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Original Article

# Monocyte-derived Macrophages from Crohn's Disease Patients Are Impaired in the Ability to Control Intracellular Adherent-Invasive *Escherichia coli* and Exhibit Disordered Cytokine Secretion Profile

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**Special note:** This paper is dedicated to the memory of Arlette Darfeuille-Michaud who sadly passed away on June 28, 2014.

## Abstract

**Background:** Ileal lesions of Crohn's disease [CD] patients are colonised by adherent-invasive *Escherichia coli* [AIEC] able to survive in macrophage cell lines. We analysed the ability of monocyte-derived macrophages [MDM] from CD patients to control AIEC intracellular replication and the pro-inflammatory cytokine response of the infected-MDM.

**Methods:** Peripheral blood MDM were obtained from 24 CD genotyped for *NOD2* and *ATG16L1* mutations, 5 ulcerative colitis [UC] patients and 12 healthy controls [HC]. The numbers of intracellular bacteria were determined using gentamicin assay. Cytokine secretion was quantified by ELISA assay.

**Results:** We observed that higher levels of bacteria were internalised within MDM from CD patients than MDM from HC or UC patients. MDM from CD patients were unable to restrict AIEC intracellular replication. Infection of MDM from CD patients with AIEC resulted in significantly increased secretion of IL-6 and tumour necrosis factor alpha [TNF  $\alpha$ ] than did infection with non-pathogenic *E. coli*. AIEC-infected MDM from CD patients exhibited a disordered cytokines secretion compared with MDM from UC patients and HC. AIEC-infected MDM from patients with quiescent CD released significantly higher amounts of IL-6 and TNF-alpha than those with active disease or those from HC. The level of secreted TNF-alpha was correlated to the number of intracellular AIEC in MDM from CD patients. Treatment of MDM with infliximab did not change the MDM behaviour.

**Conclusions:** MDM from CD patients are unable to restrict intracellular AIEC replication, leading to disordered inflammatory response influenced by disease activity.

**Key words:** Macrophages; Crohn's disease; adherent-invasive *E. coli*; cytokines; infliximab

## 1. Introduction

Crohn's disease [CD] and ulcerative colitis [UC] are two idiopathic inflammatory bowel diseases [IBD]<sup>1,2</sup> with increasing incidence worldwide.<sup>3,4</sup> Although aetiology of IBD has not yet been fully elucidated, it is now widely accepted that IBD result from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host. Evidence of the importance of host-microbe interactions in the pathogenesis of IBD has been widely highlighted by genetic studies. In particular, *NOD2* [nucleotide oligomerization domain 2] gene encodes an intracellular innate immune receptor, and in *ATG16L1* [autophagy-related like 1, T300A] is involved in the machinery and regulation of autophagy, an intracellular degradation pathway that delivers cytoplasmic content, including bacteria, to lysosomes. The association of CD with polymorphisms of those genes strengthens the hypothesis of the involvement of invasive bacteria in the disease.<sup>5,6,7,8,9</sup> Defects in such pathways could lead to ineffective clearance of pathogens and dysregulated inflammation.<sup>10,11</sup>

Intramucosal *Escherichia coli* or mucosa-associated *E. coli* with invasive properties in CD patients has been reported in a number of independent studies.<sup>12,13,14,15,16,17,18</sup> CD-associated *E. coli* are highly adherent and invasive, and accordingly they have been termed adherent-invasive *E. coli* [AIEC]. The presence of *E. coli* has been convincingly evidenced within macrophages in CD tissue. *E. coli* antigens have been identified in macrophages within the lamina propria and in the germinal centres of mesenteric lymph nodes in CD patients,<sup>19,20,21</sup> and *E. coli* DNA was detected in 80% of microdissected granulomas from CD patients.<sup>22</sup> In addition, AIEC can target M cells, which could allow them to interact with Peyer's patches and lamina propria macrophages.<sup>23</sup> *In vitro* studies have demonstrated that *E. coli* associated with CD are able to survive and replicate within macrophages, and infected macrophages secreted high amounts of tumour necrosis factor alpha [TNF- $\alpha$ ].<sup>24,25,26,27,28,29,30</sup> We recently showed that TNF- $\alpha$  enhances the intramacrophagic replication of AIEC LF82 bacteria and that, conversely, treatment of infected macrophages using antibodies that neutralise the bioactivity of TNF- $\alpha$  decrease the number of intracellular AIEC bacteria in J774 macrophages.

The aim of the present study was to compare the ability of peripheral blood monocyte-derived macrophages [MDM] isolated from CD patients to handle internalisation and intracellular replication of AIEC bacteria with those from UC patients and healthy controls, and the pro-inflammatory cytokine response in infected macrophages.

## 2. Materials and Methods

### 2.1. Ethical considerations

The study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice and applicable regulatory requirements. Study ethics approval was obtained on Comité de Protection des Personnes (CPP) Sud-Est 6, France [approval number AU 904].

### 2.2. Patients

A total of 24 CD patients with quiescent CD (Crohn's disease activity index [CDAI] < 150) or active CD [CDAI  $\geq$  150], 5 UC patients and 12 healthy controls were prospectively and consecutively included between February 2012 and May 2013, at the University Hospital of Clermont-Ferrand, France. Patients presenting with previous or concomitant anti-TNF therapy exposure were excluded. Blood samples [50 ml] were drawn from all participants in EDTA tubes regarding MDM separation and in dry tubes regarding genetics. All CD patients were genotyped for the main coding mutations in *NOD2* (single nucleotide polymorphism [SNP]8 [Arg702Trp], SNP12 [Gly908Arg], and SNP13 [Leu1007 fsins C]) and for *ATG16L1* [T300A].

### 2.3. Procedure

#### 2.3.1. Bacterial strains

AIEC strain LF82 was isolated from a chronic ileal lesion of a patient with CD.<sup>14</sup> The *E. coli* K-12 C600 strain [laboratory stock] was used as a non-pathogenic control.

#### 2.3.2. MDM isolation and culture

Monocytes were purified from blood by Ficoll [Eurobio] density gradient separation and by negative selection using the EasySep™ Human Monocyte Enrichment Kit [Stem Cell]. Monocytes were suspended in RPMI 1640 medium [Dutcher] supplemented with 10% heat-inactivated fetal calf serum [FCS; Dutcher], 1% L-glutamine [Life Technologies], and 0.2  $\mu$ g/ml of recombinant human macrophage colony stimulating factor [rh-M-CSF; Immunotools]. Cells were seeded into 48-well culture plates at a density of  $2.5 \times 10^5$  and were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for 6 days.

#### 2.3.3. MDM uptake, survival and replication assays

Before infection, MDM were washed twice with PBS and the medium was replaced with 1 ml of RPMI 1640 supplemented with 10% heat-inactivated FCS. MDM were infected at a multiplicity of infection [MOI] of 100 bacteria per macrophage. After 10 min of centrifugation at 1000g and a 10-min incubation period at 37°C with 5% CO<sub>2</sub>, fresh cell culture RPMI 1640 medium, supplemented with 10% heat-inactivated FCS and containing 20  $\mu$ g/ml of gentamicin, was added for a period of 40 min [1 h post-infection] or 10 h [10 h post-infection], and the numbers of intracellular bacteria were determined as previously described.<sup>28</sup>

#### 2.3.4. TNF- $\alpha$ neutralization

Macrophages were infected as described above. Infliximab at 1  $\mu$ g/ml [Remicade, Centocor, Malvern, Philadelphia, PA] was added during infection and after infection to the medium containing gentamicin.

#### 2.3.5. Enzyme-linked immunosorbent assay

At 10 h post-infection, supernatants were collected, centrifuged, and stored at -80°C. The amounts of IL-6, IL-8, and TNF- $\alpha$  released in

cell culture supernatants were determined by ELISA [R&D systems]. Cytokine concentrations were assessed according to the manufacturer's instructions.

## 2.4. Statistical analysis

Data were analysed by Mann–Whitney, Kruskal–Wallis, and Spearman tests. A  $p$ -value  $\leq 0.05$  was considered statistically significant. Data are expressed as the median [Q1, Q3] or the mean  $\pm$  SEM. Univariate analysis was performed to look for factors associated with the ability of peripheral blood MDM isolated from CD patients to handle internalisation and intracellular replication of AIEC bacteria. The studied factors are listed in Table 1.

Study data were collected and managed using REDCap electronic data capture tools hosted at the University Hospital of Clermont-Ferrand.<sup>31</sup> REDCap [Research Electronic Data Capture] is a secure, web-based application designed to support data capture for research studies, providing: 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data

downloads to common statistical packages; and 4) procedures for importing data from external sources.

## 3. Results

### 3.1. Patients' characteristics

In our cohort, median ages were 45.0 [28.0–54.5], 43.0 [39.0–48.0], and 32.0 [28.0–43.0] years, respectively, in CD, UC, and healthy controls. Overall 11 CD patients [45.8%], 1 UC patient [20.0%] and 6 controls [50.0%] were females. All the patients' characteristics are given in Table 1.

### 3.2. Increased number of AIEC internalised within MDM from CD patients

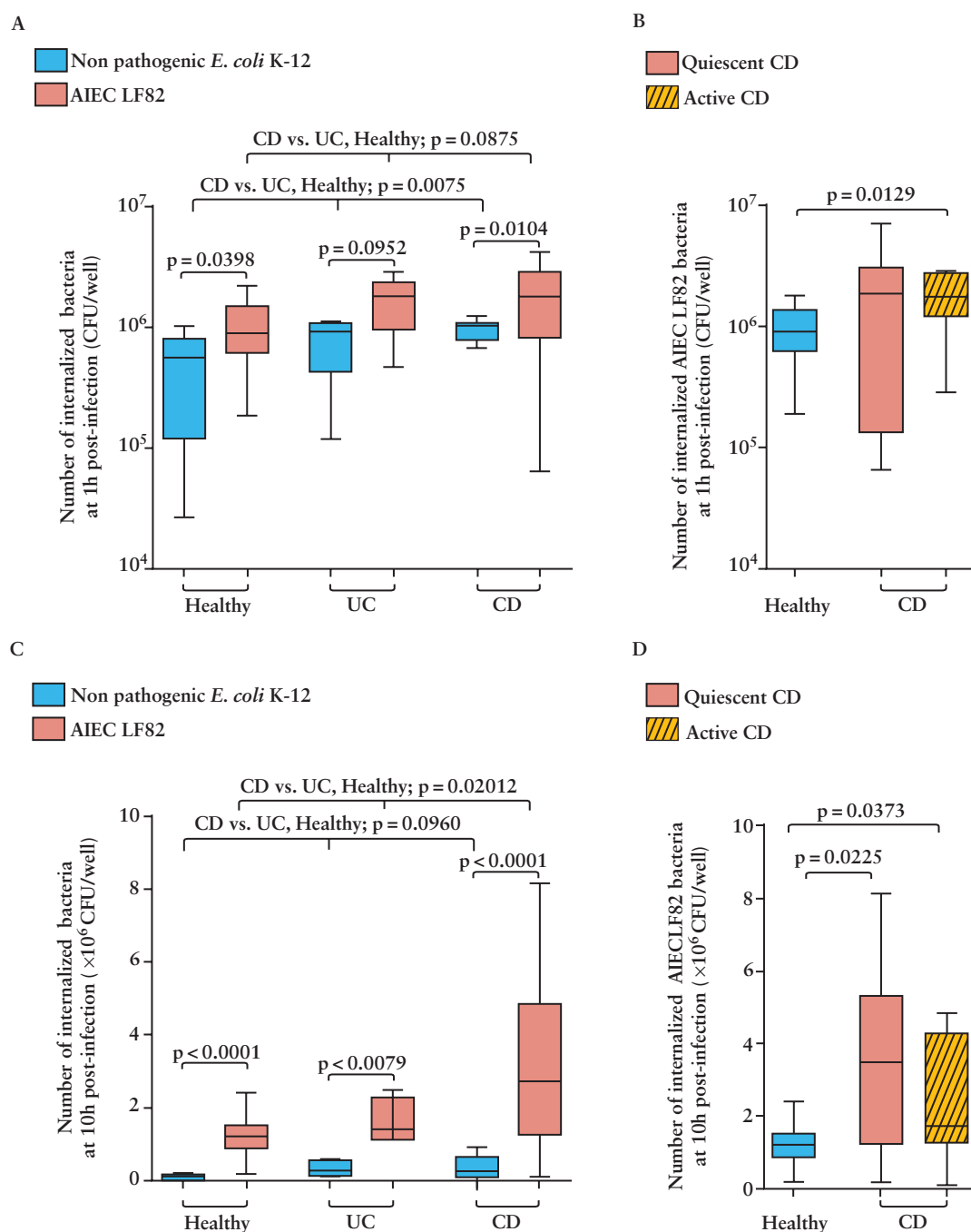
The uptake of AIEC reference strain LF82 and non-pathogenic *E. coli* K-12 C600 strain by macrophages was assessed in peripheral blood MDM obtained from healthy controls, CD, and UC patients [Figure 1A]. The numbers of intracellular bacteria within macrophages were determined at 1 h post-infection [Figure 1A].

**Table 1.** Characteristics of Crohn's disease patients, ulcerative colitis patients, and healthy controls.

Characteristics	Patients with active CD	CD patients in remission	Ulcerative colitis patients	Healthy controls
<i>n</i>	11	13	5	12
Age [years]	51[34–62]	30[28–46]	43 [39.0–48.0]	32 [28.0–43.0]
Gender				
Female [ <i>n</i> , %]	5, 45.5	6, 54.5	1, 20.0	6, 50.0
Male [ <i>n</i> , %]	6, 54.5	5, 45.5	4, 80.0	6, 50.0
Weight [kg]	71[62.5–87.5]	60[54.0–70.0]	71.0 [65.0–82.0]	68.0 [55.0–74.0]
Height [cm]	170[163–175]	166[163–170]	177.0 [173.0–180.0]	172.5 [165.5–175.5]
Body mass index [kg/m <sup>2</sup> ]	25.9[19.4–28.2]	20.4[19.8–25.6]	23.9 [23.7–24.8]	22.2 [20.3–25.0]
Smoking status				
Active smokers [ <i>n</i> , %]	5, 45.4	4, 30.7	2, 40.0	3, 25.0
Former smokers [ <i>n</i> , %]	1, 9.1	6, 46.2	2, 40.0	3, 25.0
Never smoked [ <i>n</i> , %]	5, 45.4	3, 23.1	1, 20.0	6, 50.0
Familial history of IBD [ <i>n</i> , %]	0, 0.0	3, 23.1	0, 0.0	0, 0.0
Previous appendectomy [ <i>n</i> , %]	5, 45.4	6, 46.2	0, 0.0	6, 50.0
Montreal classification				
Crohn's disease				
L1	2, 18.2	2, 15.4	-	-
L2	5, 45.4	4, 30.7	-	-
L3	4, 36.4	7, 53.8	-	-
L4	2, 18.2	5, 38.5	-	-
B1	8, 54.5	10, 76.9	-	-
B2	3, 27.3	1, 7.7	-	-
B3	0, 0.0	2, 15.4	-	-
p	2, 18.2	2, 15.4	-	-
Ulcerative colitis				
E1	-	-	1, 20.0	-
E2	-	-	1, 20.0	-
E3	-	-	3, 60.0	-
Age at diagnosis [years]	46[21–53.5]	24[20–40]	43.0 [39.0–48.0]	-
Disease duration [years]	1[0–5]	4[0–7]	5.1 [2.7–5.3]	-
CDAI	250[188–295]	104[44–131]	-	-
SCCAI	-	-	2.0 [1.0–8.0]	-
Treatment				
5-ASA	0, 0.0	0, 0.0	2, 40.0	-
Steroids	2, 18.2	0, 0.0	1, 20.0	-
Thiopurines	3, 27.3	6, 46.2	3, 60.0	-
C-reactive protein [g/l]	19.0[4.5–24.0]	1.0[3.0–4.0]	2.9 [2.9–25.0]	3.0 [3.0–3.5]

When not specified, results are indicated in median [low interquartileupper interquartile].

*n*, number; IBD, inflammatory bowel disease; CDAI, Crohn's disease activity index; SCCAI, Simple clinical colitis activity index [Walmsley's score]; CD, Crohn's disease ; 5-ASA, 5-aminosalicylic acid.



**Figure 1.** Internalisation of AIEC LF82 bacteria and non-pathogenic *E. coli* K-12 bacteria within monocyte-derived macrophages obtained from healthy volunteers, ulcerative colitis [UC], and Crohn's disease [CD] patients. Monocyte-derived macrophages [MDM] from healthy controls, CD, and UC patients were infected with AIEC strain LF82 and non-pathogenic *E. coli* K-12 C600 strain. The numbers of bacteria internalised within macrophages were determined at 1h post-infection [A, B] and at 10h post-infection [C, D]. Results were expressed as numbers of colony-forming units [CFU] per well. Data are represented as a box plot showing the median and the lower and upper quartiles. Data were analysed by Mann-Whitney and Kruskal-Wallis tests. A  $p$ -value  $\leq 0.05$  was considered statistically significant.

Interestingly, in CD patients, the number of internalised bacteria seemed to be higher than those observed in MDM from healthy controls and UC patients. This was observed from K12 [ $p = 0.0074$ ] and was almost significant for LF82 [ $p = 0.0875$ ], indicating that MDM from CD patients are more permissive to infection than MDM from healthy controls or UC patients. No difference was observed in the numbers of internalised bacteria within MDM from CD patients according to the disease activity [Figure 1B].

In addition, we observed that a higher number of intracellular AIEC LF82 bacteria were internalised within MDM, regardless of their origin, compared with non-pathogenic *E. coli* K-12 C600 bacteria. This was significant in MDM obtained from healthy controls [ $p = 0.0398$ ] and CD patients [ $p = 0.0104$ ] but not those from UC patients [ $p = 0.0952$ ]. This result shows an enhanced ability of AIEC LF82 bacteria to enter macrophages compared with non-pathogenic *E. coli* K-12 C600.

### 3.3. Impaired ability of MDM from CD patients to control intracellular AIEC

The ability of CD-associated and non-pathogenic *E. coli* to survive and replicate within MDM was analysed by determining the number of intracellular bacteria at 10h post-infection [Figure 1C–D and Figure 2]. As shown in Figure 1C, the number of intracellular AIEC LF82 bacteria was significantly higher than that of intracellular non-pathogenic *E. coli* K-12 bacteria, whatever the origin of MDM [healthy controls,  $p < 0.0001$ ; CD patients,  $p < 0.0001$ ; UC patients,  $p = 0.0079$ ]. AIEC LF82 bacteria were able to survive within MDM from healthy controls [ $p < 0.0001$ ], CD patients [ $p = 0.0079$ ], and

UC patients [ $p < 0.0001$ ] [Figure 1C], in contrast to non-pathogenic *E. coli* K-12 C600 bacteria that were efficiently killed by macrophages [Figures 1C and 2A]. When data were analysed according to disease activity, numbers of intracellular AIEC LF82 bacteria observed in MDM from CD patients with quiescent or active disease were similar [Figure 1D]. In addition, we showed that AIEC LF82 bacteria were able to replicate within MDM from CD patients but not within MDM from UC patients or healthy controls [ $p = 0.0168$ ] [Figure 2B], showing that replication was not related to the initial phagocytosis level. These results point to the defective ability of MDM from CD patients to control intracellular AIEC bacteria replication. Univariate analysis did not show any association between clinical factors [listed in Table 1] such as age at diagnosis, disease duration, Montreal classification, concomitant therapies, smoking habits, and CRP value, and the ability of macrophages from CD patients to control AIEC bacteria.

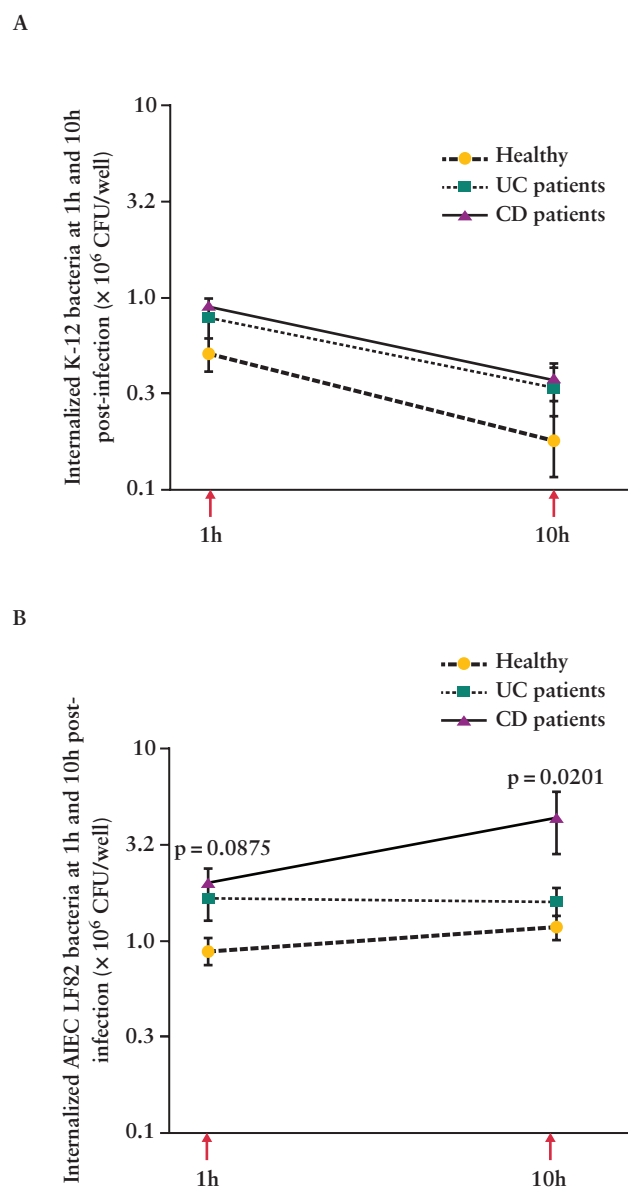
### 3.4. Association between *NOD2* and *ATG16L1* polymorphisms and the ability of macrophages from CD patients to control AIEC bacteria

Genetic analysis of patients in our cohort revealed that 14 CD patients were normal for *NOD2* and *ATG16L1*, 5 CD patients were heterozygous for *NOD2* [SNP8], and 4 CD patients were homozygous for *ATG16L1* [T300A]. No patient carried SNP12 and 13 polymorphisms in *NOD2* gene. We analysed the ability of macrophages, carrying *NOD2* [SNP8] or *ATG16L1* [T300A] genes variants or not, to control AIEC LF82 bacteria [Figure 3]. The median number of AIEC LF82 bacteria internalised within macrophages at 1h post-infection were similar between the three genetic groups [Normal =  $1.54 \times 10^6$  CFU/well; *NOD2* [SNP8] =  $1.84 \times 10^6$  CFU/well; *ATG16L1* [T300A] =  $1.74 \times 10^6$  CFU/well]. However, the numbers of intracellular bacteria at 10h post-infection seemed to be higher in macrophages from CD patients heterozygous for *NOD2* [median =  $4.28 \times 10^6$  FU/well] [ $p = 0.18$ ] or homozygous for *ATG16L1* [median =  $3.95 \times 10^6$  CFU/well] [ $p = 0.07$ ] compared with those in macrophages from CD patients normal for *NOD2* and *ATG16L1* [median =  $1.71 \times 10^6$  CFU/well] [Figure 3B] but this did not reach statistical significance.

### 3.5. Disordered cytokine secretion profile in MDM from CD patients infected with AIEC bacteria

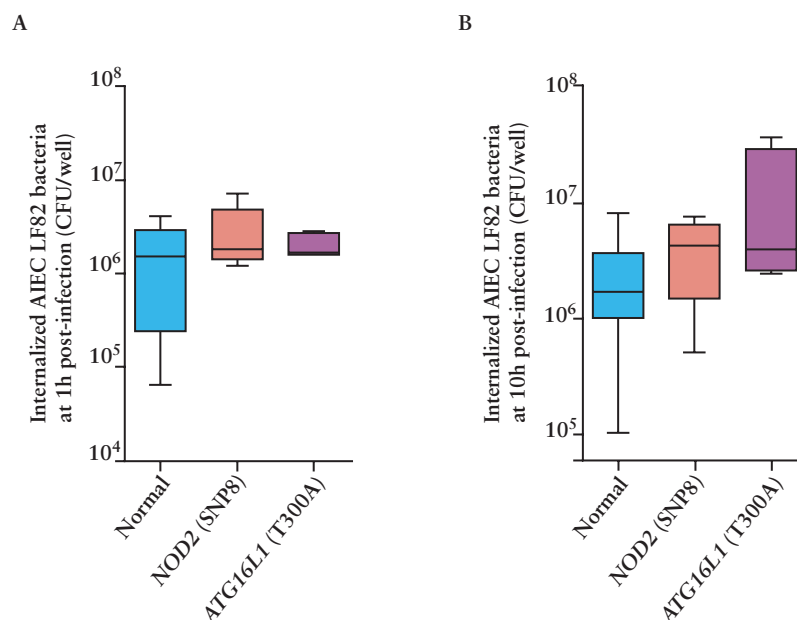
The levels of the pro-inflammatory cytokines IL-6 and TNF- $\alpha$  and of the chemokine IL-8 were determined at 10h post-infection in the supernatants of infected MDM [Figure 4]. Infection of MDM from healthy controls or UC patients with AIEC or non-pathogenic *E. coli* strain K-12 induced the secretion of similar levels of IL-8 [Figure 4A], IL-6 [Figure 4B] and TNF- $\alpha$  [Figure 4C]. However, significant increases in the levels of IL-6 [ $p = 0.0259$ ] and TNF- $\alpha$  [ $p = 0.0259$ ] were measured in the supernatants of MDM obtained from CD patients infected with AIEC LF82 bacteria compared with those infected with non-pathogenic *E. coli* strain K-12 C600. This result indicates that AIEC induced a stronger inflammatory response than non-pathogenic *E. coli*, in MDM from CD patients.

In addition, we observed that the levels of IL-8 secreted by MDM obtained from CD patients infected with AIEC bacteria [ $p = 0.0177$ ] or non-pathogenic *E. coli* K-12 C600 [ $p = 0.0462$ ] were significantly decreased compared with those of infected MDM from healthy controls and UC patients. Disease activity had no impact on the IL-8 secretion by infected MDM from CD patients [Figure 4D]. We also observed higher amounts of IL-6 [ $p = 0.0157$ ] and to a lesser extent



**Figure 2.** Ability of monocyte-derived macrophages from healthy volunteers, ulcerative colitis [UC], and Crohn's disease [CD] patients to control intracellular AIEC LF82 bacteria and non-pathogenic *E. coli* K-12 bacteria. Survival and replication abilities of non-pathogenic K-12 C600 bacteria [A] and AIEC LF82 bacteria [B] in MDM from control, UC, and CD patients. Results were expressed as numbers of colony-forming units [CFU]/well at 1h and 10h post-infection. Data are means  $\pm$  standard error of the mean [SEM] and were statistically analyzed by Mann-Whitney and Kruskal-Wallis tests. A  $p$ -value  $\leq 0.05$  was considered statistically significant.





**Figure 3.** Ability of monocyte-derived macrophages [MDM] from Crohn's disease [CD] patients to control intracellular AIEC LF82 bacteria according to genetic status. MDM from 14 CD patients normal for *NOD2* and *ATG16L1*, 5 CD patients heterozygous for *NOD2* [SNP8], and 4 CD homozygous for *ATG16L1* [T300A] were infected with AIEC strain LF82. The numbers of bacteria internalised within macrophages were determined at 1h post-infection [A] and at 10h post-infection [B]. Results are expressed as numbers of colony-forming units [CFU] per well. Data are represented as a box plot showing the median and the lower and upper quartiles. Results did not reach statistical significance ( $p = 0.18$  for CD patients heterozygous for *NOD2* [SNP8] and  $p = 0.07$  for CD homozygous for *ATG16L1* [T300A] compared with patients with no mutation).

of TNF- $\alpha$  [non significant (ns)] in the supernatants of LF82-infected MDM from CD patients than those from healthy controls or UC patients. Interestingly, when data were analysed according to disease activity, we observed that the levels of IL-6 [Figure 4E] and TNF- $\alpha$  [Figure 4F] secreted by AIEC LF82-infected MDM from CD patients with active disease were significantly lower than those secreted by AIEC LF82-infected MDM from CD patients with quiescent disease. Indeed, the levels of IL-6 and TNF- $\alpha$  secreted by AIEC LF82-infected MDM from CD patients with active disease were similar to those secreted by AIEC LF82-infected MDM from healthy controls. In contrast, the levels of IL-6 [Figure 4E] and TNF- $\alpha$  [Figure 4F] secreted by K-12 C600-infected MDM from CD patients with active disease were not significantly different from those secreted by K-12 C600-infected MDM from CD patients with quiescent disease or from healthy controls. Altogether, these results showed that the inflammatory response of MDM from CD patients to AIEC infection differs according to the disease activity.

### 3.6. Correlation of TNF- $\alpha$ secretion by infected MDM from CD patients and the number of AIEC LF82 bacteria

We investigated whether levels of the pro-inflammatory cytokines IL-6 and TNF- $\alpha$  secreted by AIEC LF82-infected MDM from CD patients could be related to the number of intracellular bacteria at 10h post-infection by performing statistical correlation analysis [Spearman test]. We observed no correlation between the amounts of IL-6 secreted and the numbers of intracellular AIEC bacteria regardless of the MDM origin [healthy controls, UC, or CD] [Figure 5A-C]. No correlation was observed either between the amounts of TNF- $\alpha$  secreted by infected macrophages and the numbers of intracellular AIEC bacteria within MDM from control and UC patients [Figure 5D-E]. However, the amount of TNF- $\alpha$  released

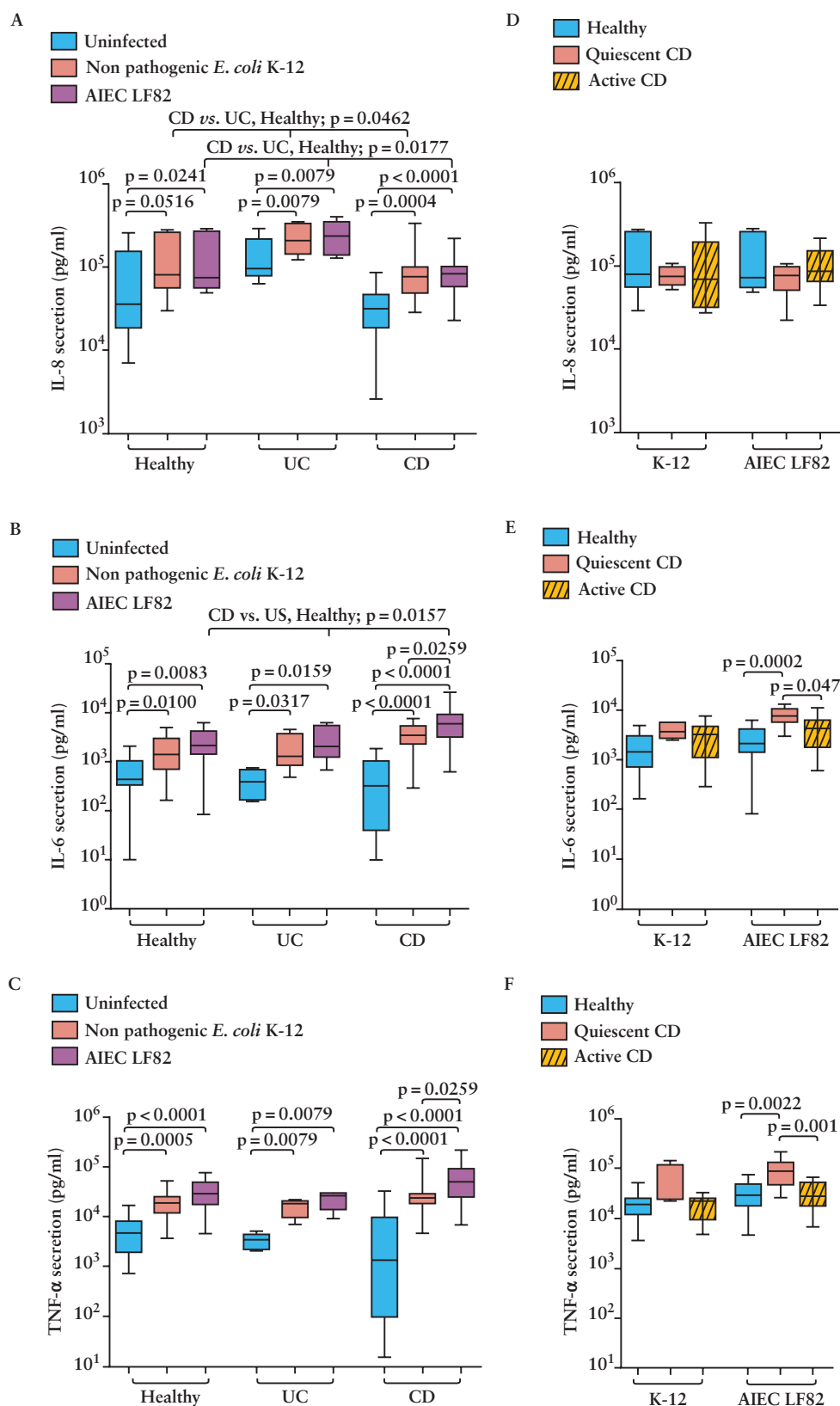
by AIEC-infected MDM obtained from CD patients was related to the number of intracellular bacteria at 10h post-infection [ $r = 0.5584$ ,  $p = 0.0069$ ] [Figure 5F].

We have previously shown that treatment of AIEC-infected J774 macrophages with antibodies that neutralise the bioactivity of TNF- $\alpha$  decreases the number of intracellular bacteria.<sup>6</sup> Here, we investigated whether infliximab, a chimeric monoclonal antibody against TNF- $\alpha$ , could impact on the intracellular survival/replication of AIEC bacteria within MDM [Figure 6]. Treatment with infliximab did not modify the number of intramacrophagic bacteria at 1h or 10h post-infection in MDM from healthy controls, CD, or UC patients.

## 4. Discussion

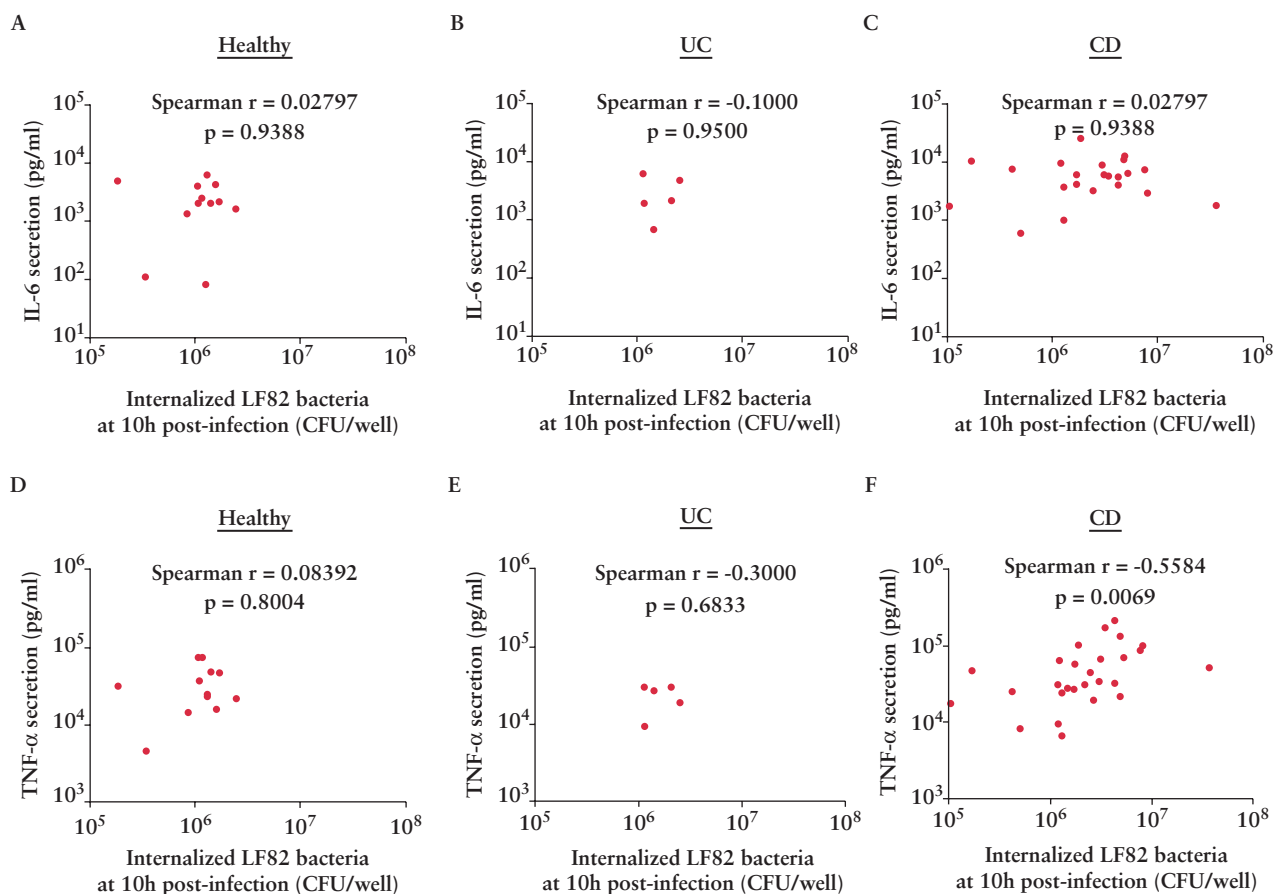
The complex aetiology of CD has not been yet fully elucidated. However, several experimental and genetic evidences point out defective innate immunity in CD. In particular, anomalies in macrophages are suspected to account for some immunological defects associated with the development of CD. Besides, due to their virulence traits, including their ability to highly colonise intestinal mucosa in inflammatory mouse models and in CD patients, to survive within macrophages and to induce severe inflammatory response, adherent-invasive *E. coli* are considered as a factor involved in CD aetiopathogenesis. In the present study, we analysed the ability of macrophages obtained from CD patients to handle AIEC and the inflammatory response associated to cell infection by these pathogenic bacteria.

Analysis of bacteria uptake by MDM revealed that cells obtained from CD patients seemed to be more permissive to both AIEC and non-pathogenic *E. coli* internalisation than MDM obtained from healthy controls or UC patients. This increased internalisation was not related to the disease activity. Conflicting results have been reported regarding phagocytosis of CD-associated monocytes/



**Figure 4.** Pro-inflammatory cytokine secretion in monocyte-derived macrophages [MDM] from healthy volunteers, ulcerative colitis [UC] and Crohn's disease [CD] patients infected with AIEC LF82 or non-pathogenic *E. coli* K-12 bacteria. Determination of the amount of IL-8 [A, D], IL-6 [B, E], and tumour necrosis factor alpha [TNF- $\alpha$ ] [C, F] released in cell culture supernatants of MDM from healthy controls, UC, and CD patients infected with AIEC LF82 or *E. coli* K-12 bacteria. Data are expressed as pg/ml of cytokines and are represented as a box plot showing the median and the lower and upper quartiles. Data were analysed by Mann-Whitney and Kruskal-Wallis tests. A  $p$ -value  $\leq 0.05$  was considered statistically significant.



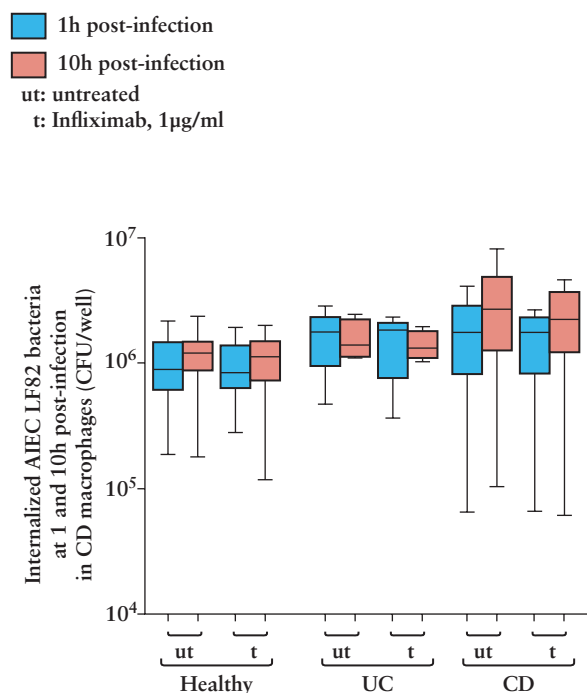


**Figure 5.** Statistical analysis of the correlation between the amounts of cytokines secreted by infected monocyte-derived macrophages from healthy volunteers, Crohn's disease [CD] or ulcerative colitis [UC] patients and the number of intracellular AIEC LF82 bacteria. Spearman correlation analysis of the number of intracellular AIEC LF82 bacteria determined at 10h post-infection and the amount of IL-6 [A-C] and tumour necrosis factor alpha [TNF- $\alpha$ ] [D-F] secreted in cell culture supernatants of infected monocyte-derived macrophages [MDM] obtained from healthy volunteers [A and D], UC patients [B and E], and CD patients [C and F]. A  $p$ -value  $\leq 0.05$  was considered statistically significant.

macrophages. Mee *et al.* have reported an increased number of *Staphylococcus aureus* internalised in monocytes from patients with UC and CD compared with controls.<sup>32</sup> Similarly, Whorwell *et al.* have observed that *Candida albicans* phagocytosis is increased in blood monocytes of CD patients compared with those from controls, independently of the disease activity.<sup>33</sup> In contrast, a decreased phagocytic activity of monocytes from CD patients with active disease as lower than that in patients with quiescent disease has been reported.<sup>34</sup> More recently, Schwarzmaier *et al.* showed that phagocytosis of non-pathogenic *E. coli* was not impaired in peripheral blood monocytes of patients with inactive CD.<sup>35</sup> This divergence in the results may be due to differences in the methodology and to the heterogeneity of the included patients in the studies [patients with quiescent or active disease] and the considered microorganisms.

We observed that a higher number of AIEC bacteria, compared with non-pathogenic *E. coli* K-12 bacteria, were internalised within MDM, irrespectively of the groups [CD, UC, HC]. Study of surface molecules on monocytes from CD patients revealed that these cells are activated and this could modulate their phagocytic function.<sup>35</sup> AIEC bacteria may express specific factors or variants that confer on them the ability, or a best affinity, to interact with receptor expressed by macrophages, as already reported regarding recognition of the CEACAM6 receptor by type 1 pili variant, expressed by AIEC.<sup>36</sup>

Our group and others have previously reported that CD-associated *E. coli* are able to survive and replicate within murine J774-A1 macrophages, human THP-1 macrophages,<sup>29</sup> and human MDM [HMDM] obtained from fresh human blood of healthy controls.<sup>24,25,26,27,28,30</sup> *In vitro* analysis of the intracellular traffic of bacteria-containing vacuoles in macrophages has revealed that AIEC persist in vacuoles with phagolysosomal traits.<sup>25</sup> In this study, we compared the ability of AIEC bacteria to persist within MDM obtained from healthy controls, UC, and CD patients. In contrast to a non-pathogenic *E. coli* strain, AIEC bacteria were able to resist MDM killing, regardless of cell origin and CD activity. However, intracellular replication of AIEC bacteria was only observed in MDM from CD patients. This result points to defects of MDM from CD patients in the ability to restrict the number of intracellular AIEC bacteria. The hypothesis of macrophage disability to degrade phagocytosed materials in the establishment of CD was already suggested by M Ward 40 years ago.<sup>37</sup> Delayed removal from the tissues has been observed of *E. coli* injected subcutaneously into the forearm of CD patients, demonstrating that bacterial clearance was impaired in CD patients.<sup>38</sup> Polymorphisms in genes encoding the innate immune receptor NOD2 involved in the recognition of invasive bacteria and the ATG16L1 and IRGM proteins involved in autophagy, a degradation system that can deliver intracellular bacteria to lysosomes for their elimination, have been identified as risk factors for CD. This supports the hypothesis of macrophage dysfunction



**Figure 6.** Impact of infliximab treatment on the number of intracellular AIEC LF82 bacteria in monocyte-derived macrophages from healthy volunteers, Crohn's disease [CD], and ulcerative colitis [UC] patients. Numbers of intracellular AIEC LF82 bacteria at 1h and 10h post-infection within MDM from healthy controls, CD patients, or UC patients untreated or treated with infliximab at 1 µg/ml. Data are expressed as colony-forming units [CFU]/well and are represented as a box plot showing the median and the lower and upper quartiles. Data were analysed by Mann-Whitney and Kruskal-Wallis tests.

in the disease. Disease-associated mutations in these genes or specific RNA interference restrict bactericidal activity of cells against AIEC in *in vitro* and *in vivo* murine models.<sup>11,39</sup> In accordance with these studies and despite restriction due to our cohort, we observed that MDM with CD-associated polymorphisms in ATG16L1 could be less efficient in controlling intracellular AIEC bacteria than those of CD patients normal for ATG16L1. However, due to the low numbers of CD patients included in this study who carried CD-associated polymorphisms in NOD2 or ATG16L1 genes, no conclusion can be drawn concerning the increase AIEC survival within macrophages isolated from patients harbouring NOD2. Thus, this association should be investigated in a larger cohort in the future.

Intracellular persistence of bacteria that invade and breach the intestinal mucosa within immune cells may induce granuloma formation, one of the hallmarks of CD, and participate in the establishment of the disease. Macrophages are prominent in granulomas. The presence of *E. coli* has been convincingly evidenced within macrophages in CD intestinal biopsies. *E. coli* antigens have been identified in macrophages within the lamina propria and in the germinal centres of mesenteric lymph nodes in patients with CD.<sup>20,21</sup> Given their ability to persist within macrophages, AIEC could favour granuloma formation. Furthermore, *E. coli* DNA was detected in 80% of microdissected granulomas from CD patients,<sup>22</sup> and AIEC were able to induce the formation of cell aggregates very similar to epithelioid granulomas in an *in vitro* model based on human blood-derived mononuclear cells.<sup>40</sup>

Whereas AIEC and non-pathogenic *E. coli* induced similar secretion levels of IL-6, TNF-α, and IL-8 in MDM from healthy controls and UC patients, the amount of IL-6 and TNF-α secreted by

MDM from CD patients infected with AIEC bacteria was significantly higher than that induced by non-pathogenic *E. coli* K-12. This could be due to difference either in immunogenicity of the bacteria or in activation of a host-specific signalling pathway, or could be related to the number of bacteria internalised within MDM. Indeed, we observed that a higher number of AIEC bacteria than non-pathogenic *E. coli* bacteria were phagocytosed and persisted within MDM from CD patients. In addition, as previously shown in J774 murine macrophages, we observed that a correlation exists between the number of intracellular bacteria in MDM from CD patients and TNF-α secretion.<sup>24</sup> Interestingly, this was not observed in AIEC-infected MDM from healthy controls or from UC patients. It has been reported that treatment of AIEC-infected J774 macrophages with antibodies that neutralise the bioactivity of TNF-α and treatment of human THP-1 macrophages infected with *Mycobacterium avium* subsp. paratuberculosis [MAP] with infliximab decreased the number of intracellular bacteria.<sup>24,41</sup> Under the conditions tested in this study, infliximab treatment of MDM from healthy controls, CD, and UC patients did not modify the number of intracellular AIEC. The discrepancy observed regarding the ability of anti-TNF-α to control intracellular AIEC replication could be due to different biological activities of the antibodies used, since Bringer *et al.*<sup>24</sup> used a goat antibody selected for its ability to neutralise mouse TNF-α. In this study, we used infliximab, a chimeric IgG1κ monoclonal antibody [composed of human constant and murine variable regions] specific for human TNF-α, for which different mechanisms of action have been described.<sup>42</sup> Sources of macrophages are also different, as mouse J774-A1 macrophages were used in Bringer *et al.* compared with human MDM in this study. To date, there are no published data on the impact of infliximab treatment on the intracellular survival/replication of bacteria within MDM. Therefore, it would be interesting to study the impact of several treatments' duration and the use of higher infliximab doses on the number of intracellular bacteria within MDM.

Conflicting results have been reported regarding the inflammatory response of monocytes/macrophages from CD patients in remission. Indeed, impaired secretion of pro-inflammatory cytokines, including TNF-α and IL-6, by macrophages from patients with quiescent CD in response to infection with live *E. coli*, MAP, and *M. avium* subsp. avium, or stimulation with heat-killed *E. coli*, or agonists of Toll-like receptors, have been reported.<sup>38,43,44</sup> In contrast, in a recent study, Schwarzmaier *et al.* observed similar levels of cytokines, including TNF-α, IL-6, and IL-8, in patients with inactive CD and healthy controls.<sup>35</sup> To our knowledge, our study is the first report comparing the inflammatory response of infected-macrophages obtained from CD patients with quiescent or active disease. We observed that MDM from CD patients with quiescent disease secreted higher levels of IL-6 and TNF-α than those from patients with active disease or healthy controls. As previously suggested by Zorzi *et al.*, pro-inflammatory cytokine secretion may possibly vary in CD, in a temporary manner.<sup>45</sup>

Further comparative studies are needed on monocytes and mucosal macrophages in CD patients, regarding the disease activity and innate immune genetic polymorphisms associated with CD. Such investigations would provide further insight into the potential of the immunological response of these cells upon stimulation by innate immune receptor agonists and commensal or CD-associated pathogenic bacteria.

The originality of this work was that we used samples retrieved from CD patients compared with UC and healthy controls. In addition, several assays have shown that autophagy defect in murine models or *in vitro* autophagy blockade could favour AIEC replication.

However our study is the first to show that MDM retrieved from CD patients with autophagy defect could be unable to control AIEC infection.

In conclusion, our observations provide new insight into the ability of macrophages from CD patients to handle pathogenic bacteria and into the immunological features of these cells according to the disease activity. In CD patients, AIEC bacteria breaching the mucosal barrier could take advantage of the poor bactericidal activity of macrophages to create an intracellular replication niche. Intracellular persistence of AIEC bacteria and immunological reactivity of the macrophages in quiescent CD patients, characterised in part by high secretion of IL-6 and TNF- $\alpha$ , could support the chronicity of the disease. These first results should lead to further investigations to search for the cause of this abnormal behaviour of macrophages in some CD patients, which could be a potential therapeutic target in the future.

## Conflict of Interest

The authors declare no conflict of interest.

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