abnormal. We were therefore interested in determining whether additional genetic variants in key innate immunity genes alter an individual's risk for developing IBD. Thus we chose to perform an association study targeting the TLR genes, as well as genes encoding their adaptor molecules and their downstream signaling and regulatory molecules. The first step was to determine whether two putative risk alleles (TLR4^{299G1y} in CD and IBD; NFKB1$^{-94delAATG}$ in UC) that had previously been reported in the literature could be confirmed. The second step was to perform a haplotype-based evaluation of a large number of candidate genes in the same biological pathway that had not been thoroughly examined for association to IBD.

Table 2: *Pooled analysis of studies exploring the role of TLR4^{299Gly} in IBD*

Sample	***Total number of cases***	***Odds ratio (95% CI)***		
		IBD	***CD***	***UC***
Original observation				
1. Belgian C/C #1[24]	610	2.27 (1.29-4.00)	2.34 (1.32-4.18)	2.05 (1.07-3.93)
Published replication studies				
2. Belgian trios #1[24]	318	1.68 (1.13-2.52)	1.79 (1.14-2.82)	None
3. Dutch C/C #1[33]	112	2.05 (1.08-3.89)	2.05 (1.08-3.89)	None
4. Dutch C/C #2[32]	591	1.44 (0.92-2.25)	1.51 (0.94-2.44)	1.30 (0.73-2.34)
5. German C/C #1[34]	200	2.08 (1.19-3.98)	1.71 (0.77-3.77)	2.34 (1.10-1.98)
6. German C/C #2[28]	204	1.95 (1.03-3.70)	1.95 (1.03-3.70)	None
7. Greek C/C[29]	205	2.10 (0.85-5.20)	2.78 (1.09-7.10)	1.18 (0.37-3.74)
8. Hungarian C/C[30]	527	0.81 (0.50-1.32)	0.81 (0.50-1.32)	None
9. Scottish C/C[26]	499	0.99 (0.60-2.07)	1.20 (0.76-1.90)	0.77 (0.47-1.27)
Novel *replication studies performed as part of our TLR screen*				
10. Canadian trios	160	1.00 (0.57-1.74)	1.00 (0.56-1.78)	Not included
11. Canadian C/C	114	0.86 (0.32-2.27)	0.86 (0.32-2.27)	None
12. Belgian C/C #2	249	1.11 (0.65-2.07)	1.39 (0.99-1.95)	1.09 (0.66-1.80)
13. Belgian trios #2	104	1.00 (0.57-1.74)	0.89 (0.34-2.30)	Not included
14. NIDDK trios	912	1.02 (0.77-1.35)	1.00 (0.71-1.41)	1.03 (0.64-1.66)
Summary statistics (OR and 95% CI)				
Novel replication (10-14)	1539	1.04 (0.85-1.26)	1.12 (0.92-1.36)	1.04 (0.82-1.31)
Replication only (2-14)	4195	1.22 (1.06-1.40)	1.27 (1.10-1.47)	1.08 (0.91-1.28)
All studies (1-14)	4805	1.26 (1.10-1.43)	1.33 (1.16-1.54)	1.14 (0.97-1.34)
Summary statistics (P-value)				
Novel replication (10-14)	1539	0.21	0.27	0.38
Replication only (2-14)	4195	0.0020	0.00064	0.20
All studies (1-14)	4805	0.00017	0.000035	0.058

Abbreviations: CD, Crohn's disease; CI, confidence interval; IBD, inflammatory bowel diseases; NIDDK, National Institute of Diabetes and Digestive and Kidney; UC, ulcerative colitis.
A Pearson χ^2 goodness-of-fit test reveals no significant evidence of genetic heterogeneity among the 14 sample collection *(P* = 0.055). 'None' is used when collections reported no data on UC samples. 'Not included' is used when collections had too few UC samples to make a meaningful contribution to the UC only analysis. They were therefore not considered as part of the pooled analysis. For the two Belgian sample collections (12 and 13) used in our replication effort, only samples not previously analyzed in collections 1 and 2 were included in the replication analysis; thus, we excluded 359 subjects with IBD from collection #12 in this pooled analysis. All of the subjects in collection #13 are new and were therefore all included. For further details regarding the subjects included in each study, please see Supplementary Table 1.

In an attempt to confirm the published associations of TLR4 and NFKB1 genes with IBD, we typed a large number of samples for these variants and also pooled these results with all published replication studies. In doing so, we have found evidence to confirm the association of $TLR4^{299G1y}$ with CD and IBD as well as the association of $NFKB1^{-94delATTG}$ with UC. It should be highlighted that not all of the individual studies of the TLR4 and NFKB1 variants had significant evidence of association, despite the fact that the pooled analyses demonstrated significant association (Table 2). These seemingly contradictory results are consistent with the known challenges of replication studies: (1) the modest effect size of an allele on disease risk, (2) the modest number of subjects involved in both initial and replication studies, (3) the non-homogeneous inclusion and exclusion diagnostic criteria for complex diseases across studies, and (4) population-specific or -enriched effects, such as the difference in risk allele frequency, in different populations. The latter problem is of particular concern in pooled analyses, and we have therefore used a Pearson χ^2 goodness-of-fit test to demonstrate that there are no significant differences in allele frequencies of $NFKB1^{-94delATTG}$ or $TLR4^{299Gly}$ in the various sample collections considered in the pooled analyses. The pooled analyses therefore represent statistically robust assessments that provide a more accurate evaluation of the role of these alleles in risk of disease given the much larger combined sample size of the pooled analyses. Lohmueller *et al.*[36] systematically evaluated this approach and demonstrated that such pooled analyses offered convincing evidence of replication for 8 out of 25 (32%) of associations that had a history of inconsistent replication results in 301 publications. This evaluation was consistent with that Ioannidis *et al.,*[37] who used a slightly different approach. The pooled analyses provided herein, therefore, provide statistically significant evidence that the $TLR4^{299G1y}$ allele is a true risk factor for CD and IBD; on the other hand, we provide more modest evidence that $NFKB1^{-94delATTG}$ is a risk allele for UC.

Table 3: *Association results from family- and population-based screening of the TLR pathway*

Gene	***Location HG16***	***Size (kb)***	***Tests of association***		***Screening phase results meeting P<0.05***	***T:U trios (IBD-1)***	***Frequency controls (%) (IBD-2)***	***Frequency cases (%) (IBD-2)***	***Combined P-value***
			SNPs	***Haplotypes***					
IKKA	10q24.31	15.0	2	1	None				
IKKB	8pll.21	42.3	2	1	None				
IRAK2	3p25.3	121.1	20	11	H.B.1: GA[a]	48:31	13.1	16.4	0.042
					H.B.2: GTA[a]	33:16	13.2	16.8	0.017
LY96	8q21.11	353.6	5	None	None				
MAP3K1417	q21.31	38.1	4	5	None				
NFkBl	4q24	209.2	10	3	None				
TIRAP	llq24.2	26.7	4	5	Rs671492-G	67:39	82.8	81.7	0.033
TLR1	4pl4	20.0	6	3	None				
TLR2	4q31.3	42.3	5	4	None				
TLR3	4q35.1	8.6	4	5	H.B.1: CT[a]	40:49	26.2	15.3	0.037
TLR4	9q33.1	71.1	13	3	H.B.1: GCC[a]	79:70	17.4	30.6	0.045
TLR5	1q41-q42	55.8	11	7	None				
TLR6	4pl4	0.16	2	None	None				
TLR9	3p21.2	9.4	3	2	None				
TLR10	4pl4	15.9	7	2	None				
TOLLIP	11pl5.5	67.3	10	3	None				
TRAF6	11pl2	46.7	7	4	None				

Abbreviations: IBD, inflammatory bowel diseases; SNP, single nucleotide polymorphism; TDT, transmission disequilibrium test; TLR, Tolllike receptors.
This table summarizes the results of our analysis of 115 tag SNPs in 17 genes involved in TLR signaling. Only those results meeting our criteria for replication are listed in this table. The total number of hypotheses tested (both SNPs and haplotypes) is noted at the bottom of the table. As noted in out Materials and methods section, only those haplotypes with a frequency >0.l were tested in this analysis. In the third column from the right, we report the transmitted:untransmitted (T:U) ratio in our TDT analysis of sample IBD-1.
[a]The haplotypes passing our screening criteria are composed of the following alleles: (1) IRAK 2 haplotype block 1 (H.B.1) - $rs779805^{c}$ and $rs458952^{A}$; (2) IRAK2 H.B.2 - $rs708030^{G}$, $rs2306696^{T}$ and $rs1177614^{A}$; (3) TLR3 H.B.1 - G9862a1[c] and $rsl519312^{T}$; TLR4 H.B.1 - $rsl252041^{G}$, $rs2770150^{c}$ and $rsl927911^{c}$.

Given the significant genetic evidence implicating $TLR4^{299Gly}$ in disease risk, it is important to examine how this allele may influence biological function and disease pathogenesis. Interestingly, it has been reported that the $TLR4^{299Gly}$ variant leads to decreased airway responsiveness to inhaled LPS, decreased NFKB1 activation and decreased *in vitro* IL1α expression in airway epithelial cells.[38] However, several other groups report no

significant evidence for a functional difference in *ex vivo* or *in vitro* characterization of cytokine expression by peripheral blood mononuclear cells between subjects having either the Asp or Gly allele at position 299.[39-45] It should be noted, however, that binding of the TLR4 receptor leads to more than just the activation of NFkB. For example, signaling via TLR4 can also lead to the activation of the STAT family of proteins and the triggering of gene expression of the IRF family of proteins[46] - raising the possibility that TLR4^{299Gly} may affect the function of TLR4 signaling by another mechanism. Alternatively, since TLR4 is found within a large block of LD (Figure 1), the TLR4^{299Gly} allele may simply be highly correlated with the causal allele. In fact, at least one other non-synonymous coding polymorphism, TLR4^{Thr399} was found to be on the same haplotype as TLR4^{299Gly} The strong correlation between these two coding polymorphisms (r^2>0.9 in our data) makes it difficult to resolve their genetic effects. The OR associated with each variant are virtually identical, and a previous study indicates that both alleles appear to have an effect on NFKB1 activation and IL1α expression.[38]

These findings are supported by recent evidence that both TLR4^{299Gly} and TLR4^{399Ile} demonstrate decreased responsiveness to LPS and other TLR4 ligands in an *in vitro* reconstituted TLR4 signaling apparatus when compared to the TLR4 proteins bearing the major allele of each SNP (TLR4^{AsP299} and TLR4^{Thr399}).[47] A construct containing both minor alleles had a significantly lower response to TLR4 stimulation than all other constructs, and all of these differences in responses were noted to be dependent on the stochiometry of TLR4, MD-2 and CD14. Thus, we have several clues that could implicate either or the combination of both minor alleles as being causal for susceptibility to IBD, but additional work is required to reconcile the results of these *in vitro* and *ex vivo* experiments and to confirm that no other polymorphism in this large TLR4 haplotype contributes to the susceptibility phenotype.

The second part of our study consisted of a broad screen of the TLR pathway for association with susceptibility to IBD, followed by a replication study in an independent set of samples. Using this approach, we identified a genetic variant in the gene encoding TIRAP that may be associated with IBD, although its genetic effect is quite modest (OR = 1.15). Secondary analyses suggest that this allele may play a more important role in UC (OR = 1.38) than in CD (OR=1.10). However, the evidence that we present does not meet a threshold of genome-wide statistical significance (P<10 ^{8}); even within the confines of our study, this result is not formally significant when one takes into account the multiple hypotheses being tested. This is not surprising given the very modest OR of this putative risk allele in TIRAP, and several thousand more cases of IBD from other collections will need to be investigated before we can come to a firm conclusion on the role of this allele in IBD. For now, we present our own data as being suggestive of replication for the association of TIRAP with IBD susceptibility since the P-value in the replication stage of this study was 0.032, which is just above the 0.01 Bonferroni-corrected level of significance for the five hypotheses tested in our replication effort. It is intriguing to note that the TIRAP gene encodes a protein with a critical role as an adaptor molecule at the juncture of several signaling pathways, including that of TLR4. The association that we detect with TIRAP and IBD further suggests that the TLR4 pathway plays an important role in IBD pathogenesis.

It is interesting to note that the genetic associations reported herein only have a modest influence on a person's risk to developing IBD (OR = 1.1-1.3) for the NFKB1, TLR4 and TIRAP variants. These genetic effects are in the same range as that observed for CTLA4^{risk} in type I diabetes (OR ~1.18)[48] and are typical of many other chronic inflammatory and autoimmune disease risk alleles.[49] We can speculate that these common alleles with modest effects on gene function are tolerated in the innate immune system and can provide diversity in a population's response to the microbial flora, whereas alleles with stronger effects on function may only be able to survive as rare alleles in a population, a pattern observed with the CARD15 IBD risk alleles that are less common but have strong effects on disease risk.[50] Regardless of the genetic model that can explain these effects, it is clear that determining the functional consequences of these effects and mechanistic link to disease will remain a considerable challenge.

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Table 4: *Replication study results - follow-up of the TLR pathway screen*

Phenotype	*Putative risk allele or haplotype*	*Replication data*			*Association with IBD (P-value)*		
		Collection IBD-3 Belgium case and control	*Collection IBD-4 Belgium trios*	*Collection IBD-5 NIDDK trios*	*Screening sample (Canada)*	*Combined Replication samples (IBD-3 to -5)*	*All tested samples P-value and odds ratio (95% CI)*
IBD	IRAK2-H.B.1: GA				0.042	0.954	0.783
	T:U		20:30	187:215			0.93 (0.81-1.06)
	Frequency cases	14.4					
	Frequency controls	15.1					
	IRAK2-H.B.2: GTA				0.017	0.751	0.488
	T:U		27:27	288:306			1.00 (0.89-1.213)
	Frequency cases	18.5					
	Frequency controls	18.8					
IBD	TIRAP-rs671492-G				0.033	0.032	0.007
	T:U		36:39	341:295			1.15 (1.03-1.28)
	Frequency cases	76.5					
	Frequency controls	74.1					
IBD	TLR3-H.B.1: CT				0.037	0.463	0.207
	T:U		41:43	375:407			0.96 (0.86-1.07)
	Frequency cases	71.0					
	Frequency controls	69.4					
IBD	TLR4-H.B.1: GCC				0.045	0.887	0.622
	T:U		37:41	358:382			0.97 (0.88-1.08)
	Frequency cases	26.5					
	Frequency controls	27.9					

Abbreviations: CI, confidence interval; IBD, inflammatory bowel diseases; NIDDK, National Institute of Diabetes and Digestive and Kidney; T:U, transmitted:untransmitted. The frequencies reported in the table are derived either from healthy control subjects (Canada and Belgium columns) or from parents (NIDDK column). In the case of Belgian and Canadian samples, there was no significant difference in allele frequencies between healthy control subjects and subjects who are parents of a trio (data not shown). The results deemed to be statistically suggestive are highlighted in bold.

Materials and methods

Subjects

All affected subjects fulfill clinical criteria for IBD[51,52] and were consented using protocols approved by the Institutional Review Boards of each institution contributing samples. All DNA samples were extracted from the blood of the consented subjects. The complete collection assembled for this study consists of 1919 subjects with IBD. In most cases, a clinical subtype of CD or UC was assigned using standard clinical criteria;[51] few cases were deemed to be cases of 'indeterminate colitis' and were not used in the CD- or UC-only analyses. In total, five different collections (IBD-1 to -5) were used in the analysis (Table 1). Collections IBD-1 and -2 were collected at multiple sites in the province of Quebec, Canada; these subjects are all of European ancestry. In the IBD-2 collection, self-declared healthy control subjects were matched to cases on the basis of ethnicity[53] Collection IBD-3 is described below. Collection IBD-4 consists of Belgian subjects of European ancestry collected at the Erasme Hospital, Brussels. Collection IBD-5 was established by the National Institute of Diabetes and Digestive and Kidney (NIDDK) Diseases IBD Genetics Consortium and consists of samples from several different collections from the United States and Canada. Out of 933 trios in collection IBD-5, the vast majority of probands are of European ancestry; there were 17 probands of Asian ancestry, four African-American probands, five probands with 'other' ancestry and six of unknown ancestry. For the phenotypic analyses of the IBD-5 collection, we used the CD and UC phenotypes available in the 'trio pheno release 1.2' of the NIDDK-sponsored database. The clinical features of this sample collection has recently been published.[54] Of note, sample collection 'IBD-3' consists of samples from four independent collections. Collection 1 from the Erasme Hospital, Brussels, Belgium: 179 subjects affected with CD.[24] Collection 2 from the University of Liege, Belgium: 429 subjects affected with IBD and 96 healthy control individuals.[55] Collection 3 from the University of Leuven, Belgium: 191 healthy control individuals collected as controls for another IBD study[56] Collection 4

from University of Leuven, Belgium: 192 healthy control individuals collected to serve as a control population for a multiple sclerosis project.[57] All affected and control subjects were collected within a 100 km radius and are all of European ancestry. Because of the disparate origins of its component sample collection, the pooled collection of cases and controls from Belgium is not precisely matched. It should be viewed as a pooled collection of subjects with IBD and unrelated individuals collected from the same population. To assess for the possibility of population stratification, allele frequencies in the three different healthy control samples (collections 2-4*)* were compared, and marginal heterogeneity was noted at only 1 (rs4986790) out of 24 SNPs genotyped between collection 1 and 4 (χ^2 test: $P = 0.043$). Allele frequencies between the affected subjects from collection 1 and 2 showed no evidence of genetic heterogeneity. As a result, there is no strong evidence for population stratification among the control subjects and cases; all cases and all controls from these four collections were pooled for our analyses; the marginal heterogeneity at rs4986790 in control subjects is noted and is taken into account in our interpretation of results regarding this SNP.

Study design: TLR pathway screen

This part of the study consists of a screening phase and a replication phase to evaluate alleles and haplotypes of the genes of interest. At the initiation of this study the haplotype data from the International HapMap project were not yet available, and therefore we genotyped 86 Canadian subject-parent trios to characterize the LD patterns surrounding these genes to identify tag SNPs.

Specifically we searched the UCSC Genome Browser and dbSNP for SNPs providing a target SNP density of 1 SNP every 2.5 kb in the selected genomic segments. For each of the 23 genes studied, we selected SNPs between the transcription initiation site and the end of the 3' untranslated region (genic region) as well as in at least 20 kb of flanking sequence upstream and 20 kb of flanking sequence downstream of the genic region. SNP selection for this study took place in 2002, before the availability of the Haplotype Map, and many of the SNPs that we selected based on our coverage goal of 1 SNP/2.5 kb were either not polymorphic or genotyped poorly in the subset of our screening sample used for SNP validation. In all, we genotyped this validation sample of 86 Canadian IBD trios with 379 SNPs from 22 peri- and intragenic areas totaling 694 kb. The initial study-wide SNP density was therefore 1 SNP every 1.8 kb 17 genes yielded at least two SNPs that passed our quality control parameters (see Genotyping section) and were screened in the screening phase. Despite two rounds of SNP testing, no useful SNP could be found at the completion of validation efforts in four genes: IKBKG, IRAK1, MYD88 and TLR7. TAB2 and TLR8 each yielded a single usable SNP. These six genes were therefore not studied further. Overall, 115 SNPs in 17 genes were characterized as tag SNPs.

In the association screen of this biological pathway, we typed the 115 tagging SNPs in the entire set of Canadian collections of affected subject/parent trios (collection IBD-1) and matched case and control subjects (collection IBD-2; Table 1). Given the size of our sample set for the screening phase, the SNPs and haplotypes tested in our analysis were limited to those found at a frequency >0.l in the healthy Canadian population. To illustrate this point, we estimate that we have 80% power to detect an allele associated with an OR of 1.7 for IBD and found at a frequency of 0.1 in our screening sample given a multiplicative model of disease and an $\alpha = 0.05$ as a threshold for replication. Thus, our statistical power to explore the role of rarer SNPs and haplotypes is limited, and we did not pursue their assessment as part of this study. A combined Z-score and P-value were calculated as described in the Statistical Analysis section for each SNP and haplotype, which had a frequency >0.1 in the IBD-1 and -2 populations (Table 2). We considered all SNPs or haplotypes, of at least 10% frequency and having a combined two-tailed nominal $P<0.05$ for association to IBD as being putatively associated.

We tested all putatively associated SNPs and haplotypes in an independent replication study. Specifically, the relevant SNPs were typed in the combined replication panel that consists of three different sample collections: IBD-3 to -5 (Table 1). A combined Z-score is calculated as described in the Statistical Analysis section, and the resulting one-tailed P-value is the primary outcome of the replication effort. To be considered replicated, a hypothesis tested in the replication cohort must have a $P<0.01$, which is derived from an $\alpha = 0.05$ with Bonferroni correction for testing five hypotheses.

Genotyping

All samples were genotyped using the Sequenom MassArray system as described previously[53] All genotypes on collections IBD-1 to -5 were generated by the genotyping center of the Whitehead Institute Center for Genome Research (now called the Broad Institute). We used data only from those SNPs that showed >90% genotyping success, did not deviate from Hardy-Weinberg equilibrium ($P> 0.001$) in the control subjects, and had one or fewer Mendelian error in each trio collection (IBD-1 to -5).

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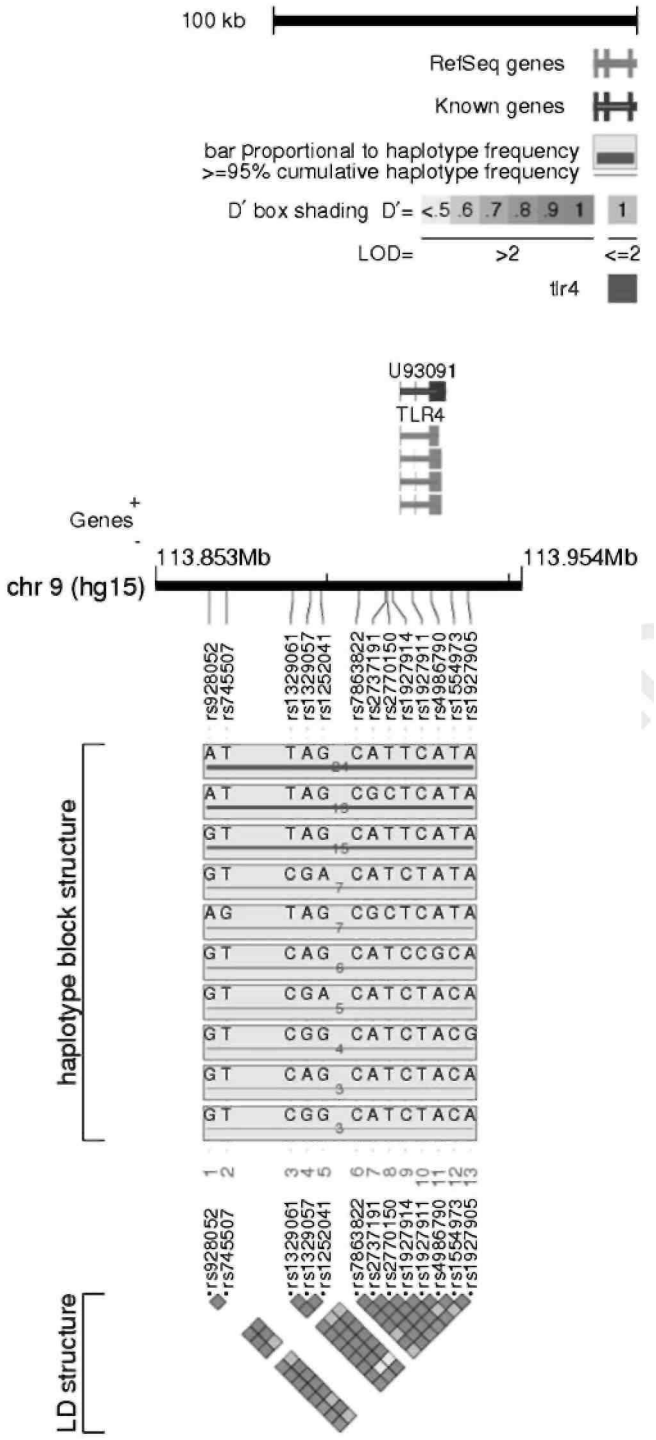
Figure 1: *Haplotype structure of TLR4. This diagram illustrates the haplotype structure of the genomic segment investigated during our study.* It was created using the LocusView software. The genomic segment of interest is outlined in black in the middle of the figure, and its physical boundaries are noted in megabases (Mb). Human genome assembly 16 (hg16) is the reference selected for these physical positions. Above the genomic segment, the position of the TLR4 gene is noted in pink and blue. Below the genomic segment, each of the thirteen SNPs used in the study is listed, and its position is indicated. Below the SNPs, the various haplotypes with >0.0l frequency in our subject sample IBD-1 (Canadian trios) are noted. Each haplotype is found within a gray box with its component alleles; the haplotype frequency is noted within each box and is also demonstrated visually by the blue bar. The thickness of the blue bar is directly proportional to the frequency of the haplotype. The SNPs are listed again below the haplotype portion of the diagram and serve as reference points for the demonstration of pairwise linkage disequilibrium; the color scheme is illustrated below; the intensity of the red color being proportional to the extent of D'.



Determining haplotypes

Many different protocols can be used to define boundaries of blocks of LD. We used the 'solid spine of LD' definition available in the Haploview program as the standard for this study[58] This definition allows for certain intervening polymorphisms not to be in LD with each other as long as the first and last markers are in strong LD with each other. The algorithm embedded in Haploview constructs multimarker haplotypes using the expectation maximization statistical algorithm[59] modified to process large data sets using the partition-ligation approach.[60] hg17 is used as the reference for all SNP positions.

Statistical analyses

Association testing. Genetic association was assessed by the transmission disequilibrium test for collections consisting of trios.[61] Association in cases and controls was determined by a standard χ^2 test done on a 2 x 2 contingency table. In both cases, the Haploview program was used as the primary analytical platform to implement these tests.[58]

Combining analyses.

For the primary outcome of the study, the results of each sample collection were produced independently and combined by calculating a Z-score and subsequently a P-value. Specifically, the number of risk alleles transmitted in trios or found in cases and controls was reformatted as a mean or expected count (E_T and E_c), observed count (O_T and O_c), and variance (var_T and var_c). A combined Z-score was then calculated by summation as $Z = [(O_T + O_c)-(E_T + E_c)]/\sqrt{(var_T + var_c)}$. Collections IBD-1 and -2 were combined in the screening phase of the study, and a two-tailed P-value was calculated from the combined Z-score. Collections IBD-3 to -5 were combined in the replication phase; as only the over-representation of a single allele or haplotype (based on the phase 1 results) is studied in the replication phase of the study, a one-tailed P-value was calculated for the replication Z-score.

Pooled analysis

We combined data from our study and from each relevant published study by calculating a Z-score as described in the preceding section. Since these pooled analyses are testing a single hypothesis (that a specific allele is associated with disease susceptibility), a one-tailed P-value was calculated from this Z-score to assess the statistical significance of the analysis. A Mantel-Haenszel approach was used to calculate a pooled OR.[36]

Assessing heterogeneity.

A Pearson χ^2 goodness-of-fit test to assess the level of genetic heterogeneity among sample populations was applied to both the study's data and data used in the pooled analyses as described previously[62]

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Web resources

PubMed (http://www.ncbi.nlm.nih.gov/entrez/quer-y.fcgi)
dbSNP (http://www.ncbi.nlm.nih.gov/entrez/quer-yfcgi?db= Snp)
UCSC Genome Browser (http://genome.ucsc.edu/)

Locus View (http://www.broad.mit.edu/mpg/locus-view/)
Haploview (www.broad.mit.edu/mpg/haploview/)
Genetic Power calculator (http://statgen.iop.kcl.a-c.uk/gpc/)

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