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Escherichia coli Isolates from Inflammatory Bowel Diseases Patients Survive in Macrophages and Activate NLRP3 Inflammasome

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Abstract

Crohn's disease (CD) is a multifactorial pathology associated with the presence of adherent-invasive *Escherichia coli* (AIEC) and NLRP3 polymorphic variants. The presence of intracellular *E. coli* in other intestinal pathologies (OIP) and the role of NLRP3-inflammasome in the immune response activated by these bacteria have not been investigated. In this study, we sought to characterize intracellular strains isolated from patients with CD, ulcerative colitis (UC) and OIP, and analyze NLRP3-inflammasome role in the immune response and bactericidal activity induced in macrophages exposed to invasive bacteria. For this, intracellular *E. coli* isolation from ileal biopsies, using gentamicin-protection assay, revealed a prevalence and CFU/biopsy of *E. coli* higher in biopsies from CD, UC and OIP patients than in controls. To characterization of bacterial

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isolates, pulsed-field gel electrophoresis (PFGE) patterns, virulence genes, serogroup and phylogenetic group were analyzed. We found out that bacteria isolated from a given patient were closely related and shared virulence factors; however, strains from different patients were genetically heterogeneous. AIEC characteristics in isolated strains, such as invasive and replicative properties, were assessed in epithelial cells and macrophages, respectively. Some strains from CD and UC demonstrated AIEC properties, but not strains from OIP. Furthermore, the role of NLRP3 in pro-inflammatory cytokines production and bacterial elimination was determined in macrophages. *E. coli* strains induced IL-1β through NLRP3-dependent mechanism; however, their elimination by macrophages was independent of NLRP3. Invasiveness of intracellular *E. coli* strains into the intestinal mucosa and IL-1β production may contribute to CD and UC pathogenesis.

Keywords

E. coli; Crohn's disease; IL-1β; NLRP3; inflammasome

INTRODUCTION

Inflammatory bowel diseases (IBD) comprise a variety of relapsing and remitting clinical conditions, all a result of chronic inflammation of the gastrointestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) are the major forms of IBD (Abraham and Cho, 2009). High IBD incidence has been reported in Europe and North America, and although there is sparse data from South America, clinical experience suggests increasing rates of IBD in recent years (Cosnes et al., 2011; Figueroa et al., 2005).

CD can affect any area of the gastrointestinal tract; however, the terminal ileum and colon are more frequently involved. Characteristics of CD are patchy inflammation, full-thickness lesions of the intestinal wall, and granulomas (Louis et al., 2001; Odze, 2003). UC, unlike CD, is characterized by continuous and symmetrical damage of the colonic intestinal mucosa (Abraham and Cho, 2009; Louis et al., 2001; Odze, 2003). Both pathologies present elevated proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6. In CD, there is a predominance of Th1 cytokines, whereas UC seems to be a Th2-like cytokine-mediated disease (Strober and Fuss, 2011). Although the aetiology of IBD remains poorly understood, several environmental, immunological, and genetic factors have been linked to the disease risk (Abraham and Cho, 2009). Recent studies have suggested that intestinal microbiota composition contribute to CD pathogenesis. Compared to normal individuals, CD patien's microbiota display reduced diversity and altered composition (Walker et al., 2011). *E. coli* are commensal bacteria that colonize the human gastrointestinal tract. However, some *E. coli* pathovars have acquired virulence factors, presumably increasing their propensity to cause enteric disease. Six categories of classic diarrheagenic *E. coli* (DEC) have been described (Kaper et al., 2004). Likewise, analysis of CD patient-derived tissue has identified bacteria named adherent-invasive *E. coli* (AIEC) (Hansen et al., 2010) as potential contributors to CD pathogenesis (Carvalho et al., 2009; Darfeuille-Michaud et al., 2004; Nash et al., 2010). Previous studies showed that 22-65% of CD patients harbour AIEC, compared to 6-9% in controls (Darfeuille-Michaud et al., 2004; Glasser et al., 2001; Sasaki

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et al., 2007). These *E. coli* strains are characterized by the absence of specific virulence factors characteristic of classic DEC, similarity to extra-intestinal *E. coli* pathovars and capability to adhere and to invade intestinal epithelial cells and macrophages *in vitro* (Glasser et al., 2001; Martinez-Medina et al., 2009a; Nash et al., 2010). The mechanism by which AIEC accesses to the mucosa is not completely defined yet. It has been proposed that AIEC adhere via FimH, the terminal subunit of the type 1 pilus, to carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) abnormally expressed in ileal mucosa of CD patients (Barnich et al., 2007). Additionally, AIEC has been also shown to adhere and translocate through Peye's Patches via long polar fimbriae (Chassaing et al., 2011). The above findings have led to the hypothesis that AIEC represents a bacterial pathotype associated with CD (Eaves-Pyles et al., 2008; Glasser et al., 2001), however a pathogenic role in CD of these bacteria is controversial. AIEC strains NRG857c, HM605, and LF82, isolated from CD patients, have been sequenced and used as reference pathotype (Clarke et al., 2011; Miquel et al., 2010; Nash et al., 2010). However none of them show defined virulence determinant genes. On the other hand, changes in intestinal microbiota have also been observed in other inflammatory pathologies (OIP) of the intestine, such as irritable bowel syndrome (IBS) or diverticulitis, whose aetiology has not been completely elucidated (Strate et al., 2012). However, presence of intracellular *E. coli* with adherent-invasive properties has not been studied in OIP.

Multiple variants of pattern-recognition receptor (PRR) genes that sense pathogen-associated molecular patterns (PAMPs) have been associated with IBD (Kaser et al., 2010; Shih and Targan, 2008), including NOD2 (Nucleotide-binding oligomerization domain containing 2), TLR4 (Toll-like receptor 4) and NLRP3 (NOD-like receptor family, pyrin domain containing 3) (Peeters et al., 2007; Shen et al., 2010; Villani et al., 2009). Several genetic variants in the non-coding region of NLRP3 and decreased expression of the receptor have been associated with increased susceptibility to CD (Villani et al., 2009). NLRP3 is one of the sensors capable to induce the formation of the multi-protein complex inflammasome, which activates caspase-1 through adaptor protein ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD) (Franchi et al., 2012), promoting IL-1β and IL-18 processing and secretion. Different microbial stimuli, such as *E. coli* RNA and enterotoxin, have been shown to activate NLRP3 (Brereton et al., 2011; Sander et al., 2011). Although the inflammasome has been involved in some bacterial elimination by macrophages (Pereira et al., 2011), a link between NLRP3-inflammasome and mucosa-associated *E. coli* driven-inflammatory responses has not been investigated.

This study aimed to assess intracellular *E. coli* prevalence in mucosal from patients with CD, UC, OIP and controls as well as to characterize virulence genes, clonality among strains and AIEC properties. Moreover, the relationship between NLRP3-inflammasome and intracellular *E.coli* driven-inflammatory responses by macrophages, cytokine induction and microbicidal ability was determined.

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MATERIALS AND METHODS

Patients

E. coli strains included in this study were isolated from ileal specimens of Chilean patients: 34 with CD; 57 with UC; 17 with other intestinal pathologies (OIP), and 22 control individuals, subjected to colonoscopy for colon cancer evaluation. Patients were diagnosed based on standardized clinical, endoscopic and histological criteria. All patients undergoing colonoscopy were older than 18 years and were not under antimicrobial treatment for at least 2 weeks before the procedure. Clinical characteristics of patients included in the study are shown in **Supplementary Table 1**. In the case of UC, endoscopic activity was determined in the damage area using the endoscopic Mayo Score and for CD the Simple Endoscopic Score for Crohn's Disease (SES-CD).

Patients were recruited at Clínica Las Condes and Hospital Clínico Pontificia Universidad Católica de Chile in Santiago, Chile from July 2010 to January 2011. The study was approved by the Institutional Review Board of Clínica Las Condes, Faculty of Medicine Universidad de Chile and Hospital Clínico Pontificia Universidad Católica de Chile, as well as the Ethics Committee of the North Metropolitan Health Service, Santiago, Chile, and informed consent was obtained for all patients and control subjects.

Bacterial strains and culture conditions

E. coli strain isolation from intestinal biopsies of patients with CD, UC, OIP and controls for this study is detailed in "*E. coli* isolation" section. *E. coli* reference strains HS, HB101, NRG857c and HM605 were used to compare genotypic and phenotypic characteristic with clinical isolates. HS strain (Levine et al., 1978) (GenBank CP000802) is a commensal bacteria and was used as negative control to invasiveness of epithelial cells. HB101 is a non-pathogenic *E. coli* and was used as negative control of adhesiveness as it lacks fimbriae to adhere to epithelial cells (Boyer and Roulland-Dussoix, 1969; Del Canto et al., 2012; Saldaña et al., 2009). AIEC reference strains NRG857c and HM605 were kindly provided by A. Torres and I. Henderson, respectively (Nash et al., 2010; Clarke et al., 2011). All strains were cultured in LB agar at 37°C with 5% CO2 or Luria Bertani broth (LB) Miller medium (Sigma-Aldrich, St Louis, MO, USA).

E. coli isolation

Intracellular bacteria were isolated from ileal mucosa biopsies by gentamicin protection assay. Tissues were suspended in Hank's balanced salt solution (HBSS) 1X (Gibco BRL, Grand Island, NY, USA) supplemented with 100 μg/ml gentamicin (Sigma-Aldrich, St Louis, MO, USA), and incubated for 1 h at 37°C, to eliminate extracellular microbiota and favour invasive bacteria isolation. Samples were then washed with phosphate buffered saline (PBS) 1X (Calbiochem, Merck Biosciences Ltd, Nottingham, Darmstadt, Germany) and lysed in 100 μl of 1% Triton-X-100/PBS. Dilutions of tissue homogenate were made, and the total volume of homogenate and dilutions were plated on MacConkey agar (Oxoid ltd, Wade Road, Basingstoke, Hampshire, UK) and incubated for 18 h at 37°C. Individual bacterial colonies were grown in Luria Bertani broth (LB) Miller medium (Sigma-Aldrich,

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St Louis, MO, USA) under aerobic conditions for 18 h at 37°C. Grown bacteria were biochemically analysed for *E. coli* identification and the stored in 30% glycerol at −80°C.

Cells

To determine bacterial invasion of epithelial cells and survival in macrophages, Caco-2 and RAW264.7 cell lines were used, respectively. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Gibco BRL, Grand Island, NY) at 37°C and 5% CO_2.

To determine cytokine production and bacterial clearance by macrophages exposed to *E. coli* strains, bone marrow-derived macrophages (BMDMs) from $Asc^{-/-}$, $Nlrp3^{-/-}$, and wild-type (WT) mice were used, as has been previously described (Kanneganti et al., 2006; Ozören et al., 2006). Animal manipulation was conducted according approved University of Michigan Committee on the use and care of animal's protocols. Mice housed in a pathogen-free facility were used for bone-marrow-derived macrophages isolation, as previously described (Davies and Gordon, 2005). Briefly, femurs and tibias were removed from euthanized mice, marrow cavity plugs were washed out, and bone marrow cells were suspended in DMEM supplemented with 25% L cell-conditioned medium, 15% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, plated and cultured at 37°C and 5% CO_2. After 5-6 days, resulting bone-marrow-derived macrophages were replated one day before the experimental procedure. Immortalized BMDMs from *Nlrp3* deficient and WT mice, previously described (Halle et al., 2008), were maintained in RPMI-1640 (HyClone, Laboratories Inc, Utah, USA) supplemented with 10% FBS, and antibiotics at 37°C and 5% CO_2.

Virulence genotyping by PCR

For virulence genotyping, a multiplex PCR protocol was used to determine genes specifically associated with defined classic DEC (EPEC, ETEC, EIEC, EHEC, DAEC, EAEC) pathotypes (Vidal et al., 2005). In addition, extra-intestinal pathogenic *E. coli* (ExPEC) associated genes, such as fimH, papC, neuC, ibeA, hlyA, cnfI, sfaDE, iucD, fimA, and *cdtB*, previously described for AIEC strains (Martinez-Medina et al., 2009b) were assessed by monoplex PCR (Blanco et al., 1997; Ewers et al., 2007; Johnson and Stell, 2000; Moulin-Schouleur et al., 2006; Tiba et al., 2008).

Pulse-Field Gel Electrophoresis

The genetic relationship among isolated *E. coli* strains, commensal HS, and reference AIEC strains NRG857c and HM605 was analysed by pulse-field gel electrophoresis (PFGE) as described previously (Céspedes et al., 2011). *Salmonella braenderup* was used as a gel loading control. Briefly, genomic DNA (gDNA) was subjected to enzymatic digestion using *Xba*I. DNA fragments were separated by PFGE on a CHEF-DRIII system (Bio-Rad Laboratories, Richmond, CA, USA) using 1% agarose gel (pulsed-field certified agarose; BioRad Laboratories, Richmond, CA, USA) and 0.5X TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.0]) at 6 V/cm and 14°C. The Gel Compare software (Applied Maths, Kortrijk, Belgium) was used for gel analysis. Similarity indices were estimated using the Dice method, with a band position tolerance of 1 or 1.5%, based on the Unweighted Pair

Group Method with Arithmetic Mean (UPGMA). The similarity percentage cut-off to distinguish clonally distinct groups was 95%.

Phylogenetic group and serogroups determination

The major *E. coli* phylogenetic group (A, B1, B2, and D) was determined by PCR (Clermont et al., 2000). O antigen was determined using antisera raised against O1 to O185 serogroups, as previously described (Guinée et al., 1972). All antisera, kindly provided by Dr. Blanco Laboratory, LREC, Universidad de Santiago de Compostela, Lugo, Spain, were adsorbed with cross-reacting antigens to remove non-specific agglutinins. *E. coli* isolates that did not react with any O antisera were classified as nontypeable (NT).

Infection assays

Cell invasion analyses were carried out in Caco-2 cells cultured in DMEM without antibiotics, and maintained in 5% CO_2 and 37°C. Cell monolayers were infected with *E.coli* strains at multiplicity of infection (MOI) of 10, for 3h at 37 °C. After the infection period, cells were washed with 1× PBS and placed in fresh medium supplemented with gentamicin (3 mg/ml), incubated for 1 h at 37°C, and lysed with 1% Triton-X-100/PBS. Lysate serial dilutions were plated on LB agar (Merck KGaA, Darmstadt, Germany) and incubated at 37°C overnight. Colonies were counted the next day and invasion percentage was determined as intracellular bacterial content at 3 h post-infection (h.p.i) in relation to the initial inoculum. We considered invasive strains those with an invasion level that exceeded by at least a factor of five that of commensal strain HS (0.65%), which is within the invasion range previously described. (Darfeuille-Michaud et al., 2004) Cell adhesion analysis was also carried out in Caco-2 cells using similar infection conditions as described for invasion assays, but omitting the gentamicin treatment. The *E. coli* strain, HB101 was used as a negative control. Adhesion percentage was determined as recovered bacterial content at 30 min post-infection in relation to the initial inoculum.

RAW 264.7 cells, bone marrow-derived or immortalized macrophages from *Asc, Nlrp3* deficient and WT mice were infected with *E. coli* strains (MOI = 20) for 2h at 37°C. Cells were then washed in PBS and placed in fresh medium supplemented with gentamicin (3mg/ml). Intracellular bacterial content was determined at 3, 24, and 48 h.p.i. at 37°C and the ratio between bacterial content at each period and content at 3 h.p.i. was determined.

Cytokine induction by intracellular *E. coli* strains

Bone marrow-derived or immortalized macrophages from *Asc, Nlrp3* deficient and WT mice were infected with mucosa-isolated *E. coli*, HS, or NRG857c strains and TNF-α and IL-1β induction was determined in cell supernatants by enzyme-linked immunosorbent assay (ELISA) kit (R&D System, Minneapolis, MN, USA). *Asc, Nlrp3* deficient or WT macrophages were infected with *E. coli* strains (MOI = 10) for 2h at 37 °C, then washed in PBS, placed in fresh medium supplemented with gentamicin (3mg/ml), and incubated for 24h at 37°C.

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Immunoblotting

For caspase-1 detection, cells were lysed with LDB supplemented with Complete Mini Protease Inhibitor Cocktail tablets (Roche® Applied Science, Indianapolis, IN, USA). Protein concentration was determined by Bradford assay (BioRad, Hercules, CA, USA). Proteins were resolved by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Non-specific binding was blocked with 5% non-fat milk in TBS-Tween (2 mM Tris-HCl, pH 7.6, 13.7 mM NaCl). Detection of caspase-1 was determined using rabbit anti-mouse caspase-1 that was a gift from Dr. Vandanabeele (Ghent University, Ghent, Belgium), followed by horseradish peroxidase-conjugated anti-rabbit antibody (Sigma-Aldrich, Saint Louis, MO, USA). Immuno-reactive bands were visualized with SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, N Meridian, Rockford, USA).

Statistical analysis

To compare *E. coli* content in biopsies from controls and patients with CD, UC, or OPI, the Kruskal-Wallis two-tailed test and Dunn's Multiple Comparison test were used, with a significance of <0.05.To compare the prevalence of *E. coli* in different diseases in regard to control Fisher test with two-sided p-values (p<0.05) was used. To compare cytokine levels in conditioned media of *Asc, Nlrp3* deficient or WT macrophages (n=3), an unpaired t-test was used, with a significance of <0.05 (*<0.05, **<0.01, ***<0.001).

RESULTS

Intracellular *E. coli* in intestinal mucosa: prevalence and content in patients with Crohn's disease, ulcerative colitis, and other inflammatory pathologies

The prevalence of intracellular *E. coli* isolated from ileal biopsies, defined as the percentage of patients presenting bacterial colonies by gentamicin protection assay, was 14/34 (41.1%) CD, 25/57 (43.8%) UC, 10/17 (58.8%) OIP, and 1/22 (4.5%) controls (p<0.05) (**Figure 1**). Notably, the number of *E. coli* in CD, UC, and OIP patients was higher than in controls (p=0.0021) (**Figure 1**). Colony forming unit (CFU) counts were highly variable in each group of patients and between them, ranging from 1-4,700 in CD, 1-4,800in UC, and 1-3,000 in OIP patients, whereas only 2 CFU/biopsy of intracellular *E. coli* were identified in one control subject (**Figure 1**).

In terms of clinical characteristics, 36% and 60% of CD patients containing bacteria or not were women, respectively. In CD patients, there was no correlation between the presence of bacteria and disease activity or intestinal area affected. Furthermore, no association between the presence of *E. coli* and gender was identified in UC and OIP patients. However, there was an association between the presence of *E. coli* and disease activity in UC patients (p 0.0277), in which 88% of those containing bacteria were in an active state of the disease compared to 66% of those patients without bacteria in ileal mucosa.

Genetic and phenotypic characterization of clinical isolates of *E. coli* strains

PFGE analysis and virulence genes typically associated with classic DEC or ExPEC (as described in methods section) were conducted in a total of 46 intracellular *E. coli* strains, 24

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isolated from 8 CD patients, 9 from 5 patients with OIP, 8 from two UC patients, 2 from one control, as well as strains HS, NRG857c and HM605 (**Figure 2**). PFGE analysis and virulence gene identification showed that most isolates were clonal or closely related within a single patient, sharing virulence genes; however, strains were genetically heterogeneous among patients. Of the total *E. coli* strain tested, 46 were grouped into two major clusters, according to PFGE analysis (**Figure 2**); cluster I contains 42 strains derived from 8 CD, 2 UC and 4 OIP patients, control, NRG875c and HM605. Cluster II contains 3 strains, two derived from a UC patient and HS. The OIP4-a strain and *Salmonella braenderup* were excluded from both clusters according to PFGE analysis. Several genes including *fimH, papC,* and *iucD* that are characteristic of ExPEC, and previously described in AIEC reference strains, were common in most isolates (**Figure 2**). Results of phylogenetic group determination show that strains from clusters I.1.1, I.1.2 and I.2 mostly belong to groups B2, A and D, respectively **(Figure 2)**. *E. coli* strains (CD6-b and CD12-a) isolated from a CD patient (cluster I) harboured the *daaE* gene, which is characteristic of DAEC pathotype (**Figure 2**).

To evaluate phenotypic characteristics of isolated intracellular *E. coli*, Caco-2 cells were infected with 19 strains selected to represent those isolated from all group of patients and control. **Figure 3A** shows invasiveness properties of intracellular strains in Caco-2 cells, whereas the HS strain has a low invasive capacity and was considered a negative control (0.130%) and the NRG857c strain showed an invasiveness of 1.783%. Isolated *E. coli* strains from patients showed variable invasiveness; higher values were observed for CD1-a, CD2-a, CD6-b, CD6-r, CD9-a, CD14-a, UC1-b and UC3-a compared to HS strain (**Figure 3A**). Of these, CD1-a, CD2-a CD6-b and UC1-b strains were 2-15 times more invasive than NRG857c. Those obtained from different groups (C7-a, CD8-a, CD12-a, CD13-a, UC-1a, UC1-c, OIP1-a, OIP2-a, OIP4-a, OIP5-a and OIP6-a) showed a low invasive ability comparable to HS (**Figure 3A**).

We then studied another phenotypic feature of intracellular *E. coli* strains related to the adhesion capacity to epithelial cells (**Figure 3B**). HB101 strain showed a 0.442% of adhesion and was used to determine a threshold of negative response. We found that NRG857c has an adherence to Caco-2 cells of 2.657% while CD1-a, CD2-a, CD6-b, CD6-r, CD12-a and UC1b strains showed higher values (**Figure 3B**).

To further phenotypically characterize intracellular *E. coli* strains, we selected some of those from CD, UC patients and controls, and assessed their survival inside RAW 264.7 macrophages. **Table 1** shows mean recovered CFUs at 3h.p.i and the percentage of bacterial content at 8 and 24 h.p.i compared to the initial bacterial uptake (at 3h.p.i). The control HS strain did not survive inside macrophages, as was found to be eliminated at 24 h.p.i. Macrophages infected with strains CD1-a, CD2-a, CD6-b, CD6-r, CD8-a, CD9-a and UC1-b contained more than 20% of the phagocytosed bacteria at 24 h.p.i. Especially noteworthy was the CD2-a strain, that almost 100% of the phagocytosed bacteria was recovered, suggesting its strong ability to survive inside macrophages.

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Intracellular *E. coli* isolated from ileal mucosa induces release of pro-inflammatory cytokines by macrophages through NLRP3-dependent signalling pathway

We next determined if infection of BMDMs from *Asc, Nlrp3* knockout or WT mice with selected *E. coli*, with or without adherent-invasive properties, induced the activation of the NLRP3-inflammasome. We found that these bacteria induced substantial production of IL-1β and this response was abolished in NLRP3- or ASC-deficient macrophages (**Figure 4A**). In contrast, the production of TNF-α and IL-6 was partially reduced in macrophages deficient in NLRP3 or ASC (**Figure 4B and 4C**). Further analysis of the role of inflammasome in IL-1β induction by these bacteria, infection of macrophages with CD2-a or NRG857c strains showed that caspase-1 activation was significantly reduced in NLRP-3 macrophages (**Figure 4D**). In addition, and to investigate the role of NLRP3 in the ability of macrophages to eliminate *E. coli* strains, we infected WT and NLRP3-deficient macrophages with CD2-a, that highly survived in macrophages, and HS strain. However, survival of intracellular or commensal *E. coli* inside macrophages was not dependent on the NLRP3-inflammasome **(Figure 4E).**

DISCUSSION

In this work, we show that *E. coli* strains colonize the mucosa of patients with CD, UC and OIP, but AIEC characteristics were identified only in isolates from IBD patients. Moreover, *E. coli* isolated from CD are able to induce the secretion of IL-1β via NLRP3-inflammasome. It has been reported that *E. coli* with AIEC properties is found in the intestinal mucosa of a significant proportion of CD patients (Darfeuille-Michaud et al., 2004; Martinez-Medina et al., 2009b), compared to UC patients and control subjects, including those with diverticulitis and IBS (Martinez-Medina et al., 2009b). Similarly, a higher content of total mucosa-associated and intracellular *E. coli* was observed in IBD compared to control subjects (Elliott et al., 2013). In our study, *E. coli* strains were isolated, using gentamicin protection assay, from patients with CD, UC and OIP comprising diverticulitis, chronic diarrhoea, and IBS. Comparable prevalence of *E. coli* was found in all patient groups compared to controls. However, intracellular *E. coli* with invasiveness and adhesiveness capacity to epithelial cells and survival in macrophages, were those exclusively isolated from IBD patients' mucosa. It is likely that these strains may contribute to the intestinal inflammation in CD and UC, given the induction of pro-inflammatory cytokines and their ability to invade and survive within host cells. The presence of mucosa-associated *E. coli* in patients with intestinal disorders of different aetiology (IBD and OIP) may indicate that persistent inflammatory environment or changes in the microbiota could favour their accumulation in the intestine. Alternatively, epithelial barrier disruption in the different intestinal disorders studied may be associated with increased expression of CEACAM6, favouring the adhesion of *E. coli* strains having or not AIEC characteristics, via FimH.

Isolated colonies from the same patient, in most cases, were closely genetically related, whereas those from different individuals exhibited wide genetic diversity. Comparative analysis of strains by PFGE showed clusters that included bacteria isolated from patients with different intestinal pathologies carrying the same virulence genes described for AIEC

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strains, such as *fimH, papC,* and *iucD* (Martinez-Medina et al., 2009b) and most of them lack virulence genes of classic DEC (Boudeau et al., 1999), except CD6-b and CD12-a. In particular, these two strains carry the *daaE* gene, a fimbria characteristic of DAEC, and coincidently both show high adhesion capacity to epithelial cells. Furthermore, invasion capacity of CD6-b strain suggests that it could be the result of horizontal transfer of *daaE* gene. Morevoer, CD6-b is genetically related to the reference AIEC strain NRG857c as shown by PFGE analysis, reinforcing the hypothesis that it is an adherent-invasive strain with DAEC features. Moreover, as CD12-a strain contained the *daaE* virulence gene, highly adhered and poorly invaded to epithelial cells, it can be more associated with a classic DAEC pathotype.

The analysis showed that 8/19 of the strains isolated from patient-derived tissues (CD1-a, CD2-a, CD6-b, CD6-r, CD9-a, CD14-a, UC1-a, UC3-a) have AIEC properties such as invade epithelial cells and survive within macrophages as previously reported (Boudeau et al., 1999; Nash et al., 2010). These bacteria were identified in the cluster I.1.1 and I.2 in the PFGE analysis, and were categorized mainly as phylogenetic group B2 and D, similar to previously described in other AIEC strains (Martinez-Medina et al., 2009b). A remarkable capacity to invade epithelial cells and survive inside macrophages was seen for CD2-a strain, compared to NGR857c strain. However, further studies will be necessary to understand the molecular basis for the enhanced virulence displayed by CD2-a strain.

Some intracellular *E. coli* strains isolated from patient-derived ileal mucosa (CD1-a, CD2-a, CD6-r, and CD6-b) induce high levels of TNF-α and IL-6 in macrophages, and also activate caspase-1 and IL-1β secretion through NLRP3-inflammasome. However, these responses were comparable to those induced by other *E. coli* strains lacking AIEC characteristics, such as CD8-a, C7-a or commensal HS, suggesting that *E. coli* has a common feature to induce pro-inflammatory cytokines via shared PAMPs. On the other hand, NLRP3-deficient macrophages induce partially lower levels of TNF-α and IL-6 than WT cells. This response could be explained for the discrete IL-1β nduction by NLRP3-deficient macrophages, which can promote further NF-kB activation, and therefore increase TNF-α and IL-6 production, through an alternative pathway independent of PRRs. It is striking to note that strains with AIEC-like properties seem to produce less IL-1β, but higher TNF-α and IL-6 levels as compared to those with non-AIEC-like strains (HS, C7-a). However, a necessity to evaluate this trend with more strains is imminent for us to identify significant differences to associate the role of a distinctly cytokine balance evoked between *E. coli* strains holding or not AIEC features.

On the other hand, NLRP3 activation by Gram-positive bacteria can enhance microbicidal capacity of macrophages, promoting phagolysosome acidification (Sokolovska et al., 2013). However, our results show no association between NLRP3 and *E. coli* elimination in macrophages.

In conclusion, we have shown that a cohort of patients with CD, as well as UC and OIP have *E. coli* strains associated with the intestinal mucosa in a higher proportion than controls. However, only *E. coli* strains associated to IBD bear virulence genes, invasiveness and

survival inside macrophages, constituting a difficult-to-control pro-inflammatory stimulus. *E. coli* isolates induced production of IL-1β mediated via the NLRP3-inflammasome.

Since polymorphisms in NLRP3, associated with lower expression of the protein, are common in CD patients (Villani et al., 2009), it is possible that dysfunctional NLRP3 activity early in disease evolution may result in a weak innate inflammatory response to intracellular *E. coli*, inducing low levels of IL-1β (Marks et al., 2006; Moráin et al., 1981). Deficient production of this cytokine may impair the efficiency of bacterial clearance in the intestinal mucosa by a poor recruitment of neutrophil and monocytes; this, in turn, may promote chronic and sustained inflammation associated with CD and other IBD disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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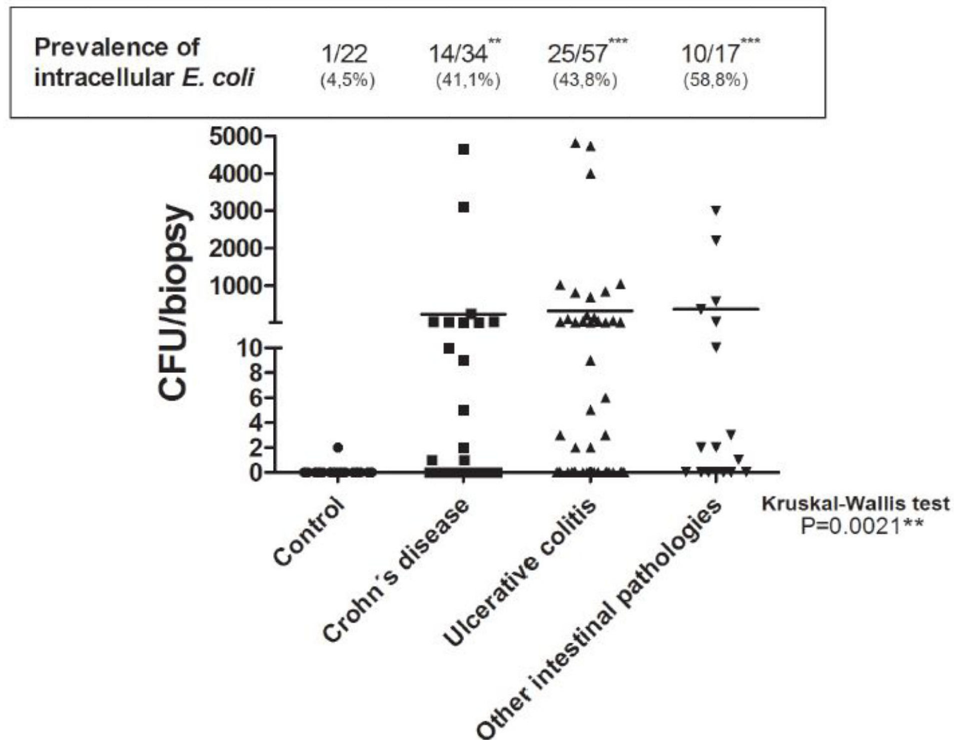

Figure 1. Intracellular *E. coli* in intestinal biopsies from CD, UC and OIP patientes

Ileal biopsies from patients with CD (n=34), UC (n=57), OIP (n=17), and controls (n=22) were incubated with gentamicin for 1h, and homogenates were then plated in MacConkey agar. Graph shows the bacterial content represented as colony-forming units (CFU) of identified intracellular *E. coli* in each biopsy and mean bacteria in each group (Kruskal-Wallis two-tailed test, Dunn's Multiple Comparison test was used, significance level set at <0.05). Prevalence of putative invasive *E. coli* is expressed as the percentage of patients with the bacteria in each group; Fisher's exact test with a two-sided p-value was used to evaluate statistical significance as compared to the control group (p<0.05, CD p**=0.0023, UC p***=0.0005, OIP p***=0.0003).

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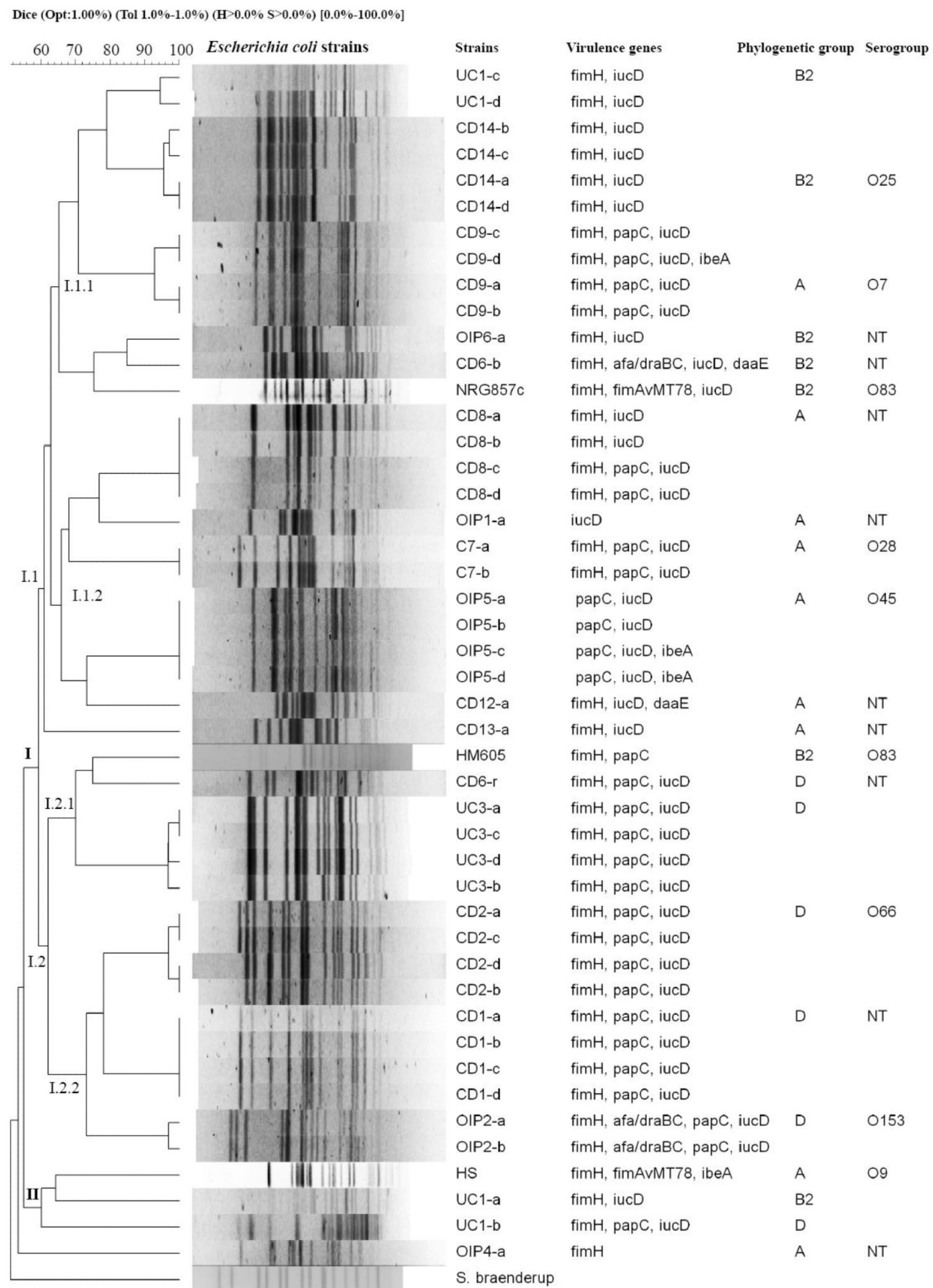

Figure 2. Genetic variability among *E. coli* isolates from patients with CD, UC and OIP

Dendrogram based on pulsed-field gel electrophoresis (PFGE) of *E. coli* strain DNA digested with *Xba*I. PFGE analysis identified two groups of genetically-related strain clusters (I and II). Cluster A harboured the greatest number of strains and was in turn divided into I.1 (I.1.1 and I.1.2) and I.2 (I.2.1 and I.2.2). The right side of the figure shows the strain name (patient code-clone), virulence genes identified in each strain by PCR, phylogenetic group and serogroup. *E. coli* isolates were compared to HS commensal bacteria, and NRG857c and HM605 reference AIEC strains.

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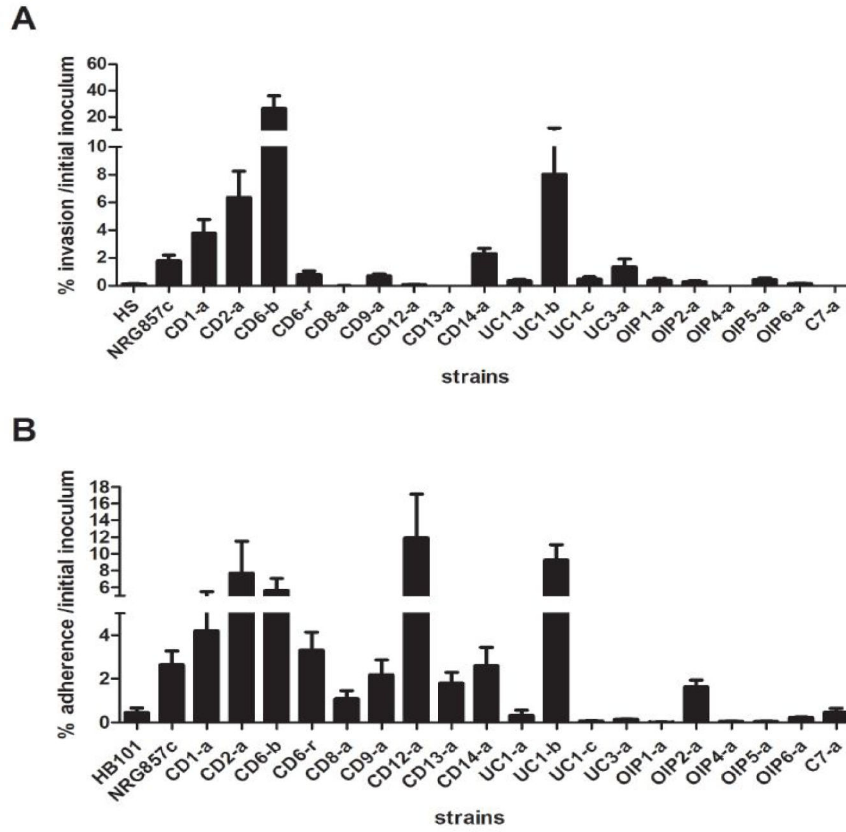

Figure 3. Invasion and adhesion of isolated *E. coli* strains in epithelial cells

(A) To evaluate invasion, Caco-2 cells were infected with representative *E. coli* strains isolated patient-derived tissue with a MOI=10 for 3h and then incubated with gentamicin for 1h. Graph shows the percentage of inoculum surviving gentamicin treatment. Graph represents mean and standard errors of three independent experiments in duplicate. (B) The capacity of isolated *E. coli* to adhere to Caco-2 cells was evaluated using a MOI=10 for 30 min. Graph shows the percentage of adhered bacteria to epithelial cells related to the initial inoculum. Data represents mean and standard errors of three independent experiments in duplicate.

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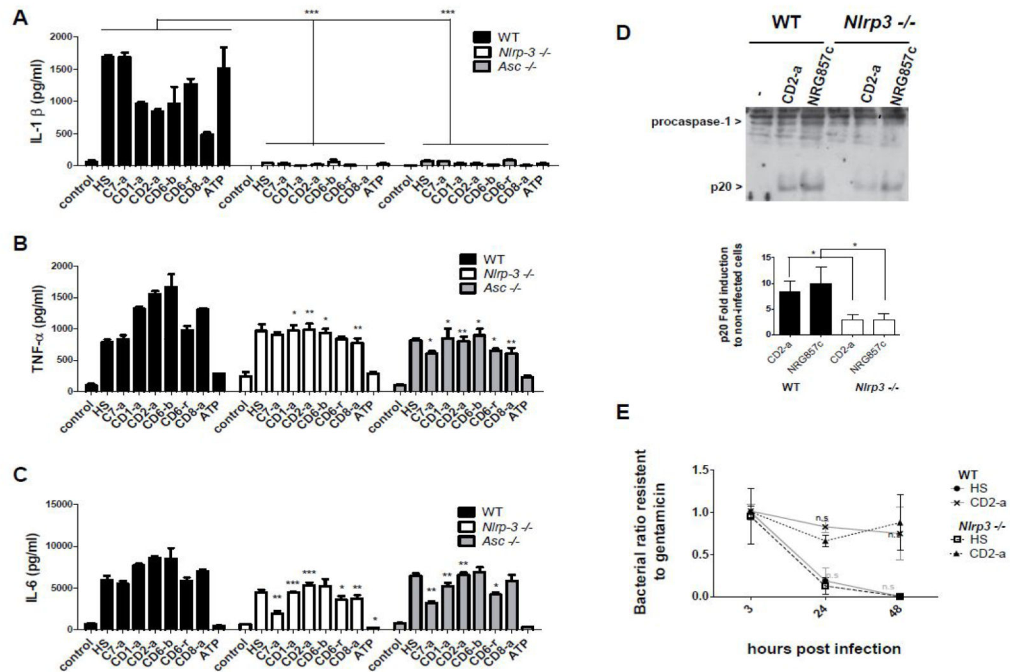

Figure 4. IL-1β production by macrophages infected with isolated *E. coli* depends on the NLRP3 inflammasome

(A) Bone marrow-derived macrophages from *Nlrp3*$^{-/-}$,*Asc*$^{-/-}$ or WT mice cells were primed with LPS for 4 h. After that, cells were infected with *E. coli* isolated from CD (CD1-a, CD2-a, CD6-b, CD6-r, CD8-a) patients, control (C7-a) or HS strain (MOI=10) for 1 h and then treated with gentamicin. Twenty-four h.p.i, supernatants were analysed for IL-1β TNF-α and (C) IL-6 secretion by ELISA. (D) As an indicator of caspase-1 activation, content of caspase-1 p20 subunit was determined in WT or *Nlrp3*$^{-/-}$ macrophages primed with LPS for 4 h, infected with CD2-a or NRG857c strains (MOI=10) for two h and then treated with gentamicin overnight. Extracts were prepared from cell and supernatant, and immunobloted using a caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. Graph bar shows content of caspase-1 p20 subunit related to procaspase-1 in each condition expressed as the fold induction in infected to non-infected cells. Graph represents mean and standard errors of three independent experiments (E) Bacterial clearance ability of *Nlrp3*$^{-/-}$ or WT macrophages infected with CD2-a or HS strains was evaluated at 3, 24 and 48 h.p.i. Graph shows the ratio of gentamicin-resistant bacteria to initial uptake (3 h.p.i). Graph represents mean and standard errors of three independent experiments, performed in duplicate. No significant differences were found in bacterial clearance between *Nlrp3*$^{-/-}$ and WT macrophages, infected with the same strain, using Fisher's exact test with a two-sided p-value.

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Table 1

Ability of *E. coli* strain isolated from patients to survive inside RAW 264.7 macrophages

The table shows the initial uptake defined as the mean CFU resisting gentamicin treatment after 3h.p.i, and the percentage of bacterial content at 8 and 24 h.p.i related to the initial uptake (*). HS strain was used as negative control. Mean and standard deviation indicated correspond to three independent experiments, performed in duplicate.

Strain	initial uptake (3 h.p.i)	% 8 h.p.i*	% 24 h.p.i*
HS	90,833 ± 10,368	54.39 ± 26.48	0.24 ± 0.22
NRG857c	1,073,333 ± 295,945	60.12 ± 19.13	0.56 ± 0.70
C7-a	26,070 ± 18,176	86.54 ± 21.23	1.39 ± 1.93
CD1-a	266,667 ± 60,422	102.08 ± 8.68	39.74 ± 14.70
CD2-a	555,556 ± 197,390	112.10 ± 35.22	98.88 ± 21.89
CD6-b	143,889 ± 87,754	100.39 ± 20.89	43.47 ± 14.45
CD6-r	272,333 ± 257,595	110.18 ± 25.74	59.76 ± 16.01
CD8-a	33,019 ± 59,267	101.59 ± 19.15	56.99 ± 23.68
CD9-a	443,333 ± 153,930	112.11 ± 30.22	20.97 ± 11.04
CD12-a	42,222 ± 33,973	43.59 ± 18.81	0.27 ± 0.24
CD13-a	62,855 ± 114,635	55.47 ± 18.05	0.41 ± 0.56
CD14-a	168,611 ± 54,410	126.74 ± 43.97	8.58 ± 8.37
UC1-b	509,815 ± 388,578	133.75 ± 60.45	20.58 ± 15.76
UC3-a	20,501 ± 21,178	103.69 ± 36.08	8.99 ± 2.13