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# Crohn's Disease-associated *Escherichia coli* LF82 Aggravates Colitis in Injured Mouse Colon via Signaling by Flagellin

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**Background:** Ileal lesions in Crohn's disease patients are colonized by pathogenic adherent-invasive *Escherichia coli* (AIEC) that harbor various virulence factors involved in adhesion to and invasion of intestinal epithelial cultured cells. We investigated in a mouse model of colonic inflammation the behavior of virulent AIEC reference bacteria LF82 compared to that of nonflagellated LF82 mutants.

**Methods:** BALBc/J mice with intact or dextran sulfate sodium (DSS)-injured colon were orally challenged daily with 10<sup>8</sup> bacteria. The severity of colitis was assessed by determining disease activity index, colonic histological score, and myeloperoxidase activity. Flagellin receptor and cytokine expression was measured by reverse-transcriptase polymerase chain reaction (RT-PCR) in colonic tissue.

**Results:** In contrast to nonpathogenic *E. coli*, virulent LF82 bacteria exacerbated colitis in DSS-treated mice, substantially reducing survival rate, greatly lowering stool consistency, inducing marked weight loss and increased rectal bleeding, and significantly increasing erosive lesions and mucosal inflammation. Nonflagellated LF82 mutants behaved like nonpathogenic *E. coli* K-12. Interestingly, oral infection with LF82 virulent bacteria, but not with a nonvirulent LF82 mutant, induced a 7.0-fold increase in the levels of *TLR5* and a 3.1-fold increase in those of *ipaf* mRNA, which encode respectively membrane and cytosolic receptors involved in the recognition of flagellin. Hence, a 5.6-fold increase in *IL-1β* and a 5.3-fold increase in mRNA of *IL-6* were observed in mice challenged with AIEC LF82 bacteria.

**Conclusions:** Crohn's disease-associated virulent AIEC LF82 bacteria, via expression of flagella, are able to potentiate an inflammatory mucosal immune response involving increased expression of TLR5 and IPAF flagellin receptors.

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**Key Words:** Crohn's disease, adherent-invasive *E. coli*, mouse model, flagellin receptor

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), are both chronic inflammatory disorders of the gastrointestinal (GI) tract. These diseases are thought to be the result of recognition of microbial antigen(s) by a dysfunctional immune system in a genetically predisposed host. Data from both humans and experimental animals underscore the critical role of intestinal bacteria in the establishment and maintenance of IBD.<sup>1</sup> Several bacteria have been implicated in the etiology of IBD.<sup>2–6</sup> In CD the ileal mucosa of patients is abnormally colonized by pathogenic *E. coli* able to adhere to and to invade intestinal epithelial cells, termed adherent-invasive *E. coli* (AIEC).<sup>7</sup> Deciphering the crosstalk at the host–microbial interface yields new insights into the ability of AIEC to colonize the intestinal mucosa in CD patients.<sup>8</sup> AIEC are able to promote their own colonization in genetically predisposed patients who develop ileal CD. They induce an increased expression of CEACAM6, which acts as a receptor for these bacteria.<sup>9</sup>

In vitro experiments using intestinal epithelial cell monolayers show that the adhesion and invasion abilities of CD-associated AIEC strains depend on the expression of various virulence factors, among which type 1 pili mediating adherence to intestinal epithelial cells play an essential role in the invasive ability of AIEC by inducing membrane extensions.<sup>10</sup> Two other virulence factors, flagellin<sup>11</sup> and the outer membrane protein C (OmpC),<sup>12–14</sup> which are bacterial antigens relevant to CD since a specific reactivity is observed in CD patients, also play key roles in the virulence of AIEC. Flagella are involved in the adhesion-invasion process of AIEC directly via motility and indirectly by downregulating the expression of type 1 pili.<sup>15</sup> OmpC, whose expression is upregulated under the conditions of high osmolarity encountered by bacteria in the GI tract, is involved in a regulatory pathway controlling the expression of flagella and type 1

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**TABLE 1.** Bacterial Strains and Plasmids Used in This Study

Strain or Plasmid	Relevant Characteristics	Source or Reference
<b>Strains</b>		
LF82	<i>E. coli</i> isolated from an ileal biopsy of a CD patient	34
LF82- $\Delta ompR$	LF82 isogenic mutant deleted of <i>ompR</i> gene	16
LF82- $\Delta fliC$	LF82 isogenic mutant deleted of <i>fliC</i> gene	15
K-12 C600	Nonpathogenic <i>E. coli</i> strain rifampicin resistant	Laboratory stock
<b>Plasmids</b>		
pPBI09	pBAD24 containing the <i>ompR</i> gene of LF82	16
pPBI04	pHSG575 containing the complete <i>fliC</i> gene of LF82	15

pili.<sup>16</sup> The expression of OmpC is under the control of the OmpR transcriptional regulator of the EnvZ/OmpR two-component system. In AIEC bacteria, deletion of the *ompR* gene leads to absence of the expression of OmpC, but also of flagella, type 1 pili, and loss of adhesion and invasion abilities.<sup>16</sup> In the present study we used the dextran sulfate sodium (DSS)-injured mouse colon model to investigate the ability of pathogenic AIEC LF82 bacteria compared to that of nonpathogenic LF82 mutants to aggravate colitis and show that flagella are the major virulence factor involved in the potentiation of inflammatory mucosal immune response.

## MATERIALS AND METHODS

### Bacterial Strains, Plasmids, and Bacterial Culture

*Escherichia coli* strain LF82, isolated from a chronic ileal lesion of a CD patient, was used as the reference strain for AIEC. All bacteria, isogenic mutants, and plasmids used in this study are listed in Table 1. Overnight bacterial cultures in Luria-Bertani (LB) broth without shaking at 37°C were harvested by centrifugation at 4000g for 15 minutes. The supernatant was discarded and the bacterial pellet was resuspended in carboxymethyl cellulose (CMC) at 0.5% (w/v) in distilled water.

### *Escherichia coli* Challenge in Injured Colon Mouse Model

Six-week-old BALBc/J male mice ( $\approx 22$  g) were purchased from the Charles River Laboratory (L'Arbresle, France). They received 2% (w/v) DSS (MW = 36,000–50,000, MP Bio-medicals, Solon, OH) in drinking water for 14 days to induce colon injury, and were orally challenged daily by  $10^8$  bacteria in CMC during the same period. Animal care and experiments were carried out in accordance with the Committee for Research and Ethical Issues of the IASP.

### Clinical Assessment of Colitis and Histological Evaluation of Colonic Damage

Colonic damage was ascertained by disease activity index (DAI) as defined in Table 2. Rectal bleeding was assessed by

Hemocult II test (SKD SARL). The scores range from 0 (healthy) to 12 (greatest activity of colitis).

On day 14 the entire mouse colon was excised and segments of the proximal colon were fixed in buffered 4% formalin, paraffin-embedded, cut into 3- $\mu$ m slices, and stained with hematoxylin/eosin/safranin (HES). The histologic severity of colitis was graded in a “blinded” fashion by a gastrointestinal pathologist. The tissue samples were assessed for the extent and depth of inflammation with a range of 0 to 3 and the extent of crypt damage with a range of 0 to 3 (Table 3).

### Determination of Myeloperoxidase Activity

Colonic samples were washed with cold phosphate-buffered saline (PBS) and immediately snap-frozen in liquid nitrogen. Myeloperoxidase (MPO) activity, as an indicator of infiltration of polynuclear cells, was measured in samples of colonic tissue taken from the distal colon according to the published

**TABLE 2.** Disease Activity Index (DAI) Assessment

	Score	Characteristic(s)
Body weight loss	0	no loss
	1	1 to 5% loss of body weight
	2	5 to 10% loss of body weight
	3	10 to 20% loss of body weight
	4	>20% loss of body weight
Stool consistency	0	normal feces
	1	loose stool
	2	watery diarrhea
	3	slimy diarrhea, little blood
	4	severe watery diarrhea with blood
Blood in stool	0	no blood
	2	presence of blood assessed by Hemocult II test
	4	visible bleeding

**TABLE 3.** Histological Grading of Intestinal Inflammation

a	Score	Characteristic(s)
Infiltration of inflammatory cells	0	rare inflammatory cells in the lamina propria
	1	increased numbers of inflammatory cells, including neutrophils in the lamina propria
	2	confluence of inflammatory cells extending into the submucosa
	3	transmural extension of the inflammatory cell infiltrate
Epithelial damage	0	absence of mucosal damage
	1	discrete focal lymphoepithelial lesions
	2	mucosal erosion/ulceration
	3	extensive mucosal damage and extension through deeper structures of the bowel wall

procedure of Maaser et al<sup>17</sup> with a minor modification consisting of the addition of a sonification step after homogenization of colonic tissue.

### Real-time mRNA Quantification

Total RNA was isolated from colonic tissues using TRIzol (Invitrogen, La Jolla, CA) according to the manufacturer's instructions. After treatment at 37°C for 30 minutes with 20–50 units of RNase-free DNase I (Roche Diagnostics, Nutley, NJ), cDNA were obtained using 2-step reverse-transcriptase polymerase chain reaction (RT-PCR) kit (MP Biochemicals) and were quantified in Light Cycler 1.5 (Roche Diagnostics) using SYBR green Taq ReadyMix (Sigma, St. Louis, MO) with specific mouse oligonucleotides. The sense and antisense oligonucleotides used were, respectively:  $\beta$ 2-microglobulin, 5'-CAGTGTGAGCCAGGATATAG-3' and 5'-TGACCGCCTGTATGCTATC-3'; interleukin (IL)-1 $\beta$ , 5'-ATGGCAACTGTTCTTGAAGTCAACT-3' and 5'-CAGGACAGGTATAGATTCTTTCTTCTT-3'; IL-6, 5'-CACAAAGCCAGAGTCCCTCAGAGA-3' and 5'-CTAGGTTTGCCGAGTAGATCT-3'; Toll-like receptor 5 (TLR5), 5'-TCTCCAACATCCAGGCGGCTG-3' and 5'-CCAGCCAACATCCTGGAGGTC-3'; IL-1 converting enzyme-protease activating factor (IPAF), 5'-ACGACTTTTCTTTCGAAGGC-3' and 5'-GTCAAAGCGTCCACGTTCC-3'. Each sample was run in duplicate. All results were normalized to the unaffected housekeeping  $\beta$ 2-microglobulin gene.

### Transmission Electron Microscopy

Bacteria were grown overnight in LB broth without shaking, placed for 1 minute on carbon-formvar copper grids (Electron

Microscopy Sciences, Hatfield, UK) and negatively stained for 1 minute with acid phosphotungstic, pH 6.0. Grids were examined with a Hitachi H-7650 transmission electron microscope.

### Statistical Analysis

Statistical analysis was performed using a 2-tailed Fisher's exact test. A *P* value  $\leq$  0.05 was considered statistically significant. Data are expressed as the mean  $\pm$  SEM. ANOVA was used for intergroup comparison.

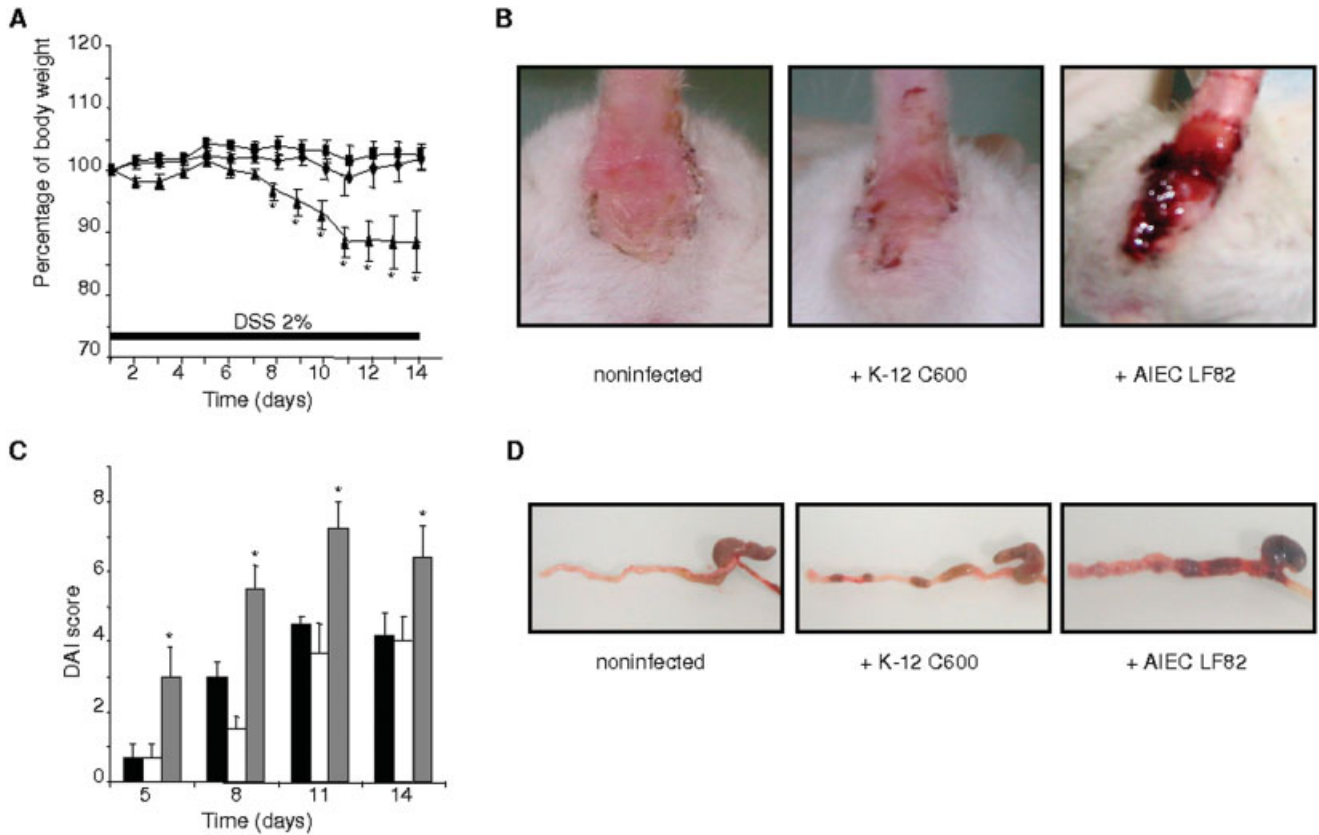
## RESULTS

### AIEC LF82 Bacteria Significantly Aggravate the Clinical Symptoms of Colitis in Injured Colonic Model

In order to estimate whether the presence of virulence factors can affect the role of CD-associated AIEC LF82 bacteria in vivo, the effects of AIEC LF82 infection were compared with those of nonpathogenic *E. coli* K-12 infection in a DSS-injured colon model. The effects were compared by recording the DAI of orally challenged mice. Mice receiving *E. coli* K-12 bacteria exhibited mild colitis-associated symptoms, similar to those observed in mice receiving CMC alone. In contrast, AIEC LF82 bacteria significantly aggravated the clinical symptoms of colitis (*P* < 0.05 for all the parameters measured). The body weight of the mouse group receiving LF82 bacteria decreased after day 5. By day 7 the difference was statistically significant (*P* = 0.022) between mice receiving LF82 bacteria (96.6%  $\pm$  1.4%) and those receiving only CMC (101.6%  $\pm$  1.2%) or *E. coli* K-12 bacteria (103.9%  $\pm$  1.5%). This difference persisted until the end of the experiment (Fig. 1A). In addition, compared with mice challenged with *E. coli* K-12, those that received LF82 bacteria had substantially reduced survival rate (LF82 bacteria group, 84%, versus *E. coli* K-12 bacteria group, 100% survival), and increased diarrhea, frequently accompanied by rectal bleeding (Fig. 1B). As a consequence the DAI of mice orally challenged with LF82 bacteria significantly increased from day 5 (*P* = 0.010) to day 14 (*P* = 0.041) (Fig. 1C) compared to mice receiving *E. coli* K-12 bacteria. The DAI of mice challenged with *E. coli* K-12 was similar to that of noninfected mice. Macroscopic examination of intestinal gut from LF82 bacteria challenged mice showed hemorrhagic colons that were not observed in the CMC or *E. coli* K-12 bacteria challenge groups (Fig. 1D). These findings demonstrate that, in contrast to nonpathogenic *E. coli* K-12, AIEC LF82 bacteria can aggravate the clinical symptoms of colitis in a mild DSS-injured colonic mucosa model.

### Key Role of AIEC LF82 Virulence in Aggravating Clinical Symptoms of Colitis

To confirm the role of AIEC bacterial virulence factors in the increased DAI score observed after AIEC LF82 infection,



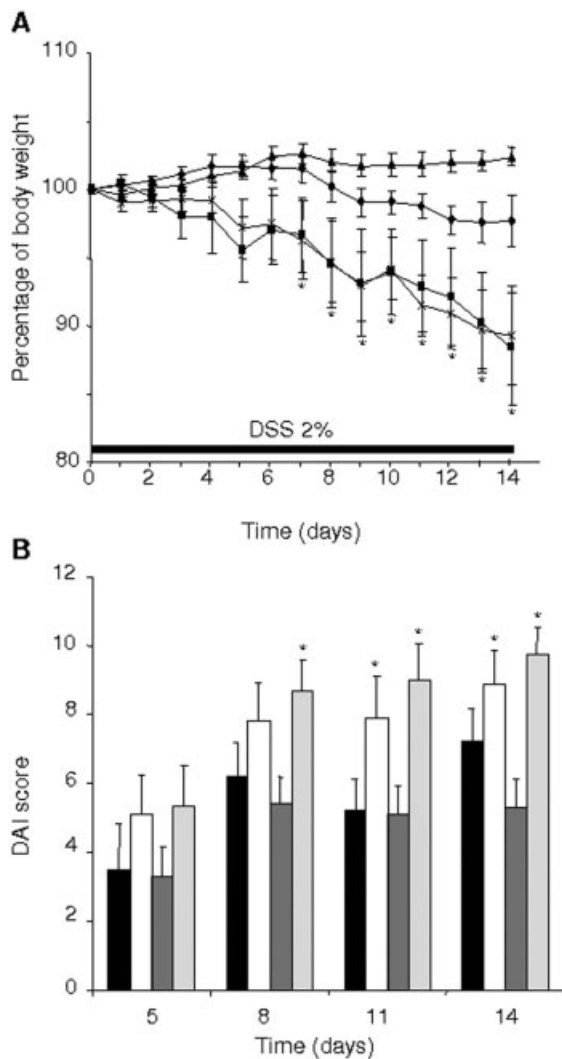
**FIGURE 1.** AIEC LF82 bacteria significantly aggravate clinical symptoms of colitis. BALBc/J mice received ad libitum water containing 2% DSS throughout the experimental period. **A:** Percentage of body weight of mice orally challenged every day with CMC alone (rhombus), CMC containing  $10^8$  AIEC LF82 bacteria (triangle) or nonpathogenic *E. coli* K-12 C600 bacteria (square). **B:** Mice exposed to LF82 bacteria showed severe rectal bleeding. **C:** DAI score was ascertained for mice that were noninfected (black), infected with AIEC LF82 bacteria (gray), or infected with nonpathogenic *E. coli* K-12 (white). **D:** Presence of hemorrhagic colon was analyzed by macroscopic examination. [BALBc/J mice: CMC alone ( $n = 6$ ), AIEC LF82 infected ( $n = 6$ ), *E. coli* K-12 infected ( $n = 6$ )]. Shown here are representative body weight evolution and DAI of three separate experiments. \* $P < 0.05$  compared with noninfected mice. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

DSS-treated mice were challenged with nonpathogenic AIEC LF82 isogenic mutants. We previously reported that the expression of most AIEC LF82 virulence factors are under the control of the two-component regulatory system EnvZ/OmpR and so the behavior of the isogenic mutant LF82- $\Delta ompR$  was compared with that of the wildtype strain. In contrast to mice challenged with wildtype LF82 bacteria, mice receiving the LF82- $\Delta ompR$  isogenic mutant did not lose body weight (Fig. 2A). Of note, their body weight substantially increased after day 11 compared to that of mice receiving CMC, which suggests that the LF82- $\Delta ompR$  isogenic mutant could protect against mild DSS-induced inflammation. In addition, the survival rate of mice challenged with AIEC LF82 bacteria was 90%, while that of mice receiving the LF82- $\Delta ompR$  isogenic mutant was 100%. The DAI scores were significantly lower ( $P < 0.001$ ) for the mouse group orally challenged with LF82- $\Delta ompR$  isogenic mutant than for those receiving wildtype AIEC LF82 bacteria (Fig. 2B). Transcomplementation of the LF82- $\Delta ompR$  isogenic mutant with cloned *ompR* gene

restored the ability of the mutant to aggravate clinical symptoms to a similar level as that observed with the wildtype LF82 bacteria. These findings confirm the role of AIEC LF82 virulence factors, whose expression is under the control of the OmpR transcriptional regulator, in the exacerbation of colitis in mice.

**Virulent AIEC LF82 Bacteria Can Exacerbate Colitis**

On histological colonic examination, DSS-treated mice challenged with AIEC LF82 bacteria, unlike those treated with 2% DSS alone, had hemorrhagic walls with multiple ulcerations, mucosal edema, neutrophil infiltrations with transmural involvement, and the presence of large erosion areas (Fig. 3A). Interestingly, this histological damage was no longer observed when mice were challenged with the nonvirulent LF82- $\Delta ompR$  isogenic mutant (Fig. 3A), but was still found after infection with the transcomplemented LF82- $\Delta ompR$  isogenic mutant expressing OmpR. Colonic histological scores were significantly higher ( $P = 0.004$ ) for mice challenged



**FIGURE 2.** Fully virulent AIEC LF82 bacteria are essential to aggravate clinical symptoms of colitis. Colon injury was achieved in BALBc/J mice by administration ad libitum of 2% DSS. A: Body weight loss was assessed for 14 days and mice were orally challenged daily with CMC alone (rhombus), with  $10^8$  AIEC LF82 bacteria (square), with  $10^8$  LF82- $\Delta ompR$  isogenic mutant (triangle) or with  $10^8$  transcomplemented LF82- $\Delta ompR$  isogenic mutant expressing OmpR (cross). B: Clinical signs of inflammation were assessed by the DAI score for noninfected mice (black), for mice infected with AIEC LF82 bacteria (white), LF82- $\Delta ompR$  isogenic mutant (dark gray), or LF82- $\Delta ompR$  isogenic mutant expressing OmpR (clear gray). [BALBc/J mice: noninfected ( $n = 10$ ), or infected with AIEC LF82 bacteria ( $n = 10$ ), with LF82- $\Delta ompR$  isogenic mutant ( $n = 10$ ), or with transcomplemented LF82- $\Delta ompR$  isogenic mutant expressing OmpR ( $n = 10$ )]. Shown here are results of a representative experiment. \* $P < 0.05$  compared with noninfected mice.

with LF82 bacteria than for noninfected mice (Fig. 3B). In accordance with histological observations, colonic histological scores were significantly lower ( $P < 0.001$ ) in mice challenged with the nonvirulent LF82- $\Delta ompR$  isogenic mu-

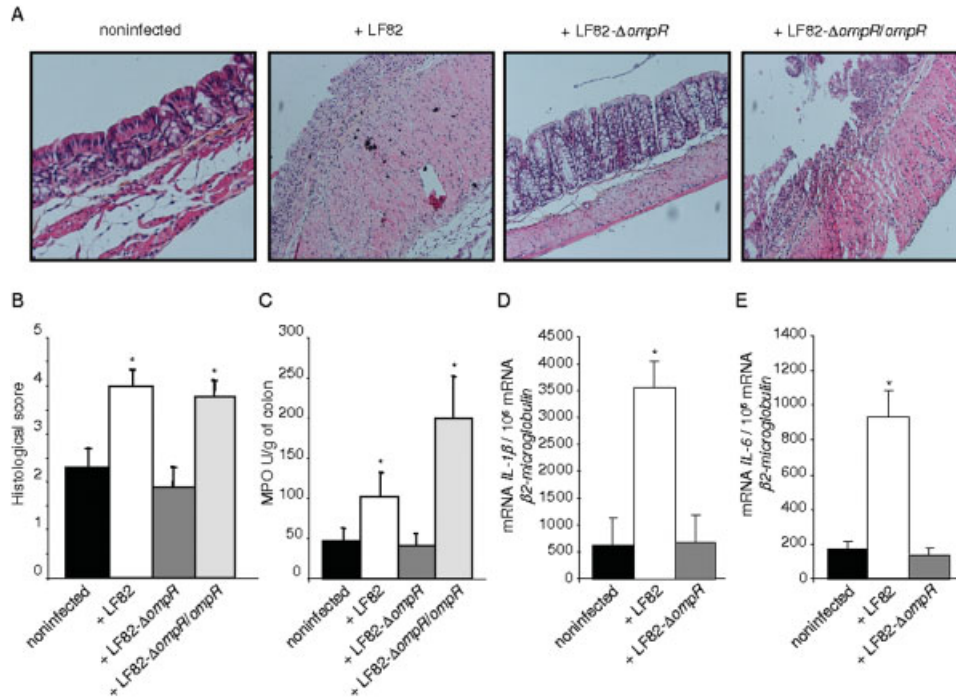
tant than with wildtype LF82. The increased infiltration of polynuclear cells was confirmed with a significant 2.2-fold ( $P = 0.05$ ) increase in MPO activity in the colonic mucosa of mice infected with AIEC LF82 bacteria compared to noninfected mice (Fig. 3C). This was not observed with nonvirulent LF82- $\Delta ompR$  mutant. Of note, a significant 4.3-fold increased MPO activity ( $P = 0.016$ ) was observed in colonic specimens of mice challenged with the fully virulent transcomplemented LF82- $\Delta ompR$  isogenic mutant expressing OmpR.

Increased levels of IL-1 $\beta$  and IL-6 mRNAs were observed in colonic specimens of mice challenged with AIEC LF82 bacteria compared to those of noninfected mice (5.6-fold and 5.3-fold, respectively) (Fig. 3D,E). In contrast, such increased cytokine levels were not observed after infection with the nonvirulent LF82- $\Delta ompR$  isogenic mutant. These results revealed that only fully virulent AIEC LF82 bacteria can exacerbate colitis by increasing expression of proinflammatory cytokines IL-1 $\beta$  and IL-6 encoding genes. Together, these results strongly suggest that exposure of injured colonic mucosa to AIEC LF82 bacteria, but not to nonvirulent LF82 mutant, substantially worsens ongoing colonic inflammation.

### AIEC LF82 Flagella Play a Key Role to Exacerbate Colitis

A consequence of the deletion of the *ompR* gene in the AIEC LF82 bacteria is the absence of flagella expression<sup>16</sup> and flagella have been described as an important factor in the aggravation of colitis in DSS-injured colon in mice.<sup>18</sup> Thus, in this murine model of colitis we investigated the behavior of an LF82- $\Delta fliC$  isogenic mutant that, like LF82- $\Delta ompR$  isogenic mutant, does not express flagella (Fig. 4). In contrast to mice challenged with wildtype LF82 bacteria, mice receiving the LF82- $\Delta fliC$  isogenic mutant did not lose body weight (Fig. 5A). The DAI scores were significantly lower ( $P < 0.001$ ) for the mouse group orally challenged with the LF82- $\Delta fliC$  isogenic mutant than for those receiving wildtype AIEC LF82 bacteria (Fig. 5B). Transcomplementation of the LF82- $\Delta fliC$  isogenic mutant with cloned *fliC* gene restored the ability of the mutant to aggravate clinical symptoms to a similar level as those observed with the wildtype LF82 bacteria. The increased levels of IL-1 $\beta$  and IL-6 mRNAs in colonic specimens observed after challenge with wildtype AIEC LF82 bacteria were no longer seen when mice were infected with the nonflagellated mutant (Fig. 5C,D). In contrast, administration of flagellated isogenic mutant LF82- $\Delta fliC$  transcomplemented with cloned *fliC* significantly increased IL-1 $\beta$  and IL-6 mRNAs levels ( $P < 0.001$ ) in colonic specimens (2.7-fold and 6.0-fold compared to those in noninfected mice, respectively). This confirms that flagella play a key role in the virulence of AIEC LF82 by exacerbating colitis.

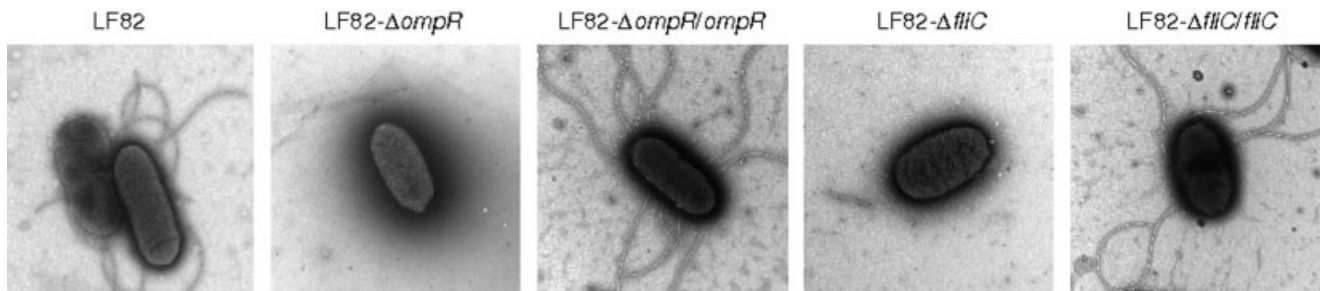
TLR5 and IPAF are 2 components of the innate immune system that detect flagellin and trigger host response.<sup>19–21</sup> Ex-



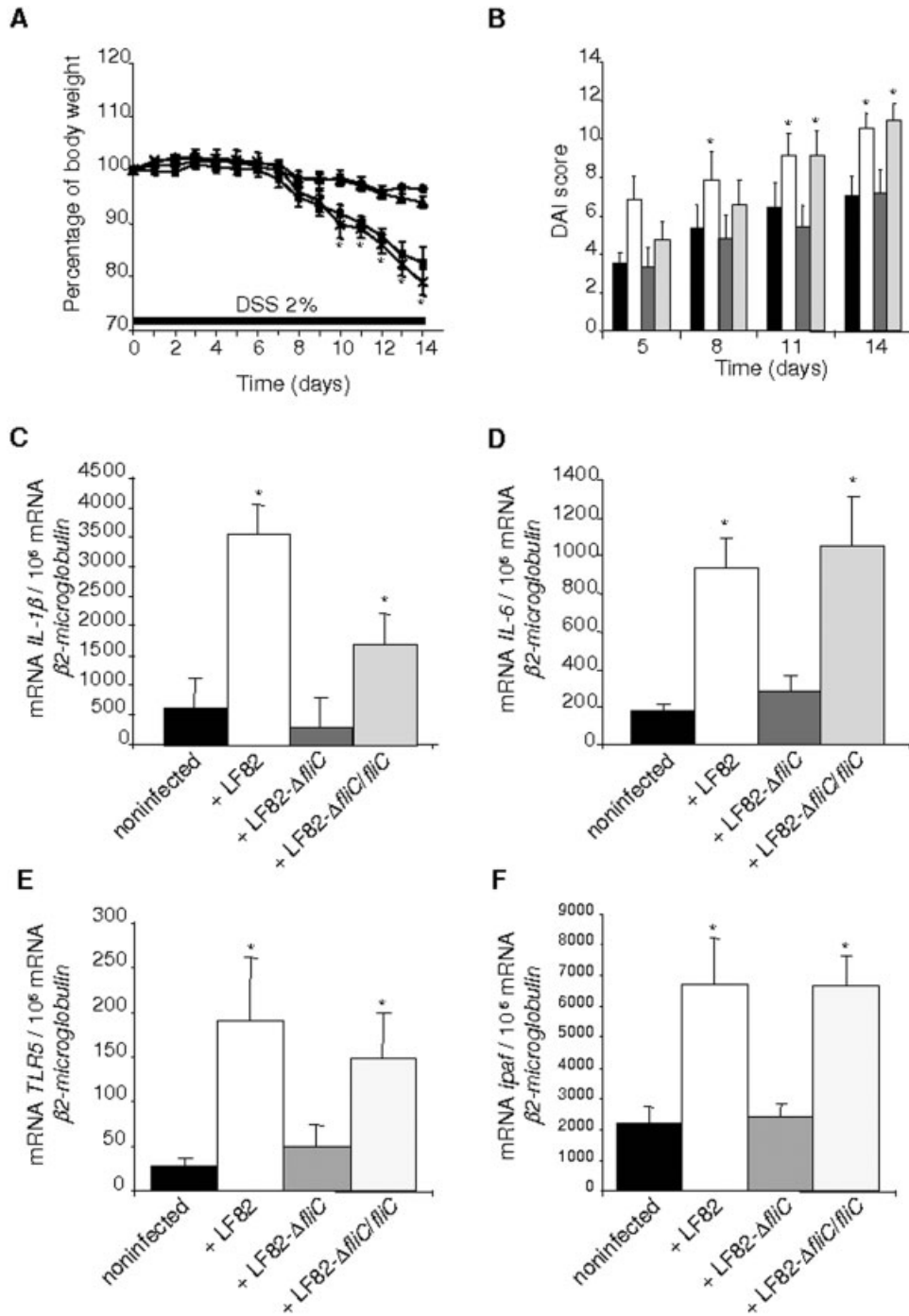
**FIGURE 3.** Fully virulent AIEC LF82 bacteria cause severe histopathological damage in colonic mucosa. Colon injury was achieved in BALBc/J mice by administration ad libitum of 2% DSS. A: Colonic tissue sections obtained from noninfected mice and mice infected with AIEC LF82 bacteria, with LF82-ΔompR isogenic mutant, or with transcomplemented LF82-ΔompR expressing OmpR at day 14 were stained with hematoxylin/eosin/safran (magnification, ×100). B: Histopathological scoring for several parameters of colonic inflammation was performed as defined in Table 3. C: Colonic myeloperoxidase activity was measured as described in Materials and Methods. D,E: Total RNA of mouse colon was isolated and *IL-6* and *IL-1* mRNA levels were quantified by RT-PCR. [BALBc/J mice: noninfected (*n* = 10), or infected with AIEC LF82 bacteria (*n* = 10), with LF82-ΔompR isogenic mutant (*n* = 10), or with transcomplemented LF82-ΔompR isogenic mutant expressing OmpR (*n* = 10)]. \**P* < 0.05 compared with noninfected mice. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com)]

pression of these 2 flagella receptors was compared in colonic specimens of mice challenged with AIEC LF82 bacteria or the nonflagellated LF82 mutant with that in noninfected mice. AIEC LF82 infection strongly enhanced *TLR5* and *ipaf* mRNA levels (7.0-fold and 3.1-fold, respectively) compared with those in noninfected mice (Fig. 5E,F). Interestingly, this was not observed in mice infected with nonflagellated mutant LF82-Δ*fliC*,

and restoration of flagella expression by transcomplementation of LF82-Δ*fliC* mutant with cloned *fliC* led to similar higher mRNA levels of both TLR5 and IPAF receptors (5.4-fold and 3.0-fold, respectively) than those observed for wildtype LF82 bacteria. This indicates that expression of flagella in AIEC LF82 bacteria can increase the expression of TLR5 and IPAF flagellin receptors.



**FIGURE 4.** Transmission electron micrographs of negatively stained LF82 bacteria, LF82-ΔompR isogenic mutant, LF82-ΔompR isogenic mutant transcomplemented with cloned *ompR* gene, LF82-Δ*fliC* isogenic mutant and LF82-Δ*fliC* isogenic mutant transcomplemented with cloned *fliC* gene.



**FIGURE 5.** AIEC LF82 flagella expression is essential in exacerbating colitis in a DSS-injured colon model. Colon injury was achieved in BALBc/J mice by administration ad libitum of 2% DSS. **A:** Body weight loss was assessed for 14 days after daily challenge with CMC alone (rhombus), or after infection with 10<sup>8</sup> AIEC LF82 bacteria (square), with 10<sup>8</sup> LF82-Δ*fliC* isogenic mutant (triangle), or with 10<sup>8</sup> transcomplemented LF82-Δ*fliC* isogenic mutant expressing flagella (cross). **B:** Clinical signs of inflammation were assessed by the DAI score for noninfected mice (black), for mice infected with 10<sup>8</sup> AIEC LF82 bacteria (white), with 10<sup>8</sup> LF82-Δ*fliC* isogenic mutant (dark gray), or with 10<sup>8</sup> LF82-Δ*fliC* isogenic mutant expressing *fliC* (light gray). **C–F:** Total RNA of mouse colon was isolated and *IL-6*, *IL-1*, *TLR5* and *ipaf* mRNA levels were quantified by RT-PCR. [BALBc/J mice: noninfected (*n* = 10), infected with AIEC LF82 bacteria (*n* = 10), with LF82-Δ*fliC* isogenic mutant (*n* = 10), or with transcomplemented LF82-Δ*fliC* isogenic mutant expressing flagella (*n* = 10)]. \**P* < 0.05 compared with noninfected mice.



## DISCUSSION

Two broad hypotheses have emerged regarding the pathogenesis of IBD. One speculates that primary dysregulation of the mucosal immune system leads to excessive immunologic responses to normal microflora and the other suggests that changes in the composition of gut microflora elicit pathologic responses from the normal mucosal immune system (for a review, see Ref. 22). It has further been suggested that IBD is characterized by an abnormal mucosal immune response to microbial factors, and we know that in CD the ileal mucosa of patients is abnormally colonized by pathogenic *E. coli*<sup>23–29</sup> that are able to adhere to and to invade intestinal epithelial cells. We therefore decided to compare the behavior of the CD-associated AIEC strain LF82 with that of nonpathogenic LF82 mutants and nonpathogenic *E. coli* K-12 using a DSS-injured colon mouse model. In contrast to nonpathogenic *E. coli* K-12, AIEC LF82 bacteria aggravated the clinical symptoms of colitis in a mild DSS-injured colonic mucosa model. Mice that received LF82 bacteria had a substantially reduced survival rate and increased diarrhea frequently accompanied by rectal bleeding compared to mice receiving nonpathogenic *E. coli* K-12. In addition, histological colonic examination revealed that only DSS-treated mice challenged with AIEC LF82 had hemorrhagic walls with multiple ulcerations, mucosal edema, neutrophil infiltrations with transmural involvement, and the presence of large erosion areas. The absence of enhanced colonic inflammation in DSS-treated mice receiving nonpathogenic *E. coli* K-12 was not surprising. It is well documented that some nonpathogenic *E. coli*, such as *E. coli* Nissle strain, have the potential to reverse some of the deleterious effects of DSS colitis.<sup>30–32</sup> Interestingly, the clinical symptoms of colitis were aggravated in DSS-treated mice challenged with enterotoxigenic *Bacteroides fragilis* associated with clinical active IBD,<sup>33</sup> as in our study with CD-associated *E. coli* bacteria. Of note, these effects were not observed in DSS-treated mice receiving nontoxicogenic *B. fragilis*.

CD-associated *E. coli* strains are able to adhere to and to invade intestinal epithelial cells in culture, and to survive and replicate within macrophages.<sup>7,34,35</sup> Under conditions of osmolarity similar to those of the GI tract, we previously reported increased ability of AIEC LF82 bacteria to interact with intestinal epithelial cells.<sup>16</sup> This response of AIEC LF82 to GI tract environment involves the two-component EnvZ/OmpR regulatory system, in which the sensor protein EnvZ senses osmolarity and phosphorylates the transcriptional regulator OmpR that regulates target gene expression. Deletion of the *ompR* gene in strain LF82 resulted in a nonvirulent mutant that had greatly reduced ability to adhere to and to invade intestinal epithelial cells, and a lack of OmpC, type 1 pili and flagella expression. In vivo experiments revealed that, in contrast to mice challenged with wildtype LF82 bacteria, mice with DSS-injured colon receiving nonvirulent

LF82- $\Delta$ *ompR* bacteria exhibited no weight loss, rectal bleeding, bloody stools, or colonic histological lesions. Interestingly, mice challenged with nonvirulent LF82- $\Delta$ *ompR* bacteria, compared to noninfected mice, gained substantial body weight, suggesting that, as reported with *E. coli* strain Nissle, nonvirulent AIEC LF82 could have a protective effect against mild DSS-induced colitis.<sup>30–32</sup>

Flagella are not expressed in the OmpR-negative mutant. As flagellin is a dominant antigen in CD and since it is documented that flagellin plays an important role in the development and progress of DSS-induced colitis in mice,<sup>11,18</sup> we investigated whether the flagella of LF82 bacteria are required to play an essential role in exacerbating colitis in DSS-treated mice. The colorectal administration of flagellin to DSS-treated mice substantially reduced survival rates, greatly lowered stool consistency, induced marked weight loss, increased rectal bleeding, and significantly increased erosive lesions and mucosal inflammation compared with DSS alone. Of note, mice receiving only flagellin had no signs of inflammation, indicating that DSS treatment is essential to observe such effects of flagellin. In contrast to mice challenged with wildtype LF82 bacteria and like mice challenged with OmpR-negative mutant, mice receiving the nonflagellated LF82- $\Delta$ *fliC* isogenic mutant showed no signs of exacerbation. The effects were dependent on the synthesis of flagellin, since restoration of flagella expression in the LF82- $\Delta$ *fliC* mutant restored the ability of the mutant to aggravate clinical symptoms to a similar level as those observed with the wildtype LF82 bacteria. Flagellin is a microbe-associated molecular pattern that is present at the bacterial surface of both pathogenic and nonpathogenic *E. coli* strains. Interestingly, a previous study, in which we dissected the regulation of flagella expression by the two-component EnvZ/OmpR regulatory system, showed that the regulation is different in AIEC strains from that of nonpathogenic *E. coli* K-12 strain.<sup>16</sup> On the basis of the observed differences in regulation, *E. coli* LF82 bacteria are probably hyperflagellated and *E. coli* K-12 bacteria are hypoflagellated or nonflagellated under GI tract conditions. These observations open up new avenues concerning the prevention of inflammation induced by AIEC bacteria in CD patients. Flagella play a major role in the gut inflammation, and we suggest that blocking flagella expression could impair the ability of AIEC bacteria to activate the host's innate immune system and to exacerbate intestinal inflammation. The expression of flagella in AIEC bacteria could be blocked by modulating the GI tract environmental conditions.<sup>16,36</sup> In addition, the development of a vaccine against flagella, as already proposed for modulating the inflammatory response in mouse lung induced by flagellated *Pseudomonas aeruginosa*,<sup>37</sup> could have beneficial effects in the treatment of CD.

TLR5 and IPAF are 2 components of the innate immune system that detect flagellin and trigger host response.<sup>20,21,38</sup>

Expression of these 2 sensors of bacterial flagellin was strongly enhanced in colonic specimens of mice challenged with AIEC LF82 bacteria compared to noninfected mice. This enhancement was not observed in mice challenged with the nonflagellated LF82 mutant. Restoration of flagella expression in LF82- $\Delta$ *flhC* mutant led to increased mRNA levels of both TLR5 and IPAF receptors similar to those observed for wildtype LF82 bacteria. This suggests that expression of flagella in AIEC LF82 bacteria can increase the expression of TLR5 and IPAF flagellin receptors. These results are consistent with those of another study that reported increased expression of TLR5 after exposure to *Salmonella* spp. or *P. aeruginosa* bacteria, or to flagellin extracted from *Salmonella typhimurium* in murine osteoblasts constitutively expressing low levels of mRNA encoding TLR5.<sup>39</sup> A recent study using mouse intestinal epithelial cell line *miC*<sub>c12</sub> showed that exposure to low concentrations of *S. enteritidis* strain 706 flagellin upregulated *TLR5* mRNA levels.<sup>40</sup> Flagellin participates in the pathophysiology of colonic inflammation in DSS-treated mice by interacting with TLR5, which is mainly expressed at the basolateral side of colonocytes since DSS, by disrupting the colonic epithelium, can give access to the basolateral-expressed TLR5.<sup>18,41</sup> Extracellular flagellin detection by TLR5 leads to increased expression of proinflammatory cytokines, including IL-6, TNF- $\alpha$ , and IL-12,<sup>42</sup> while cytosolic flagellin detection through the Nod-like receptor IPAF activates caspase-1 and leads to the secretion of IL-1 $\beta$  and IL-18.<sup>20,21</sup> We observed increased levels of *IL-6* and *IL-1 $\beta$*  mRNAs in colonic specimens in mice challenged with wildtype AIEC LF82 bacteria compared to mice infected with the nonflagellated mutants, which is consistent with the involvement of TLR5 and IPAF receptors in response to flagellin. In addition to increased access to TLR5 located at the basolateral side of intestinal epithelial cells, the observed effects could also involve TLR5 which are highly expressed by intestinal CD11c<sup>+</sup> lamina propria cells. In response to flagellin, these cells can produce inflammatory cytokines such as IL-6, IL-12, and IL-1 $\beta$ .<sup>42</sup>

In summary AIEC LF82 bacteria, but not nonpathogenic *E. coli*, significantly worsen colitis via expression of flagella and are able to potentiate an inflammatory mucosal immune response through increased expression of flagellin receptors.

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