

ORIGINAL ARTICLE

Genetic and microbial factors modulating the ubiquitin proteasome system in inflammatory bowel disease

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ABSTRACT

Objective Altered microbiota composition, changes in immune responses and impaired intestinal barrier functions are observed in IBD. Most of these features are controlled by proteases and their inhibitors to maintain gut homeostasis. Unrestrained or excessive proteolysis can lead to pathological gastrointestinal conditions. The aim was to validate the identified protease IBD candidates from a previously performed systematic review through a genetic association study and functional follow-up.

Design We performed a genetic association study in a large multicentre cohort of patients with Crohn's disease (CD) and UC from five European IBD referral centres in a total of 2320 CD patients, 2112 UC patients and 1796 healthy controls. Subsequently, we did an extensive functional assessment of the candidate genes to explore their causality in IBD pathogenesis.

Results Ten single nucleotide polymorphisms (SNPs) in four genes were significantly associated with CD: *CYLD*, *USP40*, *APEH* and *USP3*. *CYLD* was the most significant gene with the intronically located rs12324931 the strongest associated SNP ($p_{FDR}=1.74e-17$, OR=2.24 (1.83 to 2.74)). Five SNPs in four genes were significantly associated with UC: *USP40*, *APEH*, *DAG1* and *USP3*. *CYLD*, as well as some of the other associated genes, is part of the ubiquitin proteasome system (UPS). We therefore determined if the IBD-associated adherent-invasive *Escherichia coli* (AIEC) can modulate the UPS functioning. Infection of intestinal epithelial cells with the AIEC LF82 reference strain modulated the UPS turnover by reducing poly-ubiquitin conjugate accumulation, increasing 26S proteasome activities and decreasing protein levels of the NF- κ B regulator CYLD. This resulted in I κ B- α degradation and NF- κ B activation. This activity was very important for the pathogenicity of AIEC since decreased *CYLD* resulted in increased ability of AIEC LF82 to replicate intracellularly.

Conclusions Our results reveal the UPS, and *CYLD* specifically, as an important contributor to IBD pathogenesis, which is favoured by both genetic and microbial factors.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) represent two distinct forms of chronic inflammation of

Significance of this study**What is already known on this subject?**

- Alterations in the composition and diversity of the gut microbiota play a key role in the establishment and maintenance of IBD.
- Increased numbers of mucosa-associated adherent-invasive *Escherichia coli* (AIEC) are observed in patients with Crohn's disease (CD).
- Proteases and protease inhibitors tightly regulate microbiota composition, immune response and intestinal barrier function to maintain gut homeostasis.
- Pivotal to the host inflammatory responses to commensal and pathogenic bacteria is the NF- κ B pathway which is largely regulated by the ubiquitin proteasome system (UPS). The main function of the UPS is the degradation of unneeded or damaged proteins.
- *CYLD* is a de-ubiquitinase and is part of the UPS. It mainly acts as a negative regulator of NF- κ B to protect the host from an over-reactive inflammatory response.
- The expression of *CYLD* is significantly downregulated in the intestine of CD patients.

the gastrointestinal tract, collectively called IBD. Although the categories of underlying pathogenic factors (genetic, environmental, microbial and immunological factors) are roughly the same, they are evidently clinicopathologically distinct with differences in disease location, behaviour and severity;¹ and with different pathogenic mechanisms, which to date are not entirely clear. Genome-wide association studies (GWAS) and meta-analyses have identified 99 susceptibility loci.^{2–4} More recently a meta-analysis of CD and UC GWAS, with extensive validation of significant findings and a combined total of more than 75 000 cases and controls brought the total number to 163 IBD loci: 110 for both CD and UC, 30 CD-specific and 23 UC-specific.⁵ Still, these loci explain only 15%–23% of the heritability to these diseases, and for

Significance of this study

What are the new findings?

- Polymorphisms in *CYLD*, *USP40*, *APEH* and *USP3* genes are significantly associated with CD. The strongest association was with *CYLD*, a de-ubiquitinating enzyme and key regulator of the NF- κ B pathway, located adjacent to the well-established CD susceptibility gene *NOD2*.
- Polymorphisms in *USP40* and *USP3* genes, and several SNPs located at 3p21 (*USP4/DAG1/APEH/MST1*) are significantly associated with UC.
- We show that AIEC bacteria modulate the UPS turnover by reducing the accumulation of poly-ubiquitin conjugates, increasing 26S proteasome activities and decreasing the protein level of the NF- κ B regulator *CYLD*, in turn leading to I κ B- α degradation and subsequent NF- κ B activation.
- The effect of AIEC bacteria on *CYLD* protein levels is very important for the pathogenicity of AIEC bacteria since decreased *CYLD* resulted in an increased ability of AIEC LF82 to replicate intracellularly.

How might it impact on clinical practice in the foreseeable future?

- Our results indicate that proteasome inhibitor therapy targeting the UPS could be efficient in IBD. A specific example for future use in IBD could be bortezomib, which was the first therapeutic proteasome inhibitor tested in humans and which is successfully used in cancer treatment.

many of the loci the exact causal gene(s) have not yet been identified.

Alterations in the composition and diversity of the gut microbiota have shown to play a key role in IBD aetiology.⁶ Also, in patients with CD and UC, high concentrations of bacteria forming a biofilm on the surface of the gut mucosa,⁷ and increased numbers of mucosa-associated *Escherichia coli* are observed.^{8–9} These bacteria, called adherent-invasive *Escherichia coli* (AIEC), colonise the ileal mucosa of CD patients.¹⁰ They are able to adhere to and invade intestinal epithelial cells, and to survive and highly replicate within macrophages leading to the secretion of large amounts of TNF- α .^{11–12} Gut colonisation triggered by adhesion and invasion of AIEC is accompanied by a breakdown of the mucosal barrier, sustaining the inflammatory response.¹³

Besides altered microbiota composition, changes in immune responses and impaired intestinal barrier functions are observed in IBD.¹⁴ Each of these features is controlled by proteases and protease inhibitors in order to maintain gut homeostasis. Unrestrained or excessive proteolysis can lead to pathological gastrointestinal conditions: increased proteolytic activity has been found in the secretions of colonic biopsy samples from patients with IBD.¹⁵ Exactly which proteases and inhibitors are involved in the context of IBD is currently unknown. In a previously performed and published systematic review of protease (inhibitor) genes in IBD, we ranked the known protease (inhibitor) genes according to the amount of evidence present in literature for linkage and/or association of their genomic location with IBD.¹⁶ In the present study, we are following up on the identified candidates from that systematic review through a genetic association study in CD and in UC on the common variants in the top-ranked genes and functional analyses on the most promising candidate genes to explore their candidacy as

causal genes and understand their possible mode of action in IBD pathogenesis.

MATERIALS AND METHODS

Genetic association study

A genetic association study was performed in two stages. First, 182 tagging SNPs in 23 protease (inhibitor) genes (table 1) were genotyped in an exploratory cohort (University Hospital Leuven, Belgium) of 650 CD and 721 UC patients, and 542 healthy controls (HC) using Sequenom. The candidate genes were selected based on the results from the previously published systematic review.¹⁶ Nominally significant SNPs ($p_{\text{uncorrected}} < 0.05$) were then validated in an independent cohort constituting a total of 1670 CD patients, 1391 UC patients and 1254 HC from four European countries: the Netherlands (ICC/Dutch), Hungary, the UK (Exeter) and Switzerland (table 2) using KASPar chemistry (KBioscience). An FDR corrected p ($p_{\text{FDR}} < 0.05$) was considered statistically significant.

Detailed information on the study samples, SNP selection and statistical analysis for the genetic association study is provided in online supplementary materials and methods. Overall patient characteristics are given in table S1.

Functional studies with AIEC reference strain LF82

Cell culture

The intestinal epithelial cell line T84 (ATCC, CCL-248) was maintained in an atmosphere containing 5% CO₂ at 37°C in the culture medium recommended by ATCC.

Bacterial strain and culture

E. coli strain LF82, isolated from a chronic ileal lesion of a CD patient, was used as the AIEC reference strain.⁸ Isogenic mutants

Table 1 Overview selected candidate genes for the genetic association study

Gene	Chr	Position (bp)*
<i>USP40</i>	2q37	234 384 166–234 475 428
<i>USP19</i>	3p21	49 145 479–49 158 371
<i>USP4</i>	3p21	49 315 264–49 378 145
<i>DAG1</i>	3p21	49 506 146–49 573 048
<i>APEH</i>	3p21	49 711 435–49 721 396
<i>MST1</i>	3p21	49 721 380–49 726 934
<i>PSMB8</i>	6p21	32 808 494–32 812 480
<i>PSMB9</i>	6p21	32 811 913–32 847 364
<i>MEP1A</i>	6p12	46 761 127–46 807 515
<i>MMP3</i>	11q22	102 706 532–102 714 534
<i>USP15</i>	12q14	62 654 119–62 811 211
<i>USP3</i>	15q22	63 796 793–63 886 839
<i>POL35</i>	16p11	31 093 907–31 100 949
<i>PRSS8</i>	16p11	31 142 756–31 147 083
<i>PRSS36</i>	16p11	31 150 246–31 161 415
<i>LONP2</i>	16q12	48 278 207–48 395 747
<i>CYLD</i>	16q12	50 775 961–50 835 846
<i>MMP2</i>	16q12	55 512 742–55 540 603
<i>MMP15</i>	16q21	58 059 470–58 080 805
<i>MEP1B</i>	18q12	29 769 987–29 800 364
<i>ELA2</i>	19p13	852 291–856 242
<i>C3</i>	19p13	6 677 846–6 730 573
<i>QPCTL</i>	19q13	46 195 741–46 207 240

*Coordinates in NCBI GRCh37.

Table 2 Number of individuals per dataset

	CD	UC	Healthy controls
Exploratory cohort			
Leuven	650	721	542
Validation cohorts			
The Netherlands	634	528	900
Hungary	377	290	354
The UK	432	432	0
Switzerland	227	141	0
Subtotal validation cohort	1670	1391	1254
Total	2320	2112	1796

CD, Crohn's disease.

of LF82 deleted for the *FimH*, *lpfA*, *FliC*, *OmpA*, *OmpC*, *OmpF* and the *lpfA* genes^{17–21} were also used in this study.

siRNA tr3ansfection

The siRNA oligonucleotides CYLD-si2 (CGCTGTAA CTCTTTAGCAT) were used in this study.²² For control siRNA treatment, siRNAs targeting luciferase (TTCUCCGAAC GTGTCACGT) were used. Cells were transfected with siRNAs using Oligofectamine (Invitrogen) according to the manufacturer's protocol.

Plasmids and transfection

T84 cells were used to amplify CYLD by PCR with the platinum Taq DNA polymerase (Invitrogen) according to the manufacturer's instructions. The PCR reaction was performed with the primers, CYLDF1 (5'-GACAGCATGGACACCACGTT-3') and CYLDR1 (5'-TTGCCTTTCCCGATGACCC-3'), designed on the full sequence of CYLD (transcript ID=ENST00000569418) and with the following temperature cycle: 94°C for 2 min followed by 40 cycles of 94°C for 30 s, annealing at 50°C for 30 s, and 72°C for 3 min. A final extension incubation of 72°C for 5 min was followed by 5 min at 5°C. The PCR product was purified with the Nucleospin extract II kit (Macherey-Nagel) and cloned into pCR2.1 vector with the TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. The resulting plasmid was digested with HindIII and XbaI, and the excised fragment was ligated into pEGFP-C1 cut with the same enzymes. T84 cells were transfected with plasmids pEGFP-C1 or pEGFP-C1-CYLD (100nM) and Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Invasion and intracellular survival assays

For adhesion assays, monolayers were infected for 3 h at a multiplicity of infection (MOI) of 10 bacteria per cell. Bacterial invasion of T84 cells was performed using the gentamycin protection assay.¹¹ At 1 and 24 h postinfection, the number of intracellular bacteria was determined by counting the number of colony-forming units. To determine the effect of MG132 on bacterial adhesive and invasive abilities, T84 cells were infected for 3 h at an MOI of 10 in the presence of MG132 (25 µM) or vehicle (dimethyl sulfoxide, DMSO, 0.2%). For bacterial invasion assay, T84 cells were washed five times in phosphate buffer saline (PBS) after 3 h infection in presence of MG132 or vehicle. The number of intracellular bacteria was determined by counting the number of colony-forming units without adding MG132 or vehicle during the postinfection period. One

representative dataset of at least three independent experiments was presented where appropriate.

Chymotrypsin-like activities of 26S proteasome

Monolayers were infected for 3 h at an MOI of 100 or 200 bacteria per cell and washed twice with ice-cold PBS and then scraped in ice-cold HEPES buffered saline (150 mmol/L NaCl, 5 mmol/L KCl and 10 mmol/L HEPES, pH 7.4).²² Cells were pelleted (14 000 g for 20 s at room temperature) and then resuspended in ice-cold lysis buffer (10 mmol/L Tris (pH 7.4), 5 mmol/L MgCl₂ and 50 U/mL deoxyribonuclease and ribonuclease, plus complete protease inhibitor cocktail; Roche Molecular Biochemicals). Protein concentrations were determined by Bradford assay, and samples stored at –80°C until use. Chymotrypsin-like activity of the proteasome was determined by measuring the hydrolysis of the fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (AMC) (Sigma). To measure peptidase activity, 15 µL of lysate containing 20 µg protein was added to 60 µL medium containing 50 mM Tris (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol and 300 µM succinyl-Leu-Leu-Val-Tyr-7-AMC. The peptidase activity was determined by measuring the accumulation of the fluorogenic cleavage product (AMC) using the FLX800 fluorimeter (Bio-Tek) with 380 and 440 nm excitation and emission wavelengths, respectively. The difference between arbitrary fluorescence units recorded with or without 40 or 100 µmol/L of the proteasome inhibitor MG132 (Affiniti) in the reaction medium was calculated, and the final data were corrected by the amount of protein in the reaction. The time course for the accumulation of AMC after hydrolysis of the substrate was analysed by linear regression to calculate peptidase activities, that is, the slopes of best fit of accumulated AMC versus time. Values were expressed in relative fluorescence unit per microgram of protein per minute. One representative dataset of at least three independent experiments was presented where appropriate. At each experiment, monolayers treated with MG132 (25 µM) or vehicle (DMSO, 0.2%) were treated like the uninfected or infected cells.

Western blotting

Monolayers were infected for 3 h at an MOI of 100 in presence or not of MG132 (25 µM) or vehicle (DMSO, 0.02%) and washed twice with ice-cold PBS and then scraped in NP40 lysis buffer (50 mmol/L Tris HCl, 150 mmol/L NaCl, 40 mmol/L EDTA, 1% NP40, pH 7.5 plus complete protease inhibitor cocktail; Roche Molecular Biochemicals) containing 5 mmol/L N-ethyl-maleimide to protect Ub protein conjugates. Whole cell extracts were subjected to SDS-PAGE on 12% or 7.5% (wt/vol) acrylamide gels. Protein concentrations were determined by Bradford assay. Western immunoblotting was performed according to the procedure of Towbin.²³ Proteins were electroblotted onto nitrocellulose membranes (Amersham International). The membranes were immunoblotted for CYLD (rabbit anti-CYLD, 1:1000, Thermo scientific), IκB-α (Rabbit anti-IκB-α, 1:200, Santa Cruz Biotechnology Inc), β-actin (rabbit anti-β-actin, Santa Cruz Biotechnology Inc.) and for polyUb chains (anti-FK1, 1:1000, Affiniti) Immunoreactants were detected using horseradish peroxidase-conjugated antirabbit or antimouse immunoglobulin G antibody, ECL reagents (Amersham Biosciences) and autoradiography. Image J software was used to estimate protein quantity. Results are expressed as protein amount relative to β-actin, or normalised against the amount of proteins (determined following Ponceau Red staining) for poly-ubiquitinated proteins.

Confocal microscopy for NF- κ B and poly-ubiquitin conjugates localisation

T84 cells were plated at a concentration of 2.105 per cm² in 24 well plates containing sterile cover slips and grown at 37°C for 48 h. Cells were infected at an MOI of 100 in presence or not of MG132 (25 μ M) or vehicle (DMSO, 0.2%). After infection, the cells were washed four times with PBS prewarmed to 37°C and fixed onto the cover slips by incubation in 3.7% formaldehyde for 15 min. Cells were then washed with PBS and permeabilised by incubation in 0.1% Triton for 15 min at room temperature. The cover slips were blocked in blocking buffer (5% decompemented serum, 3% bovine serum albumin (BSA), 0.025% triton) during 2 h at room temperature. Antibody to the NF- κ B p65 subunit or FK1 (1:500) was added in blocking buffer and incubated for 2 h at room temperature. The cover slips were washed six times with PBS and incubated with Hoechst solution (final concentration of 0.5 μ g/mL, Sigma) and CY3 antibody (1:300) during 1 h in the dark. Cover slips were then washed six times each with PBS and mounted onto slides using Fluorescent mounting medium (mowiol). The nuclear translocation of p65 was imaged using a Zeiss LSM 510 META confocal microscope equipped with a Zeiss 37°C incubation system. Images were analysed using the Zeiss LSM Image Examiner.

Statistical analysis

Data were analysed by Student's *t* test. A $p \leq 0.05$ was considered statistically significant. Data are expressed as the means \pm SEM.

RESULTS

Genetic association study of protease (inhibitor) genes

Of the 182 tagging SNPs in 23 candidate genes, 26 SNPs were nominally significant in the exploratory cohort. With one SNP deleted from further analysis because it failed quality control (QC), we finally had 25 SNPs with genotyping results in both the exploration and validation cohorts. Association results of these 25 SNPs in the combined cohort (2320 CD, 2112 UC and 1796 HC) are listed in table 3. Ten SNPs were significantly associated ($p_{FDR} < 0.05$) with CD. They were located in *CYLD* (16q12), *USP40* (2q37), *APEH* (3p21) and *USP3* (15q22). Five SNPs were significantly associated with UC, being located in *USP40* (2q37), *DAG1* and *APEH* (both on 3p21), and *USP3* (15q22).

In CD, the most significant finding was for rs12324931 with a combined *p* value of 1.74e-17 (OR=2.24 (1.83 to 2.74)). Rs12324931 is located intronically in the *CYLD* gene. Three other SNPs in *CYLD* (2 intronic, and 1 upstream)—all in low linkage disequilibrium (LD) with rs12324931 ($r^2 < 0.1$) but with moderate LD between them ($0.5 < r^2 < 0.7$)—were also associated with CD: rs17314544 ($p_{FDR} = 1.42e-08$, OR=0.76 (0.66 to 0.88)), rs2302759 ($p_{FDR} = 4.86e-02$, OR=0.88 (0.76 to 1.01)) and rs7205423 ($p_{FDR} = 1.60e-07$, OR=1.23 (1.05 to 1.43)). Haplotype analysis showed a significant association of this 3-SNP block with CD ($p_{omnibus} = 2.69e-14$). For none of the associated *CYLD* SNPs, a clear function is evident. They are non-coding and are not identified as being expression quantitative trait loci (eQTLs) (table 3) or transcription factor binding sites. *CYLD* is located on chromosome 16q12, 9 kb downstream of the well-established CD susceptibility gene *NOD2*. We therefore tested whether the observed signals in *CYLD* were acting independently or were driven by the three major *NOD2* CD-risk variants. Genotypes were obtained for rs2066844 (p.R702W), rs2066845 (p.G908R) and rs2066847

(p.L1007fsinsC). Overall, relatively low LD was observed between the *NOD2* and *CYLD* SNPs with an average $r^2 = 0.10$, but there is a high correlation between rs2066844 and rs12324931: $r^2 = 0.83$. Conditional on rs2066844, rs2066845 and rs2066847, a significant independent association was shown for rs12324931: $p = 5.76e-5$, $p = 2.03e-17$ and $p = 4.36e-18$, respectively. When including all three *NOD2* mutations in a single conditional logistic regression test, rs12324931 was still significantly associated ($p = 2.92e-05$). Also, when stratifying based on *NOD2* carrier status, *NOD2*-negative patients (not carrying any of the three *NOD2* risk alleles) showed a significant association with rs12324931 ($p = 0.001$, OR=4.05 (1.68 to 9.73)). None of the *CYLD* SNPs were associated with UC.

A detailed analysis of the other associated SNPs is provided in table 3 and online supplementary results (also see figure S1 and tables S2–S4). Inherent to the way the candidate protease (inhibitor) genes studied here were selected, we found that also the other associated proteases in this study are located near known susceptibility genes: *ATG16L1* for *USP40* and *MST1* for the genes located at 3p21 (*USP4*, *APEH*, *DAG1*). Although the results of the genetic studies cannot prove that these genes are definitely causal, we could show that there are probably multiple variants in these loci (allelic heterogeneity) that are important in IBD pathogenesis (see online supplementary results).

Importance of the role of CYLD in CD as revealed by CD-associated bacteria

The genetic results point towards the implication of *CYLD* in IBD pathogenesis. We therefore next assessed if this gene indeed could be functionally involved. It has been reported that some bacteria and viruses are able to modulate *CYLD*.^{23–25} We therefore first investigated if mucosa-associated bacteria known to abnormally colonise the gastrointestinal tract of IBD patients would also be able to modulate *CYLD*. As AIEC are the prototype bacteria recognised worldwide as abnormally colonising the gut of IBD patients, we tested the effect of the AIEC reference strain LF82 on *CYLD* in T84 intestinal epithelial cells. In AIEC LF82 infected T84 cells, 40% decreased *CYLD* protein levels were observed ($p < 0.00001$) (figure 1A). *CYLD* is shown to be significantly downregulated in the intestine of CD patients.²⁶ As *CYLD* could also act as a negative regulator for immune and inflammatory response against invading bacteria,^{27–28} we tested if a decrease in *CYLD* expression affects the ability of AIEC LF82 to invade and replicate intracellularly. Transfection of T84 cells with *CYLD* siRNA (figure 1B) resulted in a significant increase in the ability of AIEC LF82 to invade ($p < 0.0001$) and replicate intracellularly ($p < 0.01$), compared with cells transfected with scramble siRNA (taken as 100%), with values reaching 151% and 223% (figure 1C,D). In contrast, we observed that overexpression of *CYLD* in T84 cells transfected with cloned *CYLD* cDNA into PEGFP-C1 vector did not modify the level of internalised bacteria as shown at 1 h postinfection but significantly decreased by 22.7% the number of intracellular bacteria at 24 h postinfection ($p = 0.005$ vs T84 cells transfected with the empty vector) (figure 1E–G). Altogether, these results indicated that a decrease in *CYLD* protein level can favour the invasion and the intracellular replication of AIEC bacteria.

Importance of the role of the UPS in CD

CYLD, as well as many of the other genes where we found evidence for association with IBD (*USP40*, *USP3*, *USP4*, *APEH*), is part of the ubiquitin proteasome system (UPS). Its main function

Table 3 Significant associations with CD and UC in the combined cohort

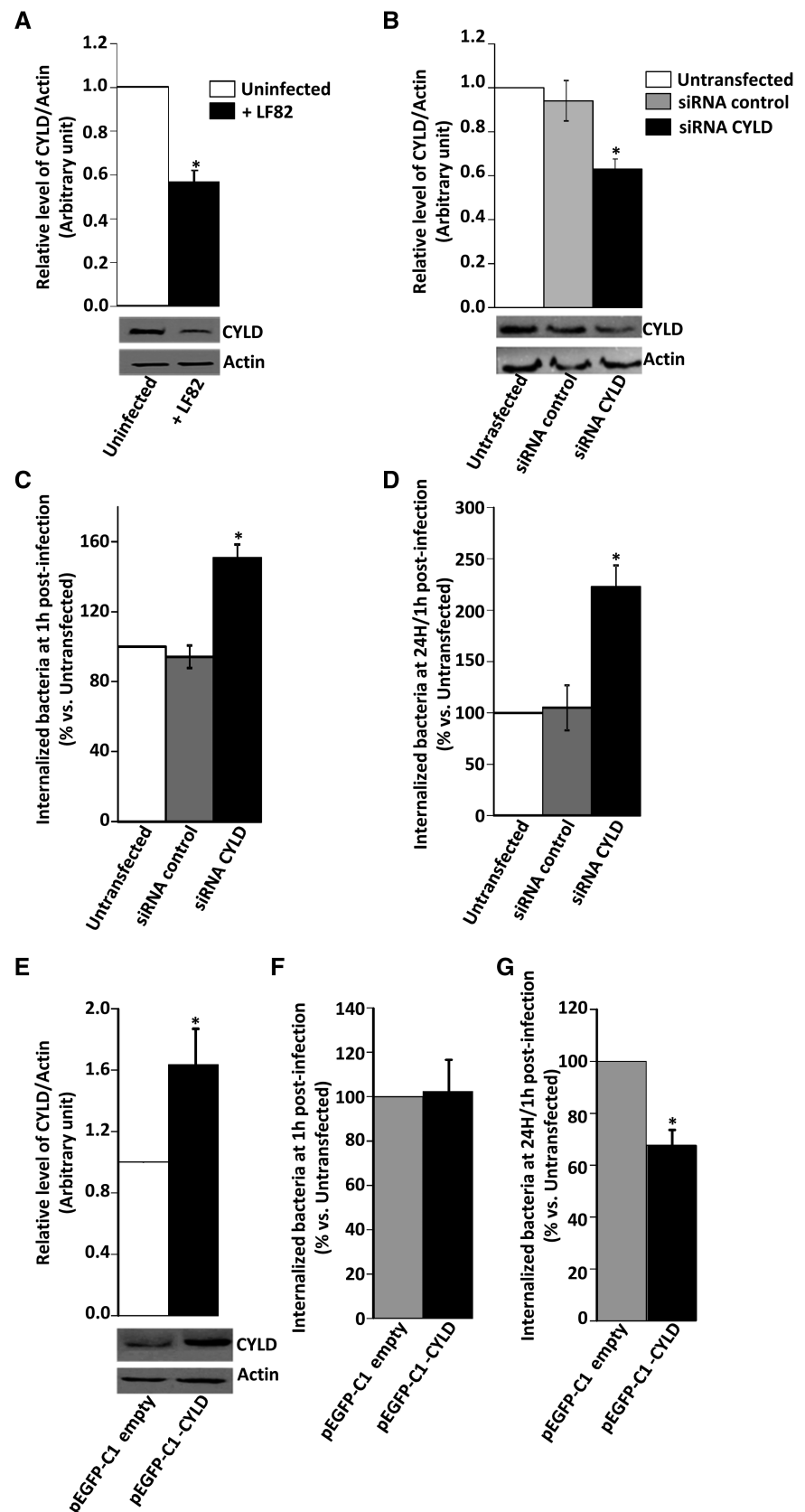
SNP	Chr	Position	Gene	Function class	eQTL for	Minor allele	MAF HC	CD				UC			
								MAF CD	P _{FDR, CD}	OR 95% CI AB><AA	OR 95% CI BB><AB	MAF UC	P _{FDR, UC}	OR 95% CI AB><AA	OR 95% CI BB><AB
rs838548	2	234092357	USP40	Intron		G	0.20	0.24	1.01E-03	1.29 (1.12 to 1.47)	1.06 (0.77 to 1.46)	0.20	8.26E-01	1.06 (0.92 to 1.22)	0.9 (0.64 to 1.27)
rs4047198	2	234119639	USP40	Intron		G	0.20	0.23	9.97E-04	1.30 (1.13 to 1.49)	0.98 (0.71 to 1.36)	0.20	8.03E-01	1.07 (0.93 to 1.23)	0.88 (0.63 to 1.24)
rs12472244	2	234131582	USP40	Intron		G	0.23	0.19	1.23E-03	0.79 (0.69 to 0.91)	0.91 (0.66 to 1.24)	0.20	4.45E-02	0.78 (0.68 to 0.89)	1.22 (0.9 to 1.65)
rs13078949	3	49336795	USP4	3'	WDR6*	G	0.42	0.41	3.09E-01	0.92 (0.80 to 1.05)	0.98 (0.83 to 1.17)	0.41	4.75E-01	0.91 (0.79 to 1.05)	1.01 (0.84 to 1.20)
rs885592	3	49472887	DAG1	Intron	WDR6, QARS	G	0.42	0.40	1.08E-01	0.85 (0.74 to 0.98)	1.02 (0.85 to 1.22)	0.40	2.05E-01	0.87 (0.76 to 1.01)	1.00 (0.84 to 1.20)
rs6766131	3	49513936	DAG1	Intron		C	0.30	0.32	7.66E-02	1.10 (0.96 to 1.26)	1.13 (0.91 to 1.41)	0.34	6.45E-03	1.14 (1.00 to 1.31)	1.28 (1.02 to 1.59)
rs3870336	3	49532861	DAG1	Intron	ARIH2, DAG1, RBM6	A	0.08	0.07	4.75E-01	0.93 (0.77 to 1.12)	0.89 (0.41 to 1.93)	0.08	9.61E-01	1.00 (0.83 to 1.21)	1.03 (0.49 to 2.19)
rs2131109	3	49679991	APEH	Intron		T	0.46	0.50	3.04E-03	1.05 (0.9 to 1.22)	1.3 (1.11 to 1.53)	0.49	4.44E-02	1.03 (0.88 to 1.20)	1.26 (1.07 to 1.48)
rs11130213	3	49687301	APEH	Intron		T	0.28	0.32	1.22E-02	1.16 (1.02 to 1.33)	1.12 (0.89 to 1.41)	0.33	5.93E-03	1.11 (0.97 to 1.28)	1.40 (1.11 to 1.76)
rs11130214	3	49710750	MST1	5'	WDR6	G	0.31	0.29	3.11E-01	0.90 (0.79 to 1.03)	1.03 (0.82 to 1.3)	0.29	1.86E-01	0.89 (0.78 to 1.02)	0.95 (0.75 to 1.2)
rs241424	6	32912912	PSMB8/ PSMB9	Intron		A	0.45	0.46	3.11E-01	1.00 (0.86 to 1.16)	1.14 (0.97 to 1.35)	0.45	9.39E-01	0.94 (0.81 to 1.09)	1.09 (0.92 to 1.29)
rs2071540	6	32920894	PSMB8/ PSMB9	5'		T	0.42	0.42	9.42E-01	0.93 (0.81 to 1.07)	1.09 (0.92 to 1.3)	0.41	6.12E-01	0.83 (0.72 to 0.96)	1.18 (0.98 to 1.41)
rs9276831	6	32940011	PSMB8/ PSMB9	Intron		G	0.13	0.14	2.05E-01	1.03 (0.89 to 1.20)	1.71 (1.06 to 2.77)	0.15	5.07E-02	1.13 (0.97 to 1.31)	1.59 (0.98 to 2.59)
rs3799858	6	46875647	MEP1A	Intron		A	0.09	0.09	6.98E-01	0.93 (0.78 to 1.10)	1.66 (0.66 to 4.17)	0.08	1.71E-01	0.84 (0.70 to 1.00)	1.10 (0.39 to 3.08)
rs11174442	12	61041898	USP15	Intron		A	0.41	0.41	8.94E-01	1.02 (0.89 to 1.18)	0.99 (0.83 to 1.18)	0.42	5.07E-01	1.05 (0.91 to 1.22)	1.05 (0.88 to 1.26)
rs17765311	15	61577005	USP3	Intron		C	0.39	0.35	1.11E-03	0.81 (0.70 to 0.92)	0.91 (0.75 to 1.1)	0.35	1.13E-02	0.93 (0.81 to 1.06)	0.75 (0.61 to 0.91)
rs2649	15	61673646	USP3	Intron		T	0.11	0.12	3.02E-01	1.11 (0.95 to 1.30)	1.01 (0.54 to 1.87)	0.11	9.32E-01	0.98 (0.83 to 1.15)	1.09 (0.58 to 2.05)
rs7205423	16	49326763	NOD2/CYLD	5'		C	0.45	0.51	1.60E-07	1.23 (1.05 to 1.43)	1.38 (1.17 to 1.62)	0.47	1.99E-01	1.05 (0.90 to 1.21)	1.12 (0.95 to 1.33)
rs12324931	16	49347659	NOD2/CYLD	Intron		C	0.05	0.10	1.74E-17	2.24 (1.83 to 2.74)	3.03 (1.05 to 8.72)	0.05	2.21E-01	1.22 (0.98 to 1.52)	0.88 (0.23 to 3.34)
rs17314544	16	49377579	NOD2/CYLD	Intron		T	0.43	0.36	1.42E-08	0.76 (0.66 to 0.88)	0.73 (0.6 to 0.88)	0.41	4.85E-01	0.90 (0.78 to 1.04)	1.03 (0.86 to 1.24)
rs2302759	16	49385102	NOD2/CYLD	Intron		A	0.18	0.16	4.86E-02	0.88 (0.76 to 1.01)	0.84 (0.58 to 1.21)	0.20	1.80E-01	1.17 (1.01 to 1.35)	0.95 (0.67 to 1.35)
rs9945133	18	28022607	MEP1B	5'		G	0.33	0.35	1.10E-01	1.06 (0.92 to 1.21)	1.19 (0.96 to 1.47)	0.33	7.45E-01	0.99 (0.86 to 1.13)	1.13 (0.91 to 1.4)
rs6506963	18	28040950	MEP1B	Intron		A	0.06	0.07	3.78E-01	1.10 (0.90 to 1.33)	1.16 (0.47 to 2.86)	0.06	8.28E-01	1.08 (0.88 to 1.31)	0.58 (0.2 to 1.71)
rs2302593	19	50888474	QPCTL	Intron		G	0.49	0.49	6.48E-01	1.16 (1.00 to 1.36)	0.90 (0.77 to 1.06)	0.49	9.50E-01	0.98 (0.84 to 1.14)	1.00 (0.85 to 1.18)
rs11668847	19	50902205	QPCTL	Intron		G	0.47	0.47	5.50E-01	0.99 (0.86 to 1.16)	0.94 (0.80 to 1.10)	0.49	2.14E-01	1.07 (0.92 to 1.25)	1.09 (0.93 to 1.28)

Bold: significant associations.

*ExonQTL.

AA, homozygous for the wild-type allele; AB, heterozygous; BB, homozygous for the minor allele; CD, Crohn's disease; eQTL, expression quantitative trait loci; HC, healthy controls; MAF, minor allele frequency.

Figure 1 Interrelation between CYLD and adherent-invasive *Escherichia coli* (AIEC) LF82 infection. T84 cells were infected with AIEC LF82 bacteria at a multiplicity of infection (MOI) of 100, and CYLD levels in T84 cells were measured by western blotting with antiserum against CYLD and β -actin as an internal control (A). T84 cells were transfected with CYLD siRNA, total proteins loaded and immunoblotted with antibody against CYLD and β -actin as an internal control (B). T84 transfected cells were infected with AIEC LF82 at an MOI of 10. The number of internalised bacteria at 1 h postinfection (C) and internalised bacteria at 24 h/1 h postinfection (D) were determined. T84 cells were transfected with pEGFP-C1-CYLD vector, total proteins loaded and immunoblotted with antibody against CYLD and β -actin as an internal control (E). T84 transfected cells were infected with AIEC LF82 at an MOI of 10. The number of internalised bacteria at 1 h postinfection (F) and internalised bacteria at 24 h/1 h postinfection (G) were determined. Results are expressed as the percentage of intracellular bacteria within transfected T84 cells relative to untransfected T84 cells, defined as 100%. Data are means \pm SEM for ≥ 3 independent experiments. * $p < 0.05$.



is to degrade unneeded or damaged proteins, but the proteasome machinery is, as is CYLD, also a key regulator of the NF- κ B pathway.^{29 30} We tested the effect of the AIEC reference strain LF82 on the UPS of T84 intestinal epithelial cells by determining

the level of accumulation of poly-ubiquitinated (Ub) proteins, which is the result of all three major UPS activities (ubiquitinase, de-ubiquitinase and proteasome activities). T84 cells were infected with AIEC LF82 bacteria during 3 h. We determined the

level of accumulation of Ub proteins by using an antibody detecting multi-ubiquitin chains (-K29-, L63- and -K48-linked chains). Infection of T84 cells with AIEC LF82 bacteria significantly decreased the level of Ub conjugates by 46% ($p < 0.001$) compared with non-infected cells (figure 2A,B). We also observed that the decrease in Ub conjugates in AIEC LF82 infected T84 cells is an active bacterial process since this was no longer found when experiments were performed with heat-killed bacteria (figure 2C). To further investigate which LF82 bacterial factor was involved in decreased Ub proteins, we performed experiments with various LF82 mutants affected in their abilities to

interact with T84 cells. We observed that when T84 cells were infected with mutants LF82- $\Delta fimH$, LF82- $\Delta fliC$, LF82- $\Delta ompA$ and LF82- $\Delta ompC$, which have decreased ability to adhere and to invade T84 cells, the decrease in Ub conjugates was not as high as observed with wild-type LF82 bacteria. In contrast, when T84 cells were infected with LF82- $\Delta lpfA$ and LF82- $\Delta ompF$ mutants, which are still able to replicate within T84 cells, the level of Ub proteins in infected cells was not significantly different from that of LF82 infected cells (figure 2C). Ub proteins are recognised and degraded by the 26S proteasome, which contains a chymotrypsin-like proteolytic activity in the β -subunits of its

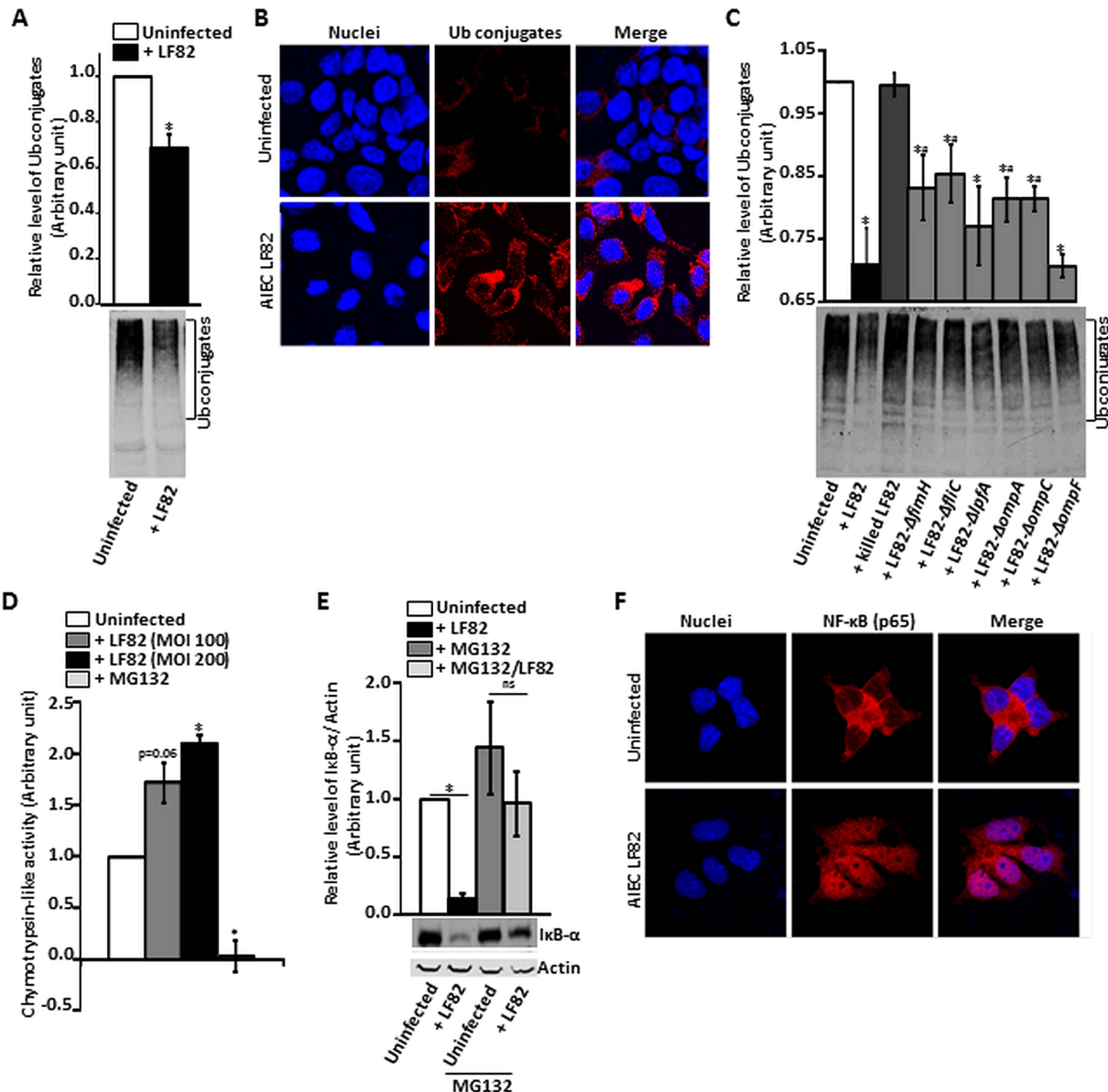


Figure 2 Regulation of ubiquitin proteasome system and NF- κ B pathways by adherent-invasive *Escherichia coli* (AIEC) LF82 infection on T84 cells. T84 cells were infected with AIEC LF82 bacteria and isogenic mutants of LF82 deleted for the *FimH*, *lpfA*, *fliC*, *OmpA*, *OmpC*, *OmpF* and the *lpf* genes at a multiplicity of infection of 100 or 200. The formation of high molecular weight biotinylated-ubiquitin (Ub) conjugates was determined by western blotting (A,C) and immune fluorescence staining (B) by using an antibody specifically recognising poly-Ub chains. Representative immunoblots are shown. Chymotrypsin-like activities of the 26S proteasome (D) were determined by measuring the accumulation of the fluorogenic cleavage product (amido-4-methylcoumarin). Values were expressed in relative fluorescence unit per microgram of protein per minute. I κ B- α protein level was determined by western blotting with antiserum against I κ B- α and β -actin as an internal control (E). Results are expressed as protein amount relative to β -actin. NF- κ B was localised in T84 cells infected with AIEC LF82 bacteria by immune fluorescence staining (F). The accumulation of Ub conjugates (A,C), chymotrypsin-like activities (D) and I κ B- α protein levels (E) are means \pm SEM for ≥ 3 independent experiments. * $p < 0.05$ versus uninfected or killed LF82 infected T84 cells, # $p < 0.05$ versus LF82 infected T84 cells.

Inflammatory bowel disease

proteolytic core complex.³¹ To see if the decrease in the accumulation of Ub proteins as observed in the presence of AIEC LF82 is in part explained by an effect on 26S proteasome proteolytic function, we compared the levels of chymotrypsin-like activity in AIEC LF82 infected T84 cells with non-infected cells. AIEC LF82 infection induced an MOI-dependent increase in chymotrypsin-like activity in T84 cells: 1.71-fold ($p=0.06$) increase at an MOI of 100, and 2.1-fold ($p<0.05$) at an MOI of 200 (figure 2D). This suggested that AIEC LF82 bacteria are able to increase the basal proteolytic level of the 26S proteasome, and consequently increase its ability to degrade Ub conjugates. In response to extracellular stimuli, the NF- κ B inhibitor I κ B- α is poly-ubiquitinated and degraded by the 26S proteasome, leading to the activation of NF- κ B.³² We tested if, in accordance with the induction of the 26S proteasome, AIEC LF82 infection favours I κ B- α degradation and subsequently activates the NF- κ B pathway. AIEC LF82 infection induced a significant ($p<0.001$) 7.7-fold decrease in I κ B- α protein level in T84 cells compared with non-infected cells. This was not observed in the presence of MG132 (figure 2E). Similarly, we observed a nuclear translocation of the NF- κ B p65 subunit in AIEC LF82-infected T84 cells (figure 2F).

DISCUSSION

The most strongly associated protease was *CYLD*, which was specific for CD. It encodes a cytoplasmic de-ubiquitinating enzyme and one of its main functions is to protect the host from an over-reactive inflammatory response by negatively regulating NF- κ B.³⁰ Interestingly, a role for *CYLD* in IBD pathogenesis can be suggested as it was identified as one of the most significantly downregulated genes in the intestine of CD patients as shown using microarrays.²⁶ In addition, *cyld*^{-/-} mice displayed more severe intestinal inflammation and intestinal tumourigenesis, compared with wild-type controls.³³ It is important to note the chromosomal location of *CYLD* adjacent to *NOD2* on 16q12. The (functional) link of *NOD2*/*CARD15* to IBD has been firmly and reproducibly established: there are several well-characterised polymorphisms in *NOD2* leading to different capacities of the protein products to regulate NF- κ B-mediated inflammatory responses to bacterial components in the gut, thus providing a causal explanation for the observed association with the disease.³⁴ CD GWAS and meta-analyses have reported strong association on 16q12,^{3 4} which is attributed to *NOD2*. Still, Hampe *et al* found that a robust association signal in this region remains after stratification

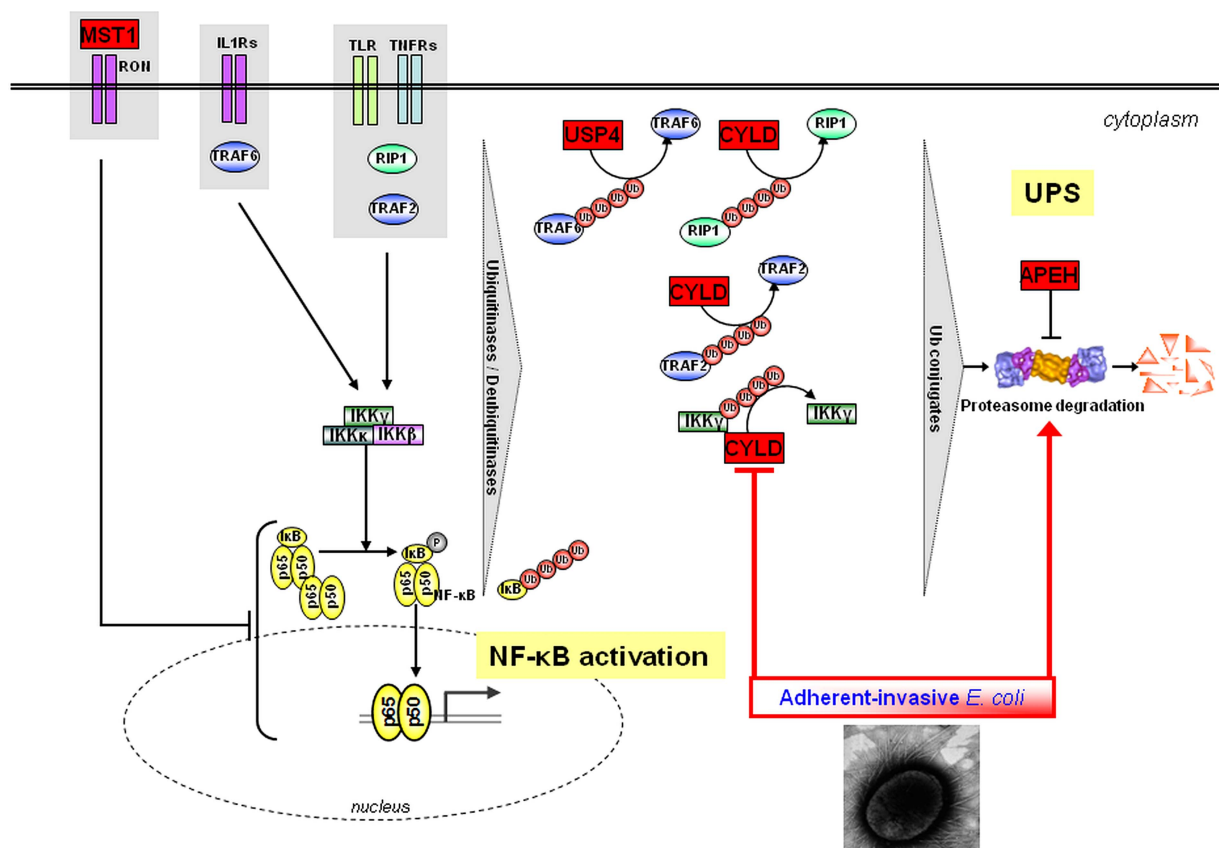


Figure 3 Proposed working model. Several of the genes found to be associated with Crohn's disease (CD) and/or UC in this study have been shown to negatively regulate NF- κ B: MST1 is a negative regulator of LPS-induced NF- κ B activation.⁴³ CYLD can de-ubiquitinate several of the NF- κ B signalling components such as TRAF2/TRAFF6 and Nemo (IKK- γ) and toll-like receptors (TLR), particularly TLR2. USP4 de-ubiquitinates TRAF2/TRAFF6 in the context of TNF- α - and IL1- β -induced NF- κ B activation,⁴⁴ and targets TAK1 to downregulate TNF- α -induced NF- κ B activation.⁴⁵ APEH is a negative effector of proteasome activity. While in normal healthy conditions commensal bacteria limit NF- κ B signalling through some of these pathways, in individuals harbouring variant alleles leading to an aberrant expression or altered function of CYLD, USP4, MST1 and/or APEH, activation of NF- κ B is no longer counteracted, resulting in aberrant immune responses and hence inflammation. In addition, CD-associated pathogenic adherent-invasive *Escherichia coli* bacteria can modulate ubiquitin proteasome system (UPS) turnover, increase 26S proteasome activities, I κ B- α degradation (directly through the UPS and by negatively regulating CYLD) and subsequently NF- κ B activation. The contribution of the UPS in IBD pathogenesis as revealed in this study could thus be modulated by both genetic and microbial factors.

by *NOD2* polymorphisms.³⁵ In the study reported here, we show a significant association of CD with *CYLD*, which appears to be independent of the association of the three major *NOD2* variants. In accordance with our findings, a recent re-analysis of the WTCCC1 CD data using a multimarker approach also revealed a significant association of *CYLD* independent from *NOD2*.³⁶ The study by Rivas *et al*³⁷ where they resequenced some of the genes identified through GWA studies suggests additional low frequency *NOD2* risk alleles. To further disentangle the independent contribution of *CYLD* and to find putative causal (functional) variants within the *CYLD* gene, the complete region needs resequencing. In addition, we found a significant association of *USP40*, the 3p21 gene region (*USP4/APEH/DAG1/MST1*) and *USP3* with both CD and UC. It should be noted however that the observed association results require further interrogation and conditional analysis of data from a dense SNP set within these and neighbouring LD blocks before firm conclusions can be drawn.

Although the genetic studies cannot prove that these genes are definitely causal (also see online supplementary results), we do show functional evidence for an important role of *CYLD*, and the UPS, in IBD pathogenesis.

Several lines of evidence suggest that one of the hallmarks of IBD is an alteration in the composition and diversity of the gut microbiota.⁶ Pathogenic bacteria like AIEC colonise the ileal mucosa of CD patients¹⁰ and favour a breakdown of the mucosal barrier, further sustaining the inflammatory response.¹³ In addition to our genetic results which point towards the implication of *CYLD* in IBD pathogenesis, *CYLD* has been shown to be downregulated in the intestine of CD patients.²⁶ *CYLD*, a key regulator of the NF- κ B pathway, can de-ubiquitinate several of the NF- κ B signalling components such as TRAF2/TRAF6 and Nemo (IKK- γ), which would otherwise lead to the degradation of I κ B- α by the proteasome.^{30 38 39} In our functional studies, we show that the pathogenic CD-associated AIEC LF82 bacteria, like some other bacteria and viruses,^{23–25} are able to modulate *CYLD* protein levels (hence a way to induce NF- κ B signalling), in turn leading to an increased or a decreased ability of AIEC LF82 to invade and replicate within intestinal epithelial cells depending on whether *CYLD* expression is decreased or increased respectively. Of note, the levels of AIEC replication within epithelial cells with decreased *CYLD* expression were similar to those seen in epithelial cells with decreased expression of autophagy-related proteins ATG16L1 or IRGM (immunity-related GTPase family M protein), for which the encoding genes are mutated in patients with CD.^{40 41}

CYLD, as well as many of the other genes where we found evidence for association with IBD (*USP40*, *USP3*, *USP4*, *APEH*), is part of the UPS. The main function of the UPS is to degrade unneeded or damaged proteins, but the proteasome machinery is, as is *CYLD*, also a key regulator of the NF- κ B pathway. In the same way as for *CYLD*, we speculated that AIEC bacteria could modulate the UPS to induce an inflammatory response. We show that the pathogenic AIEC LF82 bacteria decrease the accumulation of poly-ubiquitin conjugates and increase 26S proteasome activity in intestinal epithelial cells, indicating that AIEC bacteria stimulate ubiquitin-proteasome proteolysis. Experiments with various LF82 mutants indicated that this process is dependent on the ability of the bacteria to survive and/or replicate within host cells. Pivotal to the host inflammatory responses to commensal and pathogenic bacteria is the NF- κ B pathway which is largely regulated by the UPS.⁴² In response to extracellular stimuli, the NF- κ B inhibitor I κ B- α is poly-ubiquitinated and degraded by the 26S proteasome leading

to NF- κ B activation.³² We show that AIEC bacteria induce proteasome-dependent degradation of I κ B- α , leading to a nuclear translocation of the NF- κ B p65 subunit. AIEC bacteria can thus induce an inflammatory response involving the NF- κ B pathway, which is modulated by the effect of AIEC bacteria on the 26S proteasome activity.

In summary, we believe that the genetic data together with the functional data we provide here promote the case for the candidacy of *CYLD* as being—at least one—of the causal genes in the *NOD2*-*CYLD* gene region. Our studies identify the UPS as a major and common (both CD and UC) contributor to IBD pathogenesis. In the light of the findings of this study, we propose a working model presented in figure 3 showing that the major contribution of the UPS in IBD pathogenesis could have two origins, genetic and/or microbial, both of which being largely recognised as favouring the development and maintenance of IBD.

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Inflammatory bowel disease

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