World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i23.2829

World J Gastroenterol 2011 June 21; 17(23): 2829-2837
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

NOD2 and *ATG16L1* polymorphisms affect monocyte responses in Crohn's disease

Dylan M Glubb, Richard B Gearry, Murray L Barclay, Rebecca L Roberts, John Pearson, Jacqui I Keenan, Judy McKenzie, Robert W Bentley

Dylan M Glubb, Department of Medicine, University of Chicago, IL 60637-1470, United States
Richard B Gearry, Murray L Barclay, Rebecca L Roberts, Department of Medicine, University of Otago, Christchurch, 8140, New Zealand
Richard B Gearry, Murray L Barclay, Department of Gastroenterology, Christchurch Hospital, Christchurch, 8140, New Zealand
Rebecca L Roberts, Department of Biochemistry, University of Otago, Dunedin, 9054, New Zealand
John Pearson, Department of Pathology, University of Otago, Christchurch, 8140, New Zealand
Jacqui I Keenan, Department of Surgery, University of Otago, Christchurch, 8140, New Zealand
Judy McKenzie, Department of Haematology, University of Otago, Christchurch, 8140, New Zealand
Robert W Bentley, Department of Paediatrics, University of Otago, Christchurch, 8140, New Zealand
Author contributions: Glubb DM, McKenzie J and Bentley RW performed the research; Glubb DM, Roberts RL, Bentley RW and Gearry RB designed the research; Pearson J, Glubb DM, Roberts RL and Bentley RW analyzed the data; Barclay ML and Gearry RB provided patient samples; all authors contributed to the writing of the manuscript.
Supported by Broad Medical Research Program of The Broad Foundation, Inflammatory Bowel Disease Grant IBD-0236. RLR is the recipient of a Sir Charles Hercus Health Research Fellowship from the Health Research Council of New Zealand
Correspondence to: Robert W Bentley, PhD, Department of Paediatrics, University of Otago, PO Box 4345, Christchurch 8140, New Zealand. robert.bentley@otago.ac.nz
Telephone: +64-3-3641558 Fax: +64-3-3640009
Received: July 1, 2010 Revised: September 30, 2010
Accepted: October 7, 2010
Published online: June 21, 2011

Abstract

AIM: To assess whether polymorphisms in *NOD2* and *ATG16L1* affect cytokine responses and mycobacterium avium subspecies paratuberculosis (MAP) survival in monocytes from Crohn's disease (CD) patients.

METHODS: Monocytes were isolated from peripheral blood of CD patients of known genotype for common single nucleotide polymorphisms of *NOD2* and *ATG16L1*. Monocytes were challenged with MAP and bacterial persistence assessed at subsequent time-points. Cytokine responses were assayed using a Milliplex multi-analyte profiling assay for 13 cytokines.

RESULTS: Monocytes heterozygous for a *NOD2* polymorphism (R702W, P268S, or 1007fs) were more permissive for growth of MAP (P = 0.045) than those without. There was no effect of *NOD2* genotype on subsequent cytokine expression. The T300A polymorphism of *ATG16L1* did not affect growth of MAP in our model (P = 0.175), but did increase expression of cytokines interleukin (IL)-10 (P = 0.047) and IL-6 (P = 0.019).

CONCLUSION: CD-associated polymorphisms affected the elimination of MAP from *ex vivo* monocytes (*NOD2*), or expression of certain cytokines (*ATG16L1*), implying independent but contributory roles in the pathogenesis of CD.

Key words: Inflammatory bowel disease; Mycobacterium avium subspecies paratuberculosis; Cytokine; CARD15; Autophagy

Peer reviewers: Shashi Bala, PhD, Post Doctoral Associate, Department of Medicine, LRB 270L, 364 Plantation street, UMass Medical School, Worcester, MA 01605, United States; Giuliana Decorti, MD, PhD, Department of Life Sciences, University of Trieste, Via L. Giorgieri n° 7, Trieste 34127, Italy

Glubb DM, Gearry RB, Barclay ML, Roberts RL, Pearson J, Keenan JI, McKenzie J, Bentley RW. *NOD2* and *ATG16L1* polymorphisms affect monocyte responses in Crohn's disease. *World J Gastroenterol* 2011; 17(23): 2829-2837 Available from: URL:

http://www.wjgnet.com/1007-9327/full/v17/i23/2829.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i23.2829

INTRODUCTION

Crohn's disease (CD) has been proposed as being the product of chronic inflammation caused by a dysfunctional interaction between the intestinal immune system and commensal gut microbiota[1]. The inflammation seen in CD is characterized by pronounced Th1 and Th17/23 responses involving the cytokines interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, IFN-γ[1-3], IL-23 and IL-17[4-6].

Although the commensal intestinal microflora appear to play an important role in the etiology of CD, certain bacterial species have also been implicated as putative causal agents of CD. These include Pseudomonas maltophilia[7], Chlamydia trachomatis[8], Bacteroides fragilis[9] Yersinia species[10], adherent invasive *Escherichia coli* (*E. coli*)[11] and Mycobacterium avium subspecies paratuberculosis (MAP)[12]. The role of MAP in the etiology of CD is unclear. However, MAP causes chronic intestinal inflammation in ruminants (Johne's disease) with similar pathophysiology to CD[13] and it has been reported as a causative agent of regional enteritis with similarities to CD in a number of monogastrics, including two primate species[14,15]. MAP has also been cultured from both resected tissue[16] and peripheral blood of CD patients[17], and has been visualized inside macrophages from CD patients[18]. Meta-analyses of epidemiological studies confirm an association of MAP with CD[19,20], although it remains unknown whether MAP is pathogenic in humans, or whether this association reflects a defective host immune system permissive for the survival of MAP.

Twin and family studies have demonstrated a significant genetic component to the development and progression of CD[21-23]. Linkage analysis, candidate gene approaches and, most recently, genome-wide association studies (GWAS) have identified over 30 risk genes for CD[24-26], many of which are involved with bacterial recognition (e.g. *NOD2*) or processing and elimination of bacteria through the autophagy pathway (e.g. *IRGM*[27] and *ATG16L1*[25]).

In this study, we developed an *ex vivo* monocyte model to assess the impact of the CD-associated single nucleotide polymorphisms (SNPs) in *NOD2* (rs2066842, P268S; rs2066844, R702W; and 1007fs, rs2066847) and *ATG16L1* (rs2241880, T300A) on cytokine responses to the putative pathogen MAP. MAP can survive and replicate within phagocytic cells, and consequently we also evaluated the impact of these polymorphisms on the intracellular persistence of MAP. The use of *ex vivo* monocytes allows functional evaluation of SNPs associated with CD, and may provide a more realistic insight into the impact that genotype has on CD compared to studies which involve abrogation of protein expression or whole gene deletion.

MATERIALS AND METHODS

Patient recruitment

Patients for the current study were selected from a New Zealand population-based Caucasian inflammatory bowel disease (IBD) cohort recruited to investigate genetic and environmental factors that contribute to IBD etiology[28-32]. Inclusion criteria for the current study were a confirmed diagnosis of CD and negative MAP status as ascertained by IS900 PCR in peripheral blood[28].

The genotype combinations and patient phenotype information are detailed in Table 1. Briefly, the potential impact of *ATG16L1* and *NOD2* polymorphisms were assessed separately. For analysis of *ATG16L1*, monocytes were collected from CD patients who had a wild-type *NOD2* genotype and were homozygous for either the major (G) allele (n = 6) or minor (A) allele (n = 6) of *ATG16L1* 1138G > A (rs2241880). Conversely, for experiments evaluating the effect of *NOD2* genotype, monocytes were collected from patients who were *ATG16L1* 1138G homozygotes and were heterozygous for one of the three *NOD2* SNPs previously associated with CD; 2104C > T (R702W, rs2066844), 2722G > C (G908R, rs2066845), or 3020insC (1007fs, rs2066847), and were heterozygous for the background variant 802C > T (P268S, rs2066842). A total of 12 patients were recruited, 6 carried the polymorphisms described, and 6 were homozygous at these *NOD2* loci (Table 1). None of the patients included in this study had the SNPs rs13361189 and rs4958847. These SNPs are in complete linkage disequilibrium with a 20 kb insertion/deletion polymorphism which has been shown to alter expression of the autophagy gene *IRGM*[33].

Preparation of monocytes from peripheral blood

Blood (40 mL) was drawn into heparin tubes (Sigma-Aldrich), divided into 20 mL aliquots, and 15 mL of Phosphate Buffered Saline (PBS) was added to each. Ficoll-Paque™ PREMIUM (10 mL) (GE Healthcare Bio-Sciences Uppsala, Sweden) was layered under each aliquot and the samples centrifuged (1000 × *g*, 20 min). Mononuclear cells collected from the interface were added to 30 mL of PBS, centrifuged (350 × *g*, 10 min) and resuspended in 15 mL of PBS. In order to standardize the number of monocytes used in experiments, mononuclear cells were enumerated using a hemocytometer and a 200 μL aliquot was analyzed on a Beckman Coulter FC500 MPL flow cytometer to determine the percentage of monocytes based on forward and side scatter characteristics. After centrifugation (350 × *g*, 10 min), cells were resuspended in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) to a concentration of 4 × 10^5 monocytes/mL. Then 500 μL of this preparation were plated per well in a 24-well tissue culture plate (Nunc, Roskilde, Denmark). Monocytes were left to adhere for 1 h in a humidified incubator at 37℃ with 5% CO_2 and non-adherent cells were removed by washing three times with 1 mL of room temperature (RT) PBS.

Table 1 ***IRGM*, *ATG16L1*, and *NOD2* genotypes of Crohn's disease patients from whom monocytes were collected for *ex vivo* experiments**

	IRGM		*ATG16L1*	*NOD2*				Clinical characteristics			
Patient	rs13361189[1]	rs4958847[1]	T300A 1138G > A rs2241880	R702W 2104C > T rs2066844	P268S 802C > T rs2066842	G908R 2722G > C rs2066845	1007fs 3020insC rs2066847	Gender	Time since diagnosis (yr)	Disease location	Harvey bradshaw index[2]
1	TT	GG	AA	CC	CC	GG	00	M	53	Ileal	0
2	TT	GG	AA	CC	CC	GG	00	M	5	Ileo-colonic	0
3	TT	GG	AA	CC	CC	GG	00	F	5	Colonic	0
4	TT	GG	GG	CC	CC	GG	00	M	25	Ileo-colonic	2
5	TT	GG	AA	CC	CC	GG	00	F	6	Ileo-colonic	1
6	TT	GG	AA	CC	CC	GG	00	F	9	Ileal	3
7	TT	GG	AA	CC	CC	GG	00	M	1	Ileal	0
8	TT	GG	GG	CC	CC	GG	00	M	6	Colonic	2
9	TT	GG	GG	CC	CC	GG	00	F	10	Colonic	5
10	TT	GG	GG	CC	CC	GG	00	M	24	Ileo-colonic	1
11	TT	GG	GG	CC	CC	GG	00	F	11	Colonic	0
12	TT	GG	GG	CC	CC	GG	00	F	7	Ileo-colonic	4
13	TT	GG	GG	CT	CT	GG	00	F	5	Colonic	4
14	TT	GG	GG	CT	CT	GG	00	F	9	Ileo-colonic	0
15	TT	GG	GG	CT	CT	GG	00	M	17	Ileal	0
16	TT	GG	GG	CT	CT	GG	00	F	18	Colonic	4
17	TT	GG	GG	CC	CT	GG	0C	M	7	Ileal	1
18	TT	GG	GG	CT	CT	GG	00	F	16	Ileo-colonic	4

[1]SNPs located upstream (5') to *IRGM*; [2]Non-invasive clinical index used to assess disease activity in patients with Crohn's disease. A score of ⩾ 7 indicates active disease.

Adherent monocytes were incubated overnight in 500 μL of RPMI 1640/10% FBS.

Culture of MAP

MAP strain Dominic (ATCC 43545) was inoculated into 10 mL volumes of Difco™ Middlebrook 7H9 broth (BD Biosciences, Sparks, MD, USA) supplemented with 10% v/v BBL™ Middlebrook OADC Enrichment (BD Biosciences), 0.05% v/v Tween 80 (Sigma-Aldrich, St Louis, MO, USA) and 2 mg/mL Mycobactin J (Allied Monitor, Fayette, MO, USA) and grown at 37℃. A standard growth curve of MAP was obtained by measuring the optical density at 600 nm (OD_{600}) of an aliquot of bacterial suspension during the log phase, which had been passed repeatedly through a 25-gauge needle, and enumerating the bacteria by plating on the same medium supplemented with 1.5% agar (Invitrogen, Carlsbad, CA, USA).

Monocyte challenge experiments

Bacteria were grown to mid log phase (approximately 1×10^8 cells/mL, with reference to previously obtained growth curve data) and harvested by centrifugation (13000 × *g*, 5 min). Bacteria were resuspended in 500 μL of PBS and passed ten times through a 25-gauge needle to break up clumps of cells. One 500 μL aliquot of MAP was heat-inactivated at 90℃ for 5 min and then both aliquots were diluted to 4×10^6 cells/mL in RPMI 1640/10% FBS. The optimal temperature and incubation time for heat-inactivation had been previously confirmed by plating heat-treated MAP cells onto agar. Growth medium was removed from the monocytes and 500 μL of the MAP suspension was added to each well. Challenge experiments were performed in triplicate for each time-point/genotype combination. After incubation for 4 h at 37℃, 5% CO_2, the supernatant was removed and stored at -80℃ as a zero time-point sample for cytokine assays. Monolayers were washed three times with 1 mL of PBS to remove extracellular bacteria. A zero time-point lysate to assess bacterial uptake and persistence was removed by incubating 500 μL of a 0.1% sodium deoxycholate (Sigma-Aldrich, St Louis, MO, USA) solution with the monolayer for 5 min at RT. Lysates were centrifuged (13000 × *g*, 5 min) and resuspended in 200 μL of PBS. Serial dilutions of the lysates were made with PBS and 50 μL were spread on to a Middlebrook agar plate. The agar plates were left to incubate for four weeks at 37℃ before counting MAP colonies. Subsequent samples at time-points of 24, 48, 72 and 96 h were similarly processed.

Confirmation of genotypes of study participants

ATG16L1 genotypes were confirmed at recruitment by direct DNA sequencing of PCR products. Briefly, genomic DNA was extracted from fresh peripheral blood using GenElute™ (Sigma-Aldrich, St Louis, MO, USA) spin columns according to manufacturer's protocols. A 480 bp fragment containing the *ATG16L1* 1138G > A SNP was amplified for DNA sequencing from patient genomic DNA using the following primers: 5'-CCACAGGTTAGTGTGCAGGA-3' (forward primer) and 5'-CACAGCTGACAGAGCCAAAA-3' (reverse primer). PCR was carried out in a 20 μL volume containing 1 μL of genomic DNA, 0.3 μmol/L of each primer, 200 μmol/L dNTPs, 0.75 mmol/L $MgCl_2$, 1 × TAQ-Ti reaction buffer (Thermo Fisher Scientific, Pittsburgh, PA, USA) and 0.25 U of TAQ-Ti DNA polymerase (Thermo Fisher Scientific). After an initial denaturation step of 94℃ for 2 min, 35 cycles were performed at 94℃ for 30 s, 60℃ for 30 s and 72℃ for 30 s. Five microlitres of each PCR product was checked on 1% agarose. Another 5 μL aliquot was

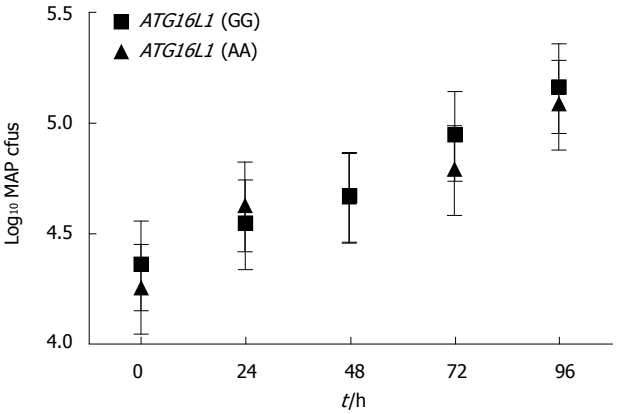

Figure 1 Effect of *ATG16L1* T300A variant on mycobacterium avium subspecies paratuberculosis numbers. Time course of mycobacterium avium subspecies paratuberculosis (MAP) growth in the context of the *ATG16L1* 1138 G > A variant. Monocytes were derived from subjects homozygous for either *ATG16L1* allele and carrying *NOD2* wild-type alleles (n = 6 for each group). MAP growth is expressed as colony forming units (cfu).

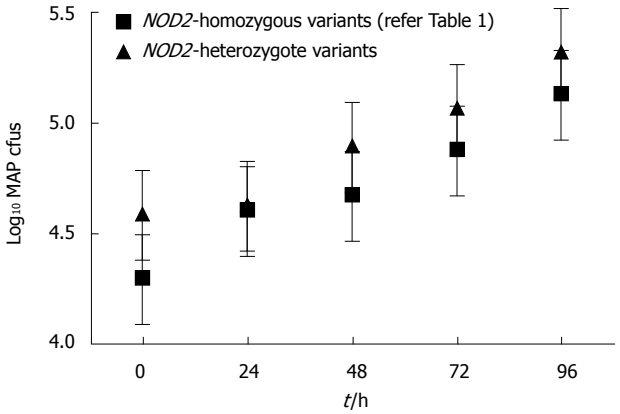

Figure 2 Effect of *NOD2* variants on mycobacterium avium subspecies paratuberculosis numbers. Time course of mycobacterium avium subspecies paratuberculosis (MAP) growth in the context of *NOD2* genetic variation. Monocytes were derived from subjects homozygous for the *ATG16L1* 1138G allele and with/without *NOD2* genetic variants (n = 6 for each group). MAP growth is expressed as colony forming units (cfu).

purified with Exo-SAP-IT (USB Corporation, Ohio, USA) and sequenced using BigDye chemistry (Applied Biosystems, California, USA) on an ABI 3730 Genetic Analyzer (Foster City, California, USA). The *NOD2* and *IRGM* genotypes of study participants were established as previously described using allele-specific PCR and pre-designed TaqMan SNP genotyping assays, respectively[34,35].

Multiplex cytokine analysis

Cytokine analysis was performed using a 13-plex MILLIPLEX™ MAP human cytokine kit according to manufacturer's recommendations (Millipore) for the following: IFNγ, IL-10, IL-12p40, IL-12p70, IL-17, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8, TNFα, TNFβ.

Statistical analysis

Data from the five time-points were analyzed by repeated measures ANOVA, with fixed effects for time (as categorical variable) and random effects for subject i.e. genotype. For cytokine analyses, where readings were below the threshold for detection (3.2 pg/mL) they were included as 3.2 pg/mL. Results were considered significant at $P \leqslant 0.05$.

Ethical considerations

Informed written consent was obtained from all participants in this study and ethical approval for this work was granted by the Upper South B Regional Ethics Committee of New Zealand.

RESULTS

Bacterial persistence ATG16L1

Numbers of MAP increased from a mean $\log_{10}$ colony forming units (cfu) of 4.24 at 0 h to 5.08 at 96 h for the AA genotype, and from log10 cfu of 4.47 to 5.29 for the GG genotype (Figure 1). There was no evidence (T-testing) for the effect of the *ATG16L1* T300A polymorphism on MAP numbers at any individual time-point, or overall (pooled time-points ANOVA, $P = 0.175$) (Figure 1).

NOD2

MAP also grew in the monocytes with different *NOD2* genotypes, from a mean $\log_{10}$cfu of 4.30 at 0 h to 5.12 for the cells homozygous for the major alleles of the *NOD2* variants, and from $\log_{10}$cfu of 4.58 to 5.42 for cells heterozygous for any *NOD2* variant (Figure 2). There was no significant effect (*T*-testing) of *NOD2* genotype on MAP numbers at any individual time-point. However, analysis of all time-points indicated that monocytes heterozygous for a *NOD2* polymorphism were more permissive for growth of MAP (ANOVA, $P = 0.045$) (Figure 2).

Multiplex cytokine panels

Thirteen cytokines were evaluated from *ex vivo* monocyte supernatants using a MILLIPLEX™ MAP human cytokine kit. Each time-point/genotype combination was assayed in triplicate. All assays passed quality controls and r^2 values for the standard curves were ⩾ 0.99.

Of the thirteen cytokines analyzed in the multiplex format, only four, IL-10, IL-6, IL-8 and TNFα, had measurable responses above the detection threshold (> 3.2 pg/mL) on the multiplex ELISA platform used. Where samples had values of < 3.2 pg/mL, they were considered to be 3.2 pg/mL for statistical purposes.

Effect of ATG16L1 genotype

Cytokine expression results are shown in Figure 3. The AA genotype of *ATG16L1* was associated with greater expression of cytokines IL-10 and IL-6 in response to challenge with MAP ($P = 0.047$ and $P = 0.019$, respectively). No significant difference was seen between *ATG16L1* genotypes AA and GG for expression of either IL-8 or TNFα ($P = 0.758$ and $P = 0.289$, respectively) (Figure 3).

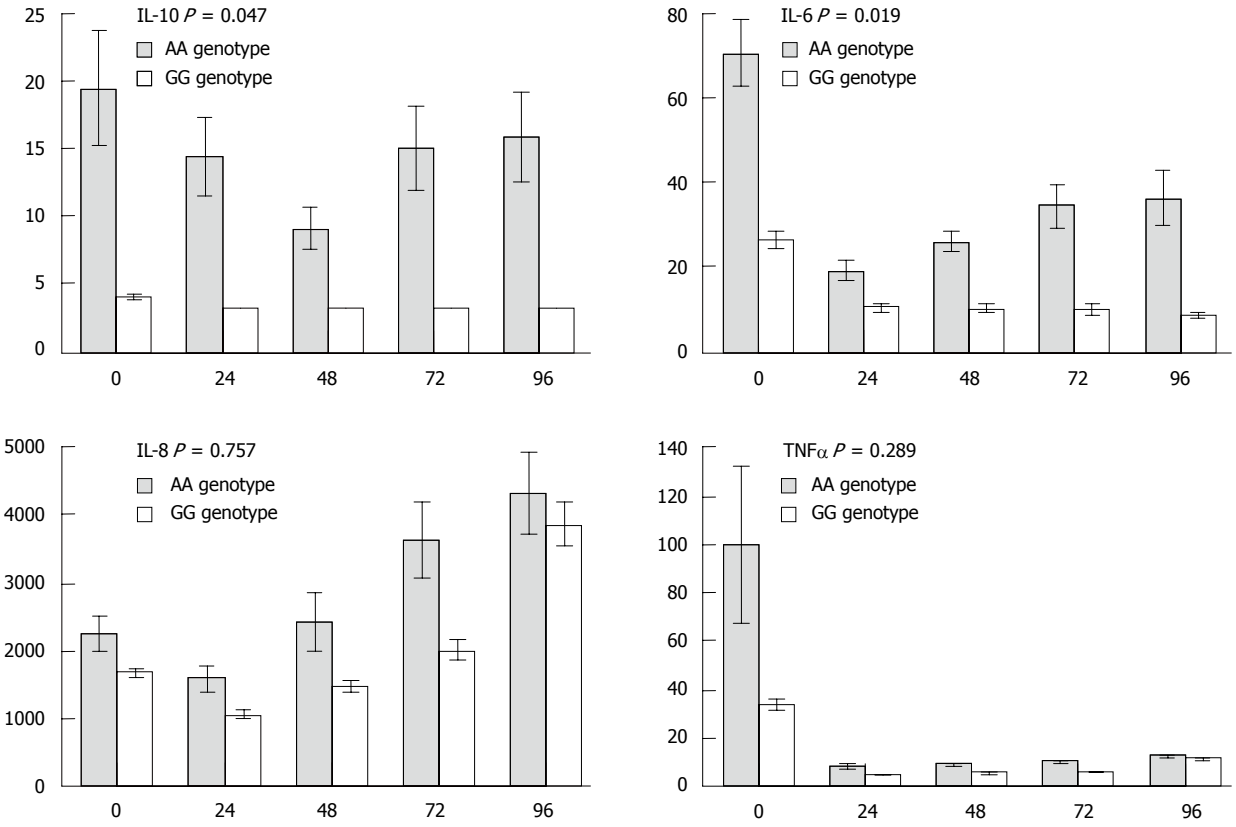

Figure 3 Effect of the *ATG16L1* T300A polymorphism on expression of cytokines following challenge with mycobacterium avium subspecies paratuberculosis. Cytokine concentrations are in pg/mL. Results are the means (and standard error of the mean) of triplicate samples from patients in Table 1. *P*-values are derived from repeated measures ANOVA testing for the effect of genotype on cytokine expression. Fixed effects for time (categorical variable) and *ATG16L1* genotype, random effect for subject. IL: Interleukin; TNF: Tumor necrosis factor.

Effect of NOD2 genotype

Cytokine expression changes for IL-10, IL-6, IL-8 and TNFα by cells of different *NOD2* genotypes are shown in Figure 4. In general, the presence of a *NOD2* polymorphism resulted in a trend of lower expression of these four cytokines throughout the time-course, compared to monocytes without *NOD2* variants.

However, this effect was not significant, with P-values of 0.56, 0.32, 0.41 and 0.97 for cytokines IL-10, IL-6, IL-8 and TNFα, respectively (Figure 4).

DISCUSSION

Numerous genetic association studies have indicated a strong role for *NOD2* and *ATG16L1* in the etiology of CD. However, functional studies have yet to fully elucidate whether, and to what extent, polymorphic variation in these genes affects bacterial clearance and inflammation in CD. Models of gene/environment interaction have mostly used epithelial or monocyte cell lines in vitro with bacterial pathogens such as Salmonella typhimurium that are not generally associated with CD. Also, the use of gene silencing or ablation techniques in these models may assist our understanding of the function of these genes, but may not be representative of the effects of SNPs which have been associated with CD. In this study we developed an *ex vivo* cellular model using monocytes from CD patients and a putative CD pathogen, MAP, in order to assess the effect of SNPs in *NOD2* and *ATG16L1* on bacterial survival and subsequent inflammatory response.

NOD2 is located in the cytoplasm, and plays an important role in cellular responses to bacterial infection[36] through recognition of muramyl dipeptide (MDP), a subunit of bacterial peptidoglycan (PGN)[37]. Furthermore, the R702W, G908R and 1007fs *NOD2* variants, which confer susceptibility to CD, have been shown to impair responses to the bacterial antigen lipopolysaccharide[38].

We found the effect of *NOD2* polymorphisms appeared to be primarily on bacterial persistence/growth, with heterozygosity at R702W or 1007fs making monocytes from CD patients significantly more permissive to growth of MAP. Indeed, a recent study has observed that these variants are associated with an impairment of monocyte phagocytosis and the development of bacteremia in intensive care unit patients[39]. Despite the increased bacterial load, no differences were seen in subsequent cytokine responses for the different host *NOD2* genotypes. Whilst there has been no prior published research with respect to MAP in this type of model of CD, the functional effects of *NOD2* SNPs have been examined for adherent invasive *E. coli* (AIEC). No differences were found in the persistence or growth of AIEC in monocytes from patients who were heterozygous or homozygous for the minor allele of *NOD2* variants R702W, G908R and 1007fs compared to

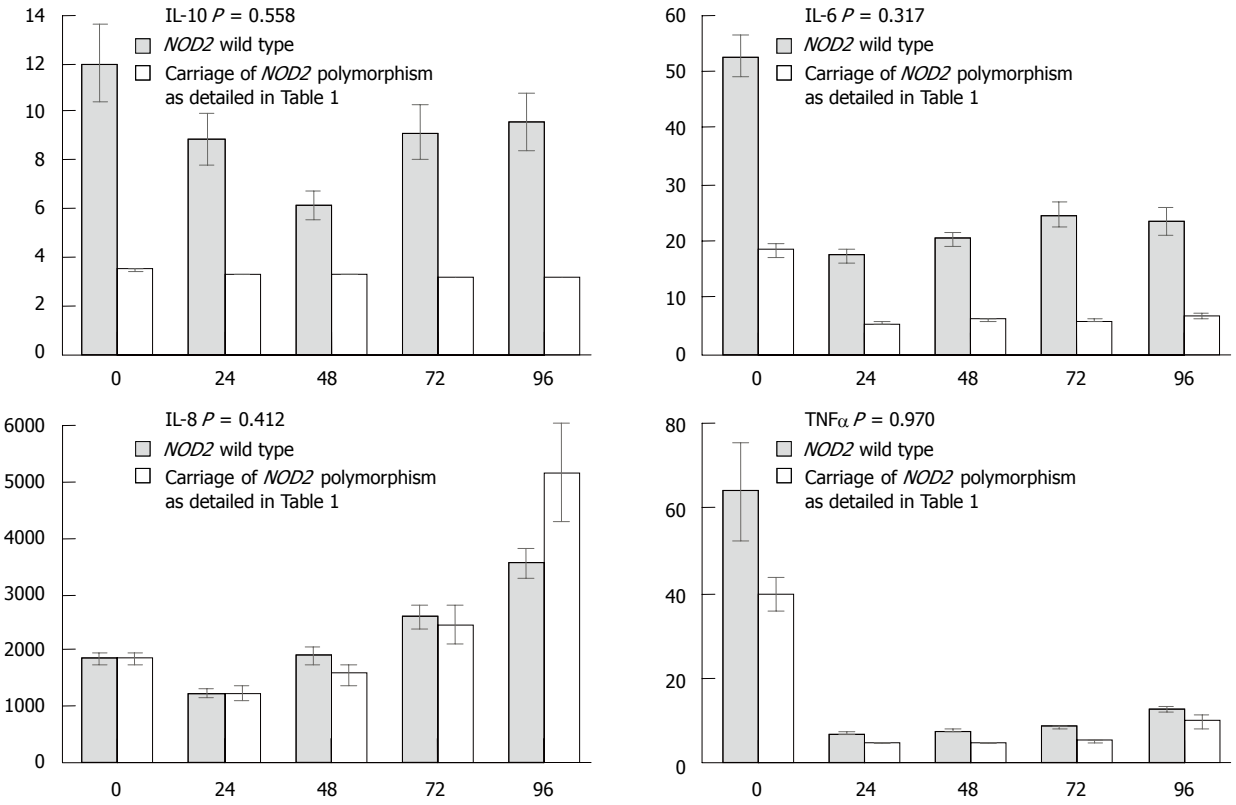

Figure 4 Effect of *NOD2* polymorphisms on expression of cytokines following challenge with mycobacterium avium subspecies paratuberculosis. Cytokine concentrations are in pg/mL. Results are the means (and standard error of the mean) of triplicate samples from patients in Table 1. *P*-values are derived from repeated measures ANOVA testing for the effect of genotype on cytokine expression. Fixed effects for time (categorical variable) and *NOD2* genotype, random effect for subject. IL: Interleukin; TNF: Tumor necrosis factor.

NOD2 major allele homozygotes[40]. It has also been shown that in peripheral blood mononuclear cells from CD patients, homozygosity for R702W does not affect the cytokine response to Gram-negative (*Helicobacter pylori*) bacterial peptidoglycan[41], although homozygosity for the 1007 fs mutation did. In our study we recruited no 1007fs homozygotes and only a single 1007 fs heterozygote so were unable to make any direct comparisons with this previous study. Comparative studies for a broader range of bacterial species in *ex vivo* cells from patients who are homozygous for these *NOD2* SNPs will help clarify the role of this genotype in disease pathogenesis.

Both MAP and AIEC are capable of intracellular survival and growth in monocytes and macrophages, and it appears that the presence of *NOD2* polymorphisms may influence their respective intracellular survival and growth in different ways[42].

Autophagy has been identified as a mechanism for clearing intracellular pathogens, and two autophagy genes, *ATG16L1* and *IRGM*, have been associated with CD[25,27]. In our study, we investigated the effect of the *ATG16L1* T300A polymorphism, and controlled for genotypic variation in *IRGM* (Table 1). *ATG16L1* T300A (rs2241880; 1138G > A) is a common non-synonymous SNP where the G major allele confers greater disease risk and results in a threonine-to-alanine substitution at amino acid position 300 of the *ATG16L1* protein (T300A). This SNP appears to account for all of the disease risk conferred by this locus[43], and functionally, this polymorphism has been proposed to contribute to defective macrophage killing of bacteria[44]. This assertion is supported by the results of two in vitro studies. Kuballa *et al*[45] found that the T300A variant impaired handling and autophagy of Salmonella within human epithelial cells, and Lapaquette *et al*[46] showed that siRNA knockdown of *ATG16L1* led to loss of autophagy of intracellular AIEC bacteria by HeLa cells. Transfection of affected HeLa cells with wild-type *ATG16L1* restored autophagic function, whereas transfection with the T300A polymorphic form did not. In contrast, *ATG16L1* T300A had no effect on the survival of either S. typhimurium or group A Streptococcus in mouse embryonic fibroblasts[47]. Although direct comparison of different model systems is complex and potentially misleading, our results with MAP in *ex vivo* monocytes generate the question as to whether the T300A polymorphism of *ATG16L1* affects autophagic clearance of certain intracellular bacteria as profoundly as indicated by knockdown or silencing models of gene function.

In our study, the T300A polymorphism was associated with significant changes in production of the cytokines IL-6 and IL-10 in response to bacterial challenge with MAP. These two cytokines are components of the Th1 (pro-inflammatory) and Th2 (modulatory) pathways of inflammation, respectively, and it is likely that CD results from an imbalance between these two pathways. It is tempting to speculate that the relative levels of the two

cytokines that were induced are indicative of an imbalance, but extrapolating from a very specific model to describe a complex disease state would be misleading and clearly further comparative work is required in this area in disease-relevant models.

Our study is the first to investigate the effect of *NOD2* and *ATG16L1* genotype on the response of *ex vivo* human monocytes to the putative CD pathogen MAP. Although our results are preliminary and need to be replicated in a larger sample, they provide novel insights into the effect of disease-associated SNPs in innate immunity genes on detection, handling, and elimination of bacteria, and ultimately CD pathogenesis. Our observations indicate that *NOD2* SNPs R702W, P268S, or 1007 fs impair the elimination of MAP yet do not impact on cytokine production. They may, therefore, increase susceptibility to prolonged intracellular bacterial infection. Conversely, the *ATG16L1* T300A polymorphism significantly alters the expression of certain Th1 and Th2 cytokines after MAP challenge, but does not seem to affect the autophagic clearance of this putative CD pathogen.

ACKNOWLEDGMENTS

We thank the people of Canterbury with IBD who generously gave of their time to take part in the study. We also thank Rhondda Brown and Judy Hoar for their assistance in coordinating the recruitment of patients to the Canterbury IBD cohort.

COMMENTS

Background

Polymorphisms of the genes *NOD2* and *ATG16L1* have been associated with susceptibility to Crohn's disease (CD). These genes are important for an effective innate immune response against potential bacterial pathogens [such as *Mycobacterium avium* subspecies *paratuberculosis* (MAP)] which may trigger or exacerbate inflammation. Monocytes from CD patients of known genotype were used to determine whether polymorphisms in *NOD2* and *ATG16L1* alter cytokine responses and bacterial survival following challenge with MAP.

Research frontiers

Previous research has investigated the role of polymorphisms in *NOD2* and *ATG16L1* in various model systems. In general, these have used gene silencing strategies that may not realistically reflect the biological consequences of single nucleotide changes in these genes, or model bacterial pathogens that have little relevance to CD. None have reported the functional consequences of the naturally occurring single nucleotide polymorphisms using patient-derived cells and bacteria such as MAP that have been implicated in CD etiology.

Innovations and breakthroughs

The authors findings indicate that monocytes heterozygous for a *NOD2* polymorphism were more permissive for the intracellular growth of MAP than those without. However, these polymorphisms did not affect subsequent cytokine expression. The T300A polymorphism of *ATG16L1* did not affect growth of MAP in our monocyte model but did result in increased expression of certain cytokines - interleukin (IL)-10 and IL-6.

Applications

By understanding how naturally occurring disease-related polymorphisms of *NOD2* and *ATG16L1* influence bacterial survival and also the production of inflammatory mediators, the authors may gain insight into the contribution of these genetic changes to the function of the host innate immune system. Development of this model system that utilizes patient cells with known single nucleotide changes in key CD-susceptibility genes will provide another research tool to assist better understanding of disease pathogenesis related to bacterial handling.

Terminology

NOD2 (CARD15) - nucleotide oligomerization domain 2 - is a cytosolic pattern recognition receptor that recognizes muramyl dipeptide, a component of bacterial peptidoglycan. Polymorphisms in *NOD2* have been associated with altered susceptibility to CD in many genetic studies. *ATG16L1* - autophagy-related 16-like 1 - is a key component of the autophagic apparatus that is involved with uptake and digestion of intracellular bacteria. The T300A polymorphism of *ATG16L1* has also been associated with altered susceptibility to CD. MAP is an intracellular bacterium that has been cited in several studies as a putative causal agent of CD.

Peer review

This is a very well-designed and well-written study, with interesting and important scientific merit. Not just simple polymorphism descriptions, but their effect on human monocyte cytokine production and intracellular pathogen survival were examined with a very functional methodology. Their *ex vivo* model is much closer to the real pathogenesis of CD than any earlier one. Using the author's concept, more descriptive polymorphism analysis of CD and other diseases may be placed into functional analysis.

REFERENCES

1 **Packey CD**, Sartor RB. Interplay of commensal and pathogenic bacteria, genetic mutations, and immunoregulatory defects in the pathogenesis of inflammatory bowel diseases. *J Intern Med* 2008; **263**: 597-606
2 **Reimund JM**, Wittersheim C, Dumont S, Muller CD, Kenney JS, Baumann R, Poindron P, Duclos B. Increased production of tumour necrosis factor-alpha interleukin-1 beta, and interleukin-6 by morphologically normal intestinal biopsies from patients with Crohn's disease. *Gut* 1996; **39**: 684-689
3 **Hisamatsu T**, Suzuki M, Reinecker HC, Nadeau WJ, McCormick BA, Podolsky DK. CARD15/*NOD2* functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology* 2003; **124**: 993-1000
4 **Annunziato F**, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, Parente E, Filì L, Ferri S, Frosali F, Giudici F, Romagnani P, Parronchi P, Tonelli F, Maggi E, Romagnani S. Phenotypic and functional features of human Th17 cells. *J Exp Med* 2007; **204**: 1849-1861
5 **Kamada N**, Hisamatsu T, Okamoto S, Chinen H, Kobayashi T, Sato T, Sakuraba A, Kitazume MT, Sugita A, Koganei K, Akagawa KS, Hibi T. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease *via* IL-23/IFN-gamma axis. *J Clin Invest* 2008; **118**: 2269-2280
6 **Kobayashi K**, Blaser MJ, Brown WR. Immunohistochemical examination for mycobacteria in intestinal tissues from patients with Crohn's disease. *Gastroenterology* 1989; **96**: 1009-1015
7 **Graham DY**, Yoshimura HH, Estes MK. DNA hybridization studies of the association of Pseudomonas maltophilia with inflammatory bowel diseases. *J Lab Clin Med* 1983; **101**: 940-954
8 **Orda R**, Samra Z, Levy Y, Shperber Y, Scapa E. Chlamydia trachomatis and inflammatory bowel disease--a coincidence? *J R Soc Med* 1990; **83**: 15-17
9 **Darfeuille-Michaud A**, Boudeau J, Bulois P, Neut C, Glasser AL, Barnich N, Bringer MA, Swidsinski A, Beaugerie L, Colombel JF. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology* 2004; **127**: 412-421
10 **Kallinowski F**, Wassmer A, Hofmann MA, Harmsen D, Heesemann J, Karch H, Herfarth C, Buhr HJ. Prevalence of enteropathogenic bacteria in surgically treated chronic inflammatory bowel disease. *Hepatogastroenterology* 1998; **45**: 1552-1558
11 **Rolhion N**, Darfeuille-Michaud A. Adherent-invasive Escherichia coli in inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**: 1277-1283
12 **McFadden JJ**, Butcher PD, Chiodini R, Hermon-Taylor J.

Crohn's disease-isolated mycobacteria are identical to Mycobacterium paratuberculosis, as determined by DNA probes that distinguish between mycobacterial species. *J Clin Microbiol* 1987; **25**: 796-801
13 **Greenstein RJ**. Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease. *Lancet Infect Dis* 2003; **3**: 507-514
14 **McClure HM**, Chiodini RJ, Anderson DC, Swenson RB, Thayer WR, Coutu JA. Mycobacterium paratuberculosis infection in a colony of stumptail macaques (Macaca arctoides). *J Infect Dis* 1987; **155**: 1011-1019
15 **Zwick LS**, Walsh TF, Barbiers R, Collins MT, Kinsel MJ, Murnane RD. Paratuberculosis in a mandrill (Papio sphinx). *J Vet Diagn Invest* 2002; **14**: 326-328
16 **Chiodini RJ**, Van Kruiningen HJ, Merkal RS, Thayer WR Jr, Coutu JA. Characteristics of an unclassified Mycobacterium species isolated from patients with Crohn's disease. *J Clin Microbiol* 1984; **20**: 966-971
17 Naser SA, Ghobrial G, Romero C, Valentine JF. Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease. *Lancet* 2004; **364**: 1039-1044
18 **Gearry RB**, Aitken JM, Roberts RL, Ismail S, Keenan J, Barclay ML. Images of interest. Gastrointestinal: Mycobacterium avium paratuberculosis and Crohn's disease. *J Gastroenterol Hepatol* 2005; **20**: 1943
19 **Abubakar I**, Myhill D, Aliyu SH, Hunter PR. Detection of Mycobacterium avium subspecies paratuberculosis from patients with Crohn's disease using nucleic acid-based techniques: a systematic review and meta-analysis. *Inflamm Bowel Dis* 2008; **14**: 401-410
20 **Feller M**, Huwiler K, Stephan R, Altpeter E, Shang A, Furrer H, Pfyffer GE, Jemmi T, Baumgartner A, Egger M. Mycobacterium avium subspecies paratuberculosis and Crohn's disease: a systematic review and meta-analysis. *Lancet Infect Dis* 2007; **7**: 607-613
21 **Orholm M**, Binder V, Sørensen TI, Rasmussen LP, Kyvik KO. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. *Scand J Gastroenterol* 2000; **35**: 1075-1081
22 **Satsangi J**, Grootscholten C, Holt H, Jewell DP. Clinical patterns of familial inflammatory bowel disease. *Gut* 1996; **38**: 738-741
23 **Spehlmann ME**, Begun AZ, Burghardt J, Lepage P, Raedler A, Schreiber S. Epidemiology of inflammatory bowel disease in a German twin cohort: results of a nationwide study. Inflamm *Bowel Dis* 2008; **14**: 968-976
24 **Barrett JC**, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, Brant SR, Silverberg MS, Taylor KD, Barmada MM, Bitton A, Dassopoulos T, Datta LW, Green T, Griffiths AM, Kistner EO, Murtha MT, Regueiro MD, Rotter JI, Schumm LP, Steinhart AH, Targan SR, Xavier RJ, Libioulle C, Sandor C, Lathrop M, Belaiche J, Dewit O, Gut I, Heath S, Laukens D, Mni M, Rutgeerts P, Van Gossum A, Zelenika D, Franchimont D, Hugot JP, de Vos M, Vermeire S, Louis E, Cardon LR, Anderson CA, Drummond H, Nimmo E, Ahmad T, Prescott NJ, Onnie CM, Fisher SA, Marchini J, Ghori J, Bumpstead S, Gwilliam R, Tremelling M, Deloukas P, Mansfield J, Jewell D, Satsangi J, Mathew CG, Parkes M, Georges M, Daly MJ. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet* 2008; **40**: 955-962
25 **Rioux JD**, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A, Green T, Kuballa P, Barmada MM, Datta LW, Shugart YY, Griffiths AM, Targan SR, Ippoliti AF, Bernard EJ, Mei L, Nicolae DL, Regueiro M, Schumm LP, Steinhart AH, Rotter JI, Duerr RH, Cho JH, Daly MJ, Brant SR. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* 2007; **39**: 596-604
26 **Hampe J**, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, Albrecht M, Mayr G, De La Vega FM, Briggs J, Günther S, Prescott NJ, Onnie CM, Häsler R, Sipos B, Fölsch UR, Lengauer T, Platzer M, Mathew CG, Krawczak M, Schreiber S. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in *ATG16L1*. *Nat Genet* 2007; **39**: 207-211
27 **Singh SB**, Davis AS, Taylor GA, Deretic V. Human *IRGM* induces autophagy to eliminate intracellular mycobacteria. *Science* 2006; **313**: 1438-1441
28 **Bentley RW**, Keenan JI, Gearry RB, Kennedy MA, Barclay ML, Roberts RL. Incidence of Mycobacterium avium subspecies paratuberculosis in a population-based cohort of patients with Crohn's disease and control subjects. *Am J Gastroenterol* 2008; **103**: 1168-1172
29 **Bentley RW**, Pearson J, Gearry RB, Barclay ML, McKinney C, Merriman TR, Roberts RL. Association of higher DEFB4 genomic copy number with Crohn's disease. *Am J Gastroenterol* 2010; **105**: 354-359
30 **Gearry RB**, Richardson A, Frampton CM, Collett JA, Burt MJ, Chapman BA, Barclay ML. High incidence of Crohn's disease in Canterbury, New Zealand: results of an epidemiologic study. *Inflamm Bowel Dis* 2006; **12**: 936-943
31 **Roberts RL**, Gearry RB, Hollis-Moffatt JE, Miller AL, Reid J, Abkevich V, Timms KM, Gutin A, Lanchbury JS, Merriman TR, Barclay ML, Kennedy MA. IL23R R381Q and *ATG16L1* T300A are strongly associated with Crohn's disease in a study of New Zealand Caucasians with inflammatory bowel disease. *Am J Gastroenterol* 2007; **102**: 2754-2761
32 **Roberts RL**, Hollis-Moffatt JE, Gearry RB, Kennedy MA, Barclay ML, Merriman TR. Confirmation of association of *IRGM* and NCF4 with ileal Crohn's disease in a population-based cohort. *Genes Immun* 2008; **9**: 561-565
33 **McCarroll SA**, Huett A, Kuballa P, Chilewski SD, Landry A, Goyette P, Zody MC, Hall JL, Brant SR, Cho JH, Duerr RH, Silverberg MS, Taylor KD, Rioux JD, Altshuler D, Daly MJ, Xavier RJ. Deletion polymorphism upstream of *IRGM* associated with altered *IRGM* expression and Crohn's disease. *Nat Genet* 2008; **40**: 1107-1112
34 **Roberts RL**, Gearry RB, Hollis-Moffatt JE, Miller AL, Reid J, Abkevich V, Timms KM, Gutin A, Lanchbury JS, Merriman TR, Barclay ML, Kennedy MA. IL23R R381Q and *ATG16L1* T300A are strongly associated with Crohn's disease in a study of New Zealand Caucasians with inflammatory bowel disease. *Am J Gastroenterol* 2007; **102**: 2754-2761
35 **Roberts RL**, Gearry RB, Barclay ML, Kennedy MA. Rapid detection of common CARD15 variants in patients with inflammatory bowel disease. *Mol Diagn* 2004; **8**: 101-105
36 **Opitz B**, Püschel A, Schmeck B, Hocke AC, Rosseau S, Hammerschmidt S, Schumann RR, Suttorp N, Hippenstiel S. Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized Streptococcus pneumoniae. *J Biol Chem* 2004; **279**: 36426-36432
37 **Rosenstiel P**, Sina C, End C, Renner M, Lyer S, Till A, Hellmig S, Nikolaus S, Fölsch UR, Helmke B, Autschbach F, Schirmacher P, Kioschis P, Hafner M, Poustka A, Mollenhauer J, Schreiber S. Regulation of DMBT1 *via NOD2* and TLR4 in intestinal epithelial cells modulates bacterial recognition and invasion. *J Immunol* 2007; **178**: 8203-8211
38 **Bonen DK**, Cho JH. The genetics of inflammatory bowel disease. *Gastroenterology* 2003; **124**: 521-536
39 **Henckaerts L**, Nielsen KR, Steffensen R, Van Steen K, Mathieu C, Giulietti A, Wouters PJ, Milants I, Vanhorebeek I, Langouche L, Vermeire S, Rutgeerts P, Thiel S, Wilmer A, Hansen TK, Van den Berghe G. Polymorphisms in innate immunity genes predispose to bacteremia and death in the medical intensive care unit. *Crit Care Med* 2009; **37**: 192-201, e1-e3
40 **Rutgeerts P**, Goboes K, Peeters M, Hiele M, Penninckx F, Aerts R, Kerremans R, Vantrappen G. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. *Lancet* 1991; **338**: 771-774
41 **Ferwerda G**, Kullberg BJ, de Jong DJ, Girardin SE, Langen-

berg DM, van Crevel R, Ottenhoff TH, Van der Meer JW, Netea MG. Mycobacterium paratuberculosis is recognized by Toll-like receptors and *NOD2*. *J Leukoc Biol* 2007; **82**: 1011-1018

42 **Chamaillard M**, Philpott D, Girardin SE, Zouali H, Lesage S, Chareyre F, Bui TH, Giovannini M, Zaehringer U, Penard-Lacronique V, Sansonetti PJ, Hugot JP, Thomas G. Gene-environment interaction modulated by allelic heterogeneity in inflammatory diseases. *Proc Natl Acad Sci USA* 2003; **100**: 3455-3460

43 **Grant SF**, Baldassano RN, Hakonarson H. Classification of genetic profiles of Crohn's disease: a focus on the *ATG16L1* gene. *Expert Rev Mol Diagn* 2008; **8**: 199-207

44 **Silverberg MS**, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, Caprilli R, Colombel JF, Gasche C, Geboes K, Jewell DP, Karban A, Loftus Jr EV, Peña AS, Riddell RH, Sachar DB, Schreiber S, Steinhart AH, Targan SR, Vermeire S, Warren BF. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005; **19** Suppl A: 5-36

45 **Kuballa P**, Huett A, Rioux JD, Daly MJ, Xavier RJ. Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated *ATG16L1* variant. *PLoS One* 2008; **3**: e3391

46 **Lapaquette P**, Glasser AL, Huett A, Xavier RJ, Darfeuille-Michaud A. Crohn's disease-associated adherent-invasive E. coli are selectively favoured by impaired autophagy to replicate intracellularly. *Cell Microbiol* 2010; **12**: 99-113

47 **Fujita H**, Eishi Y, Ishige I, Saitoh K, Takizawa T, Arima T, Koike M. Quantitative analysis of bacterial DNA from Mycobacteria spp., Bacteroides vulgatus, and Escherichia coli in tissue samples from patients with inflammatory bowel diseases. *J Gastroenterol* 2002; **37**: 509-516

S- Editor Sun H **L- Editor** Logan S **E- Editor** Ma WH