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The Adherent/invasive *Escherichia coli* (AIEC) Strain LF82 Invades and Persists in Human Prostate Cell LineRWPE-1 Activating a Strong Inflammatory Response.

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Running Head: *E. coli* (AIEC) strain LF82 behaves as an ExPEC

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ABSTRACT

Adherent/invasive *Escherichia coli* (AIEC) strains are recently receiving increased attention because they are more prevalent and persistent in the intestine of Crohn (CD) patients than in healthy subjects. Since AIEC strains show a high percentage of similarity with ExPEC NMEC and UPEC, here we compared AIEC strain LF82 with a UPEC isolate (strain EC73) to assess whether LF82 could be able to infect prostate cells, as an extra-intestinal target. The virulence phenotypes of both strains were determined by using the RWPE-1 prostate cell line. The results obtained indicated that LF82 and EC73 are able to adhere, invade and survive within prostate epithelial cells. Invasion was confirmed by immunofluorescence and electron microscopy. Moreover, cytochalasin D and colchicine strongly inhibited bacterial uptake of both strains indicating the involvement of actin microfilaments and microtubules in host cell invasion. Moreover, both strains belong to phylogenetic group B2 and are strong biofilm producers. *In silico* analysis of virulence factors reveals that LF82 shares with UPECs several virulence factors. Namely, type 1 pili, the group II capsule, the vacuolating autotransporter toxin, four iron-uptake systems and the pathogenic island PAI. Furthermore, compared to EC73, LF82 induces in RWPE-1 cells a marked increase of phosphorylation of MAPKs and of NF-κB already 5 min post-infection, thus inducing a strong inflammatory response. Our *in vitro* data support the hypothesis that AIEC strains might play a role in prostatitis and, by exploiting host-cell signaling pathways controlling the innate immune response, likely facilitating bacterial multiplication and dissemination within the male genitourinary tract.

INTRODUCTION

Escherichia coli are the most abundant facultative anaerobic bacteria of the normal human gut flora that include a variety of non-pathogenic commensals as well as a set of pathogenic variants that cause intestinal [named intestinal pathogenic *E. coli* (IPEC)], as well as extra-intestinal infections

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47 [extra-intestinal pathogenic *E. coli* (ExPEC)] (1). While IPEC are obligate intestinal pathogens, ExPEC
48 live as commensals in the digestive tract of the host (2). Compared to most commensal *E. coli*, which
49 generally belong to A and B1 phylogenetic groups, most ExPEC strains belong to the B2 or D
50 phylogenetic groups and express highly diverse virulence factors (3). ExPEC strains have been
51 classified in three major groups based on disease association, comprising uropathogenic*E. coli* (UPEC),
52 neonatal meningitis-associated *E. coli* (NMEC), and sepsis-causing *E. coli* (SEPEC). However, such
53 classification is rather restrictive, since no single virulence factor renders an ExPEC isolate capable of
54 causing site-specific disease and especially because isolates assigned to a specific ExPEC group may
55 infect different anatomic sites (3). Adherent/invasive *E. coli* (AIEC), a particular *E. coli*pathotype, has
56 been isolated from patients with Crohn's disease (CD) and several data suggest a role of these strains in
57 the pathogenesis of CD (4). Interestingly, AIEC strains have also been detected in ileal and colonic
58 specimens of healthy subjects suggesting their classification as pathobiontes (5). AIEC strains do not
59 carry virulence genes so far identified among IPEC, while the analysis of the available complete
60 genomic sequences of different AIEC strains revealed a phylogenetic linkage with ExPEC rather than
61 with IPEC (6) and in particular with the pathotypes associated with urinary tract infections (UTIs) and
62 neonatal meningitis (7). AIEC and ExPEC strains share some phenotypic traits including the ability to
63 adhere and invade host cells (8) and to induce an inflammatory response in animal models (9) as well
64 as in polarized intestinal epithelial cells (10). AIEC strain LF82 represents the prototype of AIEC
65 strains, and belongs to phylogroup B2, typical of ExPEC (3, 11, 12, 13). Adhesion and invasion of
66 intestinal epithelial cells by LF82 require the expression of several virulence determinants such as type
67 1 pili, of several outer membrane proteins (OMPs), and of the IbeA invasin (9, 14, 15, 16). In
68 particular, it has been shown that FimH, the terminal subunit of type 1 pili, interacts specifically with
69 mannosylated carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) which is
70 overexpressed in ileal CD tissue (15). It has been demonstrated that allelic variation of the *fimH* gene

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may confer significant advantage in gut colonization and to the virulence of AIEC and ExPEC (9,17). In the ExPEC uropathogenic *E. coli* (UPEC) strains, expression of type 1 pili enhances colonization of the urothelial mucosa, promotes biofilm formation, host-cell invasion and induces expression of pro-inflammatory cytokines (18, 19, 20, 21). Moreover, some UPEC strains are able to persist within infected tissues due to their ability to inhibit NF-κB activation, to modulate expression and release of pro-inflammatory cytokines (22, 23, 24) and to form intracellular bacterial communities which allows bacteria to resist to the host immune response and to antibiotic therapy (25, 26, 27, 28). On the other hand, it has been shown that AIEC strains are stronger biofilm producers (29) and can subvert the innate immune response (30) allowing them to persistently colonize the intestinal mucosa. Similarly, UPEC strains causing UTIs and prostatitis mainly belong to the B2 phylogenetic group and show a gradient of virulence traits including a greater tendency to develop biofilm-like structures (31, 32). We have recently demonstrated the ability of as many as 58 UPEC strains to adhere and invade human prostate cells with high efficiency (33). These findings, together with the carriage of some virulence-associated genes characteristic of ExPEC pathovars, led us to hypothesize that AIEC strains may also infect body sites other than the intestine. To explore this point, in this paper, we compared the behavior of AIEC strain LF82 to that of an *E. coli* strain (EC73) isolated from a subject with recurrent UTI. In the present study, the ability of LF82 and EC73 strains to adhere, invade, survive intracellularly and to induce inflammation was studied by experimentally infecting the human prostate RWPE-1 cell line. The carriage of some virulence genes were also determined and alteration of signal transduction pathways and release of pro-inflammatory cytokines IL-6 and IL-8 were studied. The results obtained clearly indicate that, like EC73, LF82 is able to invade and to survive within prostate cells. Interestingly, differently from EC73, LF82 is also able to elicit a strong inflammatory response.In conclusion our data led us to suggest that AIEC strains have the potential to invade prostate cells, potentially causing prostatitis.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The prototype adherent/invasive AIEC strain LF82 (a gift of Arlette Darfeuille-Michaud, Université of Auvergne, France) was isolated from a chronic ileal lesion from a CD patient (11). *E. coli* EC73 is one out of three UPEC strains (EC71, EC72 and EC73) collected over a 2-year time-span from a 57 years old male suffering of recurrent UTI. *E. coli* K-12 strain MG1655 was used as control in adhesion-invasion assays and enteroinvasive *E. coli* (EIEC) strain HN280 was used as a prototype of intestinal pathogen in invasion assay. All strains were grown on Brain Heart Infusion Broth (BHI, Oxoid, Rome, Italy) or on Trypticase Soy Agar (TSA, Oxoid) overnight at 37°C.

Cell lines and cell culture. The RWPE-1 cell line (derived from prostate epithelial cells isolated from the peripheral zone of a non-neoplastic human prostate and immortalized with human papilloma virus 18) were purchased from ATCC (Manassas, VA). These cells mimic normal prostate epithelial cell behavior in their response to growth factors and in the expression of PSA and androgen receptor. RWPE-1 cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C in 1% in serum free Keratinocyte-SFM (K-SFM) medium (Gibco, Life Technologies) supplemented with 0.05 mg/ml of Bovine Pituitary Extract (BPE), 5 ng/ml human recombinant Epidermal Growth Factor (EGF) and 20 µg/ml gentamicin. Human Caco-2 (ATCC HTB-37) were grown in Minimum Essential Medium (MEM, Euroclone, Milan, Italy). HEp-2 (ATCCCCL-23) were maintained in Eagle's Minimal Essential Medium (E-MEM, Sigma, Italy), both lines were supplemented with 5% heat-inactivated fetal calf serum (FCS, Euroclone Italy) and 1% penicillin/streptomycin.

PFGE typing. The genetic relationship among the three UPEC isolates was determined by PFGE typing as previously described (34). The Dice coefficient of similarity was calculated, and the

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unweighted pair group method with arithmetic averages (UPGMA) was used for cluster analysis. Strains were considered identical if no fragment differences occurred.

Biofilm assay. Biofilm formation was assayed as previously described (38). The ATCC *E. coli* strain 25922 and the UPEC strain 16 (33), were used as biofilm positive and negative controls, respectively. After 24 h of incubation at 30°C, wells were extensively washed with PBS and attached bacteria were stained with crystal violet [0.1% (vol/vol)] for 15 min. The stain was released with 150 µl of 80% (vol/vol) ethanol. Biofilms were quantified by measuring the absorbance at λ 595 nm with a microplate reader (Tecan Sunrise, X-fluor). According to their absorbance, isolates were defined strong biofilm producers (A_{595}> 0.7), medium (0.6 > A_{595}> 0.4), weak (0.3 > A_{595}> 0.1) or non-biofilm producers (A_{595}< 0.1).

Phylogenetic PCR Grouping. Phylogenetic analysis of *E. coli* strains was carried out by multiplex PCR, as previously described (35). Whole DNA bacterial extracts were prepared using Qiagen DNA extraction kit (Qiagen, Italy). Amplifications were performed with a Perkin-Elmer GeneAmp 9600 thermal cycler and amplicons were separated with electrophoresis in 2% agarose. AIEC strain LF82 was included as internal control (36).

***In silico* virulence genotyping**. The presence of 26 virulence-associated factors of ExPEC, was determined by *in silico* analysis. Gene sequences were taken from GenBankand tested against LF82, CFT073 and UTI89 strains whole genome using the BLASTn algorithm included in BLAST+ v. 2.4.0. These include: adhesins (*pap*P, *sfa*S, *foc*F1C, *fim*type I, *Afa, nfa*E and *gaf*D); capsule synthesis (*kps*MT II, *kps*MT III, and *rfc*), iron acquisition (*ent, iro, chu, Sit, fyu*A *and iut*A) toxins (*cnf*1*, cdt, cva*C*, hly*A, *vat* and *sat*), pathogenicity associated island (PAI), invasin (*ibe*A), serum resistance (*traT*), immune evasion (t*cpC).* Hits presenting a query coverage ≥ 90% and a pairwise identity percentage (percentage of pairwise residues that are identical in the alignment including gap versus non-gap residues, but excluding gap versus gap residues) ≥ 85% were considered as positive. For EC73 strain,

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142 the presence of virulence genes was tested on genomic data produced by next generation sequencing.
143 Briefly, 2 ml of an overnight bacterial culture of EC73, was used for total genomic DNA extraction
144 using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, Italy) according to manufacturer
145 instructions. DNA final elution that was performed in water. DNA was sequenced using 250 bp paired-
146 end reads on illumine MiSeq system by Bio-Fab research (Rome, Italy) producing about 7 million raw
147 sequence reads in Fastq format corresponding to about 300-fold estimated genome coverage. Raw data
148 were imported in Geneious v. 7.1.9 (Biomatters Inc., USA) and trimmed in order to remove index
149 sequences, adapter sequences and poor quality sequenced bases. Filtered data were mapped to reference
150 sequences using Bowtie2 v. 2.2.9 (37). Hits presenting a query coverage ≥ 95% were considered as
151 positive.
152 **Adhesion, invasion, intracellular survival and multiplication assays.** Adhesiveness of *E. coli* strains
153 to cultured RWPE-1 and HEP-2 cell monolayers was assayed using standard protocols (34). Bacteria
154 were considered adherent when the mean adhesion index (number of adherent bacteria/initial
155 inoculum) was ≥0.8%. To assess the role of type 1 pili in adhesion, assays were also carried out in the
156 presence of 0.5% (vol/vol) D-mannose (34, 38, 39). Cell invasion was assayed by infecting cultured
157 RWPE-1, Caco-2 and HEp-2 cell monolayers. Cells were seeded in 24-well tissue culture plates (1×10^5
158 cells/well) and incubated at 37°C 5% CO_2 for 48 h. Cell monolayers were washed and infected with
159 diluted bacterial suspensions (MOI of 10) in 0.5 ml volume of cell culture medium devoid of
160 antibiotics. Bacteria were centrifuged onto cell monolayers at 500 x *g* for 2.5 min and incubated for 2 h
161 at 37°C. Two hours post-infection, infected monolayers were washed and incubated for 60 min in
162 growth medium containing gentamicin (100 µg/ml, Fisher Scientific). In survival and multiplication
163 assays, after the incubation time, medium containing 50 µg/ml gentamicin was added and
164 multiplication was evaluated at 6, 12 and 24 h post-infection. A strain was considered invasive when
165 the ratio between the number of intracellular bacteria/initial inoculum was ≥ 0.1%. Bacteria were

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considered able to survive, when the number of intracellular bacteria recovered at different time points was comparable to that recovered 3 h post-infection (100%). Strains were considered able to replicate intracellularly when the ratio was ≥200%. All assays were performed in triplicate.

Effect of eukaryotic cytoskeletal inhibitors. Cell monolayers were pre-incubated for 30 minutes prior to the invasion assay in cell culture medium devoid of antibiotics with 1 µg/ml cytochalasin D or 0.5 µg/ml colchicine (Sigma). The inhibitors were present throughout the 2 h bacterial infection period. The inhibitory effect of each inhibitor on bacterial uptake was evaluated against control assays without inhibitors, which were defined as 100% of bacterial uptake. All of the assays were performed at least three times in separate experiments.

Immunofluorescence labelling. RWPE-1cell monolayers were infected with *E. coli* strains (MOI of 10). Cells were washed and incubated for 30 min at 37°C with goat anti-*E. coli* antibody. After washing in PBS, cells were incubated with rabbit anti-goat Alexa Fluor 564 (red) (Invitrogen) (diluted 1:500). Since this incubation occurred while the plasma membrane was still intact, antibodies only interacted with extracellular bacteria. Samples were then extensively washed with PBS, fixed 10 min in a solution of 4% paraformaldehyde and permeabilized with a solution of 0.1% Triton X-100 for 5 min. Cells were washed with PBS, blocked for 45 min with 3% milk in PBS, followed by incubation with goat anti-*E. coli* antibody and rabbit anti-goat Alexa Fluor 488 (green) (Invitrogen) (diluted 1:500) to label intracellular bacteria. Under these experimental conditions, the intracellular bacteria were stained green whereas extracellular bacteria were orange/yellow (25). Nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI, Molecular Probes). Images were acquired by a Leica DM5000B microscope equipped with the Digital FireWire Color and Black&White Camera systems Leica DFX350 and DFX300, respectively, and processed using the Leica Application Suite 2.7.0.R1 software (Leica).

Transmission electron microscopy (TEM). RWPE-1 cells infected with LF82 and EC73 strains as described above were detached by light trypsinization, yielding densely packed pellets of sphericized

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cells. These were fixed overnight in cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer, post fixed in 2% osmium tetroxide for 2 h, then treated for 30 min. with 1% tannic acid in 0.05 M cacodylate buffer, dehydrated in ethanol and processed for Epon embedding. Infected cells were identified in thin toluidine blue stained sections. Ultrathin sections were contrasted in lead hydroxide and analyzed in a Hitachi 7000 transmission electron microscope.

SDS-PAGE and Western blot analysis. Infected RWPE-1 cell monolayers were lysed 5, 30 min and 24 h post-infection as previously described (40). Immunoblot analysis was done with the following antibodies: polyclonal anti-phospho and anti-ERK1/2, anti-phospho and anti-p38, (Cell Signalling); and monoclonal antibodies anti-phospho and anti-JNK1/2, anti-phospho and anti-p65 (Santa Cruz) and anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Bio-Rad). The levels of phosphorylated proteins were quantified by densitometry (ImageJ software).

Cytokine release. RWPE-1 cell monolayers were infected with *E. coli* strains (MOI of 10) and incubated at 37°C as described above. Three and 24 h post-infection, supernatants were collected and processed for human IL-6 and IL-8 quantification by sandwich ELISA Max Deluxe Sets (BioLegend, San Diego, CA, USA), following the manufacturer's instructions.

Statistical analyses. One way and repeated measures ANOVA followed by post hoc Student unpaired and paired t-test, as needed, were used to asses statistical significance. In all cases, a P value ≤ 0.05 was considered statistically significant.

RESULTS

UPEC isolates EC71, EC72 and EC73 are the same clone. PFGE analysis of the three UPEC isolates (EC71, EC72 and EC73) showed 100% similarity. *E. coli* strain EC73 was chosen for further studies.

LF82 strain shares with EC73, CFT073 and UTI89 some important virulence factors.

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213 Four main phylogenetic groups (A, B1, B2, and D) characterize the *E. coli* population. In general
214 virulent ExPEC such as AIEC strains mainly belong to groups B2 and D**.** Furthermore, biofilm
215 production represents an important virulence factor that promotes bacterial growth and persistence at
216 the site of infection, and protects bacteria from host immune response and to antimicrobials. As shown
217 in Table 1, LF82 and EC73 such as CFT073 and UTI89 belong to phylogenetic group B2 and were
218 strong biofilm producers. The *in silico* analysis of virulence factors reveals that LF82 shares with
219 UPECs some virulence factor. Namely, i) the type 1 group of genes (*fim*).Type 1 pili is a key factor for
220 LF82 adhesion/invasion of intestinal epithelial cells and essential for successful UPEC
221 adhesion/invasion of the urinary tract epithelial cells; ii) the group II capsule (*kps*MT) II, produced by
222 the majority of the ExPECs, has been shown to be essential for the development of UTI and to protect
223 bacteria against complement-mediated killing; iii) the vacuolating autotransporter toxin (Vat*)* known to
224 contribute to during UPEC systemic infections. iv) LF82 shares four out of six of the iron-uptake
225 systems investigated: *ent* which encodes siderophore enterobactin*, chu*which encodes heme transport
226 system*, Sit* which encodes a permease involved in the uptake of iron and manganese and *fyuA* which
227 encodes the yersiniabactin receptor, indicating that multiple iron-acquisition systems are required to
228 survive and grow within infected tissues.

229 **LF82 and EC73 strains adhere, invade and survive within RWPE-1 cell monolayers.** The
230 adhesive, invasive and intracellular survival abilities of LF82 and EC73 were assayed by infecting cell
231 monolayers, as described in Materials and Methods. As for adhesiveness, both LF82 and EC73 were
232 found to efficiently adhere to RWPE-1 and HEp-2 cells. Moreover, experiments performed in the
233 presence of 0.5% D-mannose significantly reduced bacterial adhesion confirming the key role of type 1
234 pili in this phenomenon (Fig. 1A). The gentamicin-protection assay was used to assess the ability of the
235 strains to invade and to survive within the infected cells. As shown in Fig. 1, compared to the non-
236 invasive control *E. coli* K-12 strain MG1655, LF82 was able to invade (albeit at different extents)

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RWPE-1, HEp-2 and Caco-2 cells. For what concerns EC73, the results obtained showed that it is able to efficiently invade RWPE-1and HEp-2 but not Caco-2 cells. On the other hand the EIEC strain HN280 invaded with high efficiency Caco-2 cells, while a low efficiency of invasion in prostate cells was observed (Fig. 1B), confirming the characteristics of a true gut pathogen. For what concerns intracellular survival, LF82 and EC73 were able to survive and replicate within RWPE-1 cells while they showed reduced survival within HEp-2 cells (Fig. 1C). Taken together these results confirmed the pathobiont nature of LF82which is able to invade and survive within prostate cells and possibly cause uro-genital infections.

Actin polymerization and microtubule recruitment are involved in bacterial uptake. To evaluate the role of actin microfilaments and microtubules in bacterial uptake, cell monolayers were treated with either cytochalasin D or colchicine, as described in Materials and Methods. The addition of either cytochalasin D or colchicine to cell monolayers markedly inhibited bacterial entry (Fig. 1D). As control, cells were infected with the same strains in the absence of the inhibitors (100%). These results clearly indicated that the activity of actin microfilaments and microtubules was highly required for bacterial uptake of both LF82 and EC73.

Microscopic studies confirmed the intracellular localization of LF82 and EC73 strains. The intracellular localization of *E. coli* strains LF82 and EC73 in the RWPE-1 cells was also confirmed by immunofluorescence and transmission electron microscopy. Immunofluorescence analysis showed that 24 h post-infection both strains were localized within the cytoplasm of infected cells (Fig. 2). In these experiments (see Materials and Methods for details) extracellular bacteria stained orange/yellow, while intracellular LF82 and EC73 strains stained green (Fig. 2A and B). The non-invasive control strain MG1655 was found only extracellulary (Fig. 2C). Intracellular bacteria were also detected by light and transmission electron microscopy (Fig. 3). RWPE-1 cell monolayers were infected with strains LF82 (Fig. 3C and D) or EC73 (Fig. 3A and B) as described above and extracellular bacteria were eliminated

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by the addition of 100µg/ml of gentamicin. Twenty-four hours post-infection both strains appeared within vacuoles in the cytoplasm of the prostate cell line. These results are in accordance with a previous study which reported LF82 within membrane-bounded vacuoles in some intestinal epithelial cells (30).

LF82 activates MAPKs and NF-κB signaling pathways in the prostate RWPE-1 cell line.

The MAPK family represents important signal transduction machinery and plays a prominent role in a wide range of cellular responses, including inflammation (41). Therefore, to determine the activation of MAPKs, RWPE-1 cell monolayers were infected (MOI of 10) with strains LF82, EC73 and MG1655, the latter noninvasive strain known as capable to trigger MAPK cascade (42). At different time-points after infection, whole cell extracts were prepared and analyzed by Western blot using specific antibodies (see Material and Methods for details). Cells infected with strain LF82 as well as MG1655 displayed a prompt and dramatic increase in the amount of the phosphorylated forms of all MAPKs (Fig. 4A, B and C). Remarkably, cells infected with strain EC73 showed a basal level of MAPK phosphorylation throughout the time-course experiment (Fig. 4A, B and C). The levels of activated NF-κB in the same whole cell extracts were assessed. As shown in Fig. 4D, the phosphorylation of p65 was found to be much higher in LF82- and MG1655- than in EC73- infected samples. Overall, these results indicated that LF82 induces a stronger activation of both MAPKs and NF-κB pathways than EC73. This result is not surprising because it has been recently demonstrated that activation of NF-κB by LF82 is crucial for its intracellular survival (30).

LF82 infection of RWPE-1 cells induces release of IL-6 and IL-8 significantly higher than EC73.

IL-6 and IL-8 represent two of the major cytokines produced by urinary epithelial cells following UPEC infection (43). To assess the ability of LF82 to induce release of pro-inflammatory cytokines, RWPE-1 cell monolayers were infected with *E. coli* strains LF82, EC73 and MG1655 as described above. Twenty-four hours post-infection, LF82 induced the release of approximately three-fold

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increase in IL-6 secretion and five-foldincreaseIL-8 than did EC73 (Fig. 5). This result is consistent with activation of MAPKs and NF-κB by LF82 (Fig. 4) and confirms secretion of pro-inflammatory cytokines upon LF82 infection of intestinal epithelial cells and in transgenic mice expressing human CEACAM (44).

DISCUSSION

A subset of fecal *E. coli* strains, collectively called extra-intestinal pathogenic *E. coli* (ExPEC), moving out from the intestine, gain access to extraintestinal niches, exploiting their ability to colonize and to cause disease (45). A variety of virulence factors are carried out by ExPEC strains; however, they display considerable genotype and phenotype diversity. For this reason, a generally accepted protocol to unambiguously differentiate ExPEC subtypes (namely UPEC, NMEC and SEPEC) from commensals has not been established yet (47). Among ExPEC, UPEC strains are the main etiological agents of cystitis, of acute/chronic prostatitis and acute pyelonephritis (31, 46).

Adherent/invasive *E. coli* (AIEC) strain is a group of pathogenic *E. coli* that have been associated to the initiation or maintenance of chronic inflammation in CD patients (7). AIEC do not harbor common virulence factors present in enterophatogenic *E. coli* and LF82 and NRG857c are two prototypes of this class of strains (13). Recently, on the basis of theavailable sequence data, a phylogenetic linkage between AIEC and ExPEC, in particular between strains able to cause UTI and NMEC, has been reported (**7**). Here, we first compared the virulence determinants shared by LF82 and UPEC strains EC73, CFT073 and UTI89 (Table 1). In accordance with previous reports (7), all strains were found to belong to the *E. coli* phylogroup B2 and to be biofilm producers. Biofilm production is known to be a key factor that facilitates bacterial colonization and persistence in the urinary and intestinal tracts, as well as resistance to the host innate immune response and to antibiotic therapy (47, 48). *In silico* virulence genotyping (Table 1) showed that LF82 shares with UPECs several relevant virulence

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309 factors. Namely type 1 pili, group II capsule, the vacuolating autotransporter toxin, enterobactin,
310 permease and yersiniabactin receptor. These results clearly indicate that LF82 likely has the potential to
311 colonize human districts different from the intestinal tract.
312 To assess whether LF82 might behave as an ExPEC, we used the human prostate RWPE-1 cell line,
313 which shows many characteristics of the normal prostate epithelium, as an *in vitro* model to study host
314 cell/bacterial interactions. Adhesion, invasion and survival assays (Fig. 1) showed that LF82 and EC73
315 were able to adhere, invade and survive within prostate cells as well as within HEp-2 cells, chosen as
316 control. As previously reported using different cell models, adhesiveness of AIEC and UPEC isolates is
317 strongly inhibited by the presence of D-mannose (11, 49). Accordingly, we observed that the ability of
318 LF82 and EC73 to adhere to RWPE-1 and HEp-2 cell lines was significantly inhibited by D-mannose,
319 indicating a pivotal role of type 1 pili in the adhesiveness to the prostate cell line. Furthermore,
320 intracellular uptake of both strains was strongly inhibited in both cell lines by the addition of
321 cytoskeleton inhibitors indicating that invasion of prostate cells requires microtubule polymerization
322 and actin recruitment. Moreover, to compare the invasive efficiency of a true intestinal pathogen (EIEC
323 strain HN280) to that of LF82 and EC73, invasion assays were performed both in intestinal and
324 prostate cells. Interestingly, while the EIEC strain efficiently invaded Caco-2 cells, it was almost
325 unable to invade prostate cells (Fig.1B). On the other hand, LF82 invaded prostate and Caco-2 cells,
326 although at different extent. EC73 was able to invade prostate cells but failed to enter intestinal cells.
327 Taken together these results confirmed the "pathobiont" nature of LF82.
328 Immunofluorescence and electron microscopy (Fig. 2 and 3) showed that LF82 and EC73 were
329 intracellular and localized as multiple organisms within membrane-bound vacuoles of the prostate cell
330 line, suggesting that they can replicate within these compartments. Next, the inflammatory response of
331 infected prostate cells was evaluated by determining the phosphorylation of MAPKs, NF-κB factor 65
332 and the levels of secreted cytokines IL-6 and IL-8. The results obtained indicated that all strains were

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able to stimulate phosphorylation of MAPKs and NF-κB factor 65, already 5 min post-infection. In particular, highest phosphorylation was observed with cells infected with LF82 and with the K-12 non-invasive control strain MG1655. Compared to uninfected samples, RWPE-1 cells infected with LF82, EC73 and MG1655 released increased amounts of IL-6 and IL-8 24 h post-infection (Fig. 5) but, while LF82 induced a marked production of both cytokines, lower levels were detected in cells infected with EC73 and MG1655. As for EC73, these data are consistent with previous reports (22, 27, 43) indicating that some UPEC strains, in order to persist within host urothelial cells, can adopt different strategies leading to silencing TLR4 signaling and NF-κB activity, to modulate the release of pro-inflammatory cytokines. This finding led us to speculate that EC73 is likely well adapted to persist in infected urothelial and prostate cells. On the other hand, differently from EC73, AIEC strain LF82 strongly activates NF-κB signaling pathways and IL-6 and IL-8 secretion. Recently, it has been reported that LF82 utilizes two complementary mechanisms to survive within the intestinal mucosa; one is evading inflammasome activation and the other is activating the NF-κB pathways. Furthermore, it has been also showed that the blockade of LF82-induced NF-κB activation leads to a massive epithelial cell apoptosis (30). Interestingly, by activating NF-κB, LF82 could likely protect cells from apoptosis inducing favorable conditions to survive within prostate cells [LF82 can survive in RWPE-1 cells ten days post-infection without inducing any sign of apoptosis (data not shown)]. Starting from our data, a further study of the mechanisms underlying LF82 persistence in the prostate cells could have significant implications to understand how in prostate AIEC can cause an acute infection mediated by massive cytokine production, whereas UPEC strain, causing recurrent UTI, can establish persistent colonization through the induction of low levels of inflammatory mediators.In conclusion, the present work indicates that AIEC may well behave as ExPEC strains that, moving from the intestinal tract, may cause extra-intestinal infections. The present data on human prostate cells will hopefully encourage medical attention to evaluate the actual impact of AIEC infections in prostate/genitourinary tract

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samples. In parallel, animal models can be envisaged to experiment on the detailed characteristics of such extraintestinal colonization by different strains of *E. coli.*

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FIGURE LEGENDS

TABLE 1: *In silico* analysis of virulence factors typical of UPEC strains

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FIG 1: LF82 and EC73 adhesion (A), invasion (B) and ability to survive within infected cells (C) and role of host cell actin polymerization and microtubule in the invasion process (D). Data are expressed as means ± standard deviation from at least three independent experiments, in triplicate.

* Statistically significant differences (ANOVA post hoc unpaired Student's t-test) at alpha level 0.05.

**Statistically significant differences (ANOVA post hoc paired Student's t-test) at alpha level 0.05.

ND not determined

FIG 2: Immunofluorescence staining of RWPE-1 cell monolayers 24 h post-infection. (A) LF82; (B) EC73; (C) the noninvasive control strain MG1655. Intracellular bacteria stained green, while extracellular bacteria appear orange/yellow. Magnification 400x.

FIG3: Light and electron microscopyof Epon embedded RWPE-1 cells infected with LF82 and EC73 for 24 h(A) and (B), cells infected with EC73; (C) and (D), cells infected with LF82. Groups of bacteria are frequently observed to occupy cytoplasmic vacuoles suggesting active proliferation or/and clustering. (A, C) light micrograph of toluidine blue stained thin sections. Arrows indicate cells with groups of bacteria within cytoplasmic compartments. (B, D). electron micrographs of ultrathin sections. Bars: A, C= 8 µm; B, D = 0.9 µm

FIG4: Phosphorylation of MAPKs and of NF-κB p65 subunit induced by LF82 and EC73 in RWPE-1 cells. Representative Western blots of cells infected for various lengths of time with different *E. Coli* strains, as indicated (panels A, B, C and D). Stripped membranes were re-probed using antibodies anti-ERK1/2, anti-p38, anti-JNK1/2 and anti-p65 to control protein loading. The levels of phosphorylated proteins were quantified by densitometry (ImageJ software), and calculated as the ratio of phosphorylated/total kinases and phosphorilated/total p65. Data are expressed as arbitrary units and are means ± standard deviation from at least three independent experiments, in duplicate. Asterisks (*) above the bars indicates a statistically significant difference (one way ANOVA post hoc unpaired Student's t-test; P values < 0.05). CC, uninfected cells.

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FIG 5 Levels of cytokines IL-6 and IL-8 release in RWPE-1 cell monolayers infected with *E. coli* LF82, EC73 and MG1655 strains (MOI of 10). Cytokines were measured 3 and 24 h post-infection by ELISA. Data are expressed as mean ± standard deviation of at least three independent experiments. A P value ≤0.05 was considered statistically significant.

** Statistically significant differences (repeated measures ANOVA post hoc paired Student's t-test) at alpha level 0.05.

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TABLE 1 *In silico* analysis of virulence factors typical of UPEC strains

Virulence gene		EC73	LF82	CFT073	UTI89
Adhesins	*pap* (P)	+	-	+	+
	sfa (S)	-	-	+	+
	foc (F1C)	-	-	+	-
	m (type I)	+	+	+	+
	Afa	-	-	-	-
	*nfa*E	-	-	-	-
	*gaf*D	-	-	-	-
Capsule	*kpsMT* II	+	+	+	+
	kpsMT III	-	-	-	-
	rfc	-	-	-	-
Iron acquisition system	*ent*	+	+	+	+
	iro	+	-	+	+
	chu	+	+	+	+
	Sit	+	+	+	+
	*fyu*A	+	+	+	+
	*iut*A	-	-	+	-
Toxins	*cnf*1	-	-	-	+
	cdt	-	-	-	-
	*cva*C	+	-	-	-
	*hly*A	-	-	+	+
	vat	+	+	+	+
	sat	-	-	+	-
Invasin	*ibe*A	-	+	-	+
Pathogenic island	*PAI*	+	+	+	+
Resistence to serum	*traT*	-	-	-	+
Evasion of immune response	*tcpC*	-	-	+	-
Phylogroup		B2	B2	B2	B2
Biofilm production[c]		strong	strong	nd	Nd

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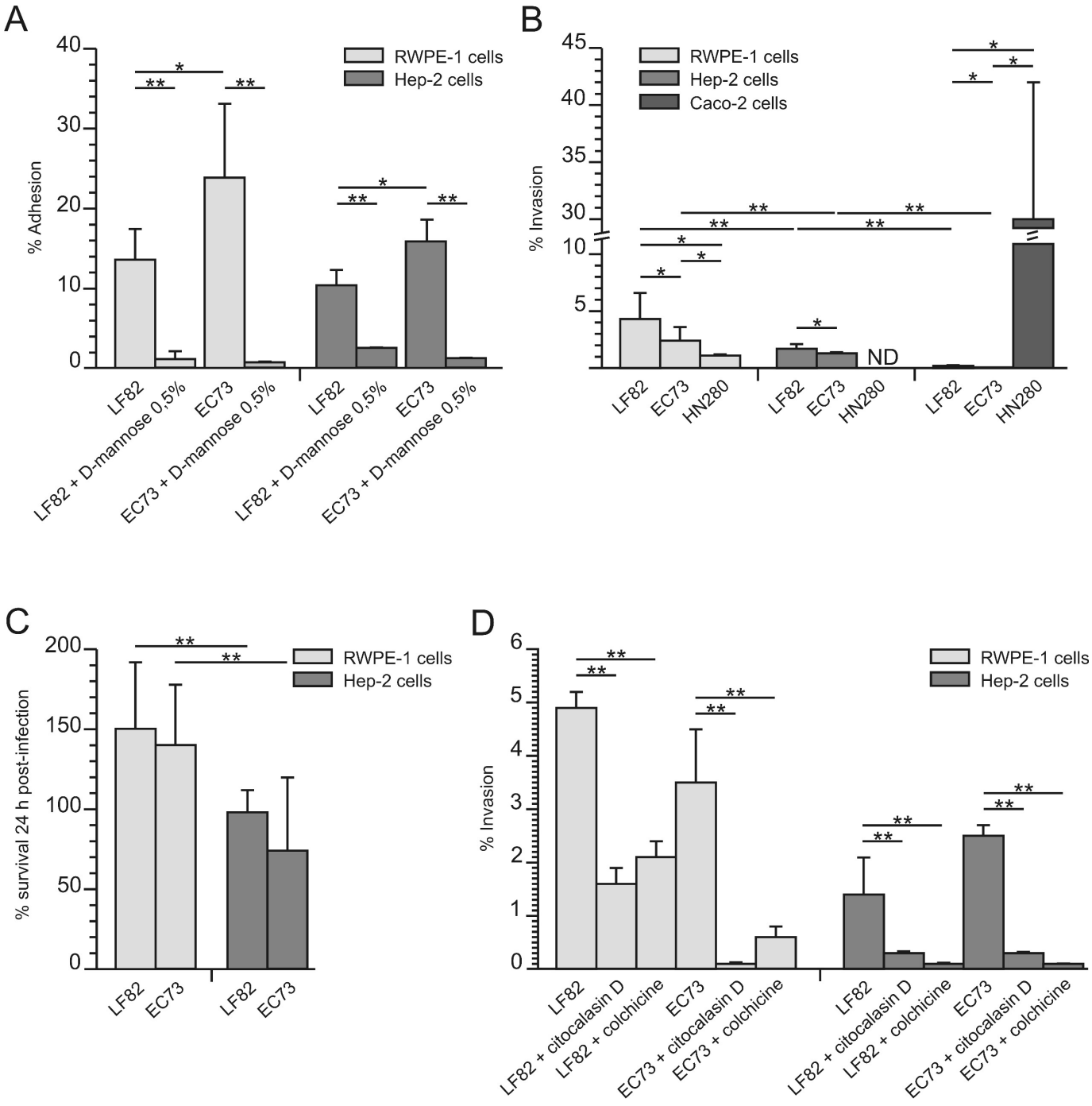

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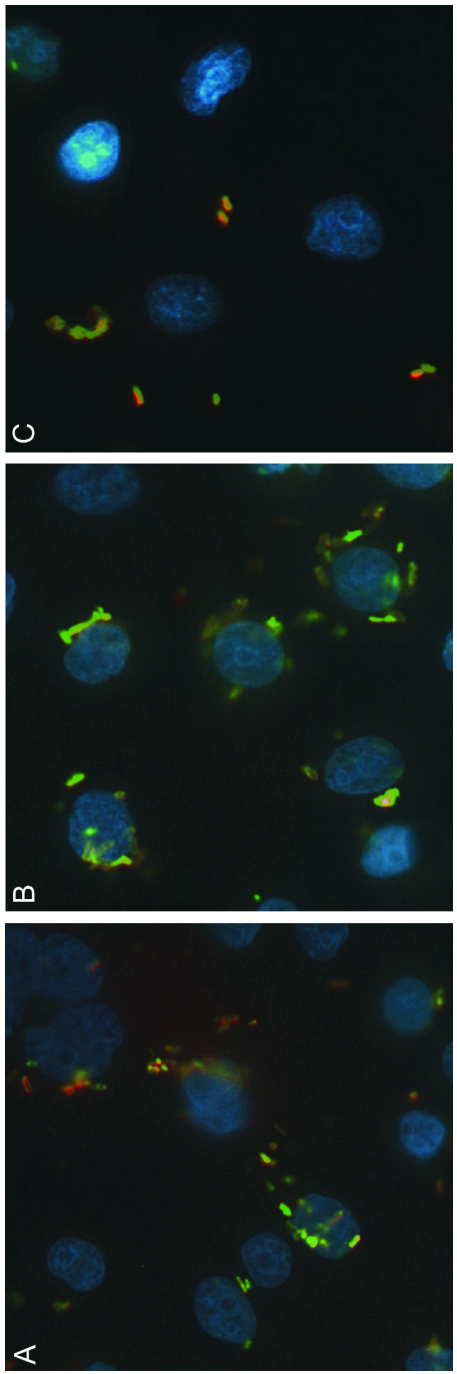

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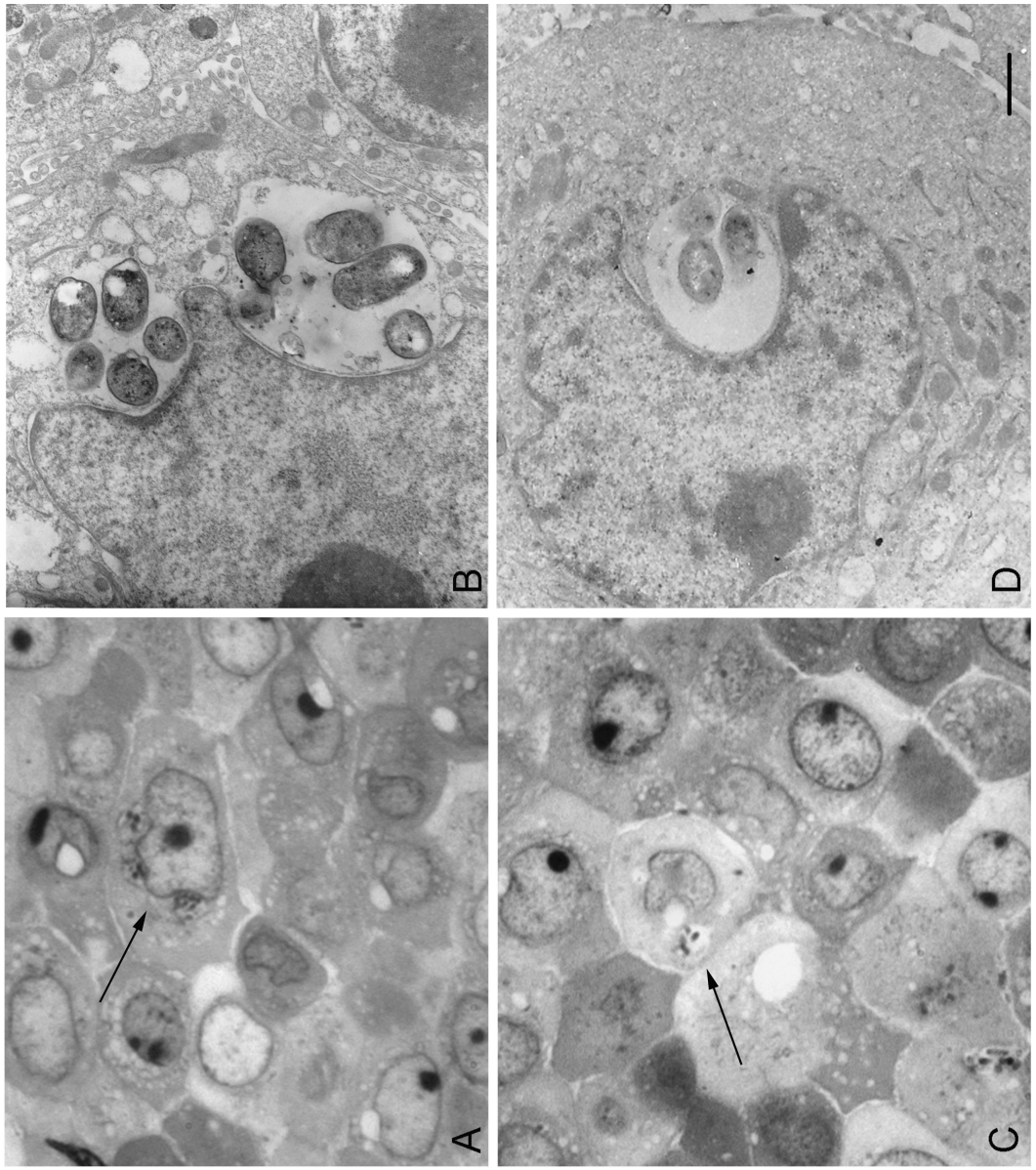

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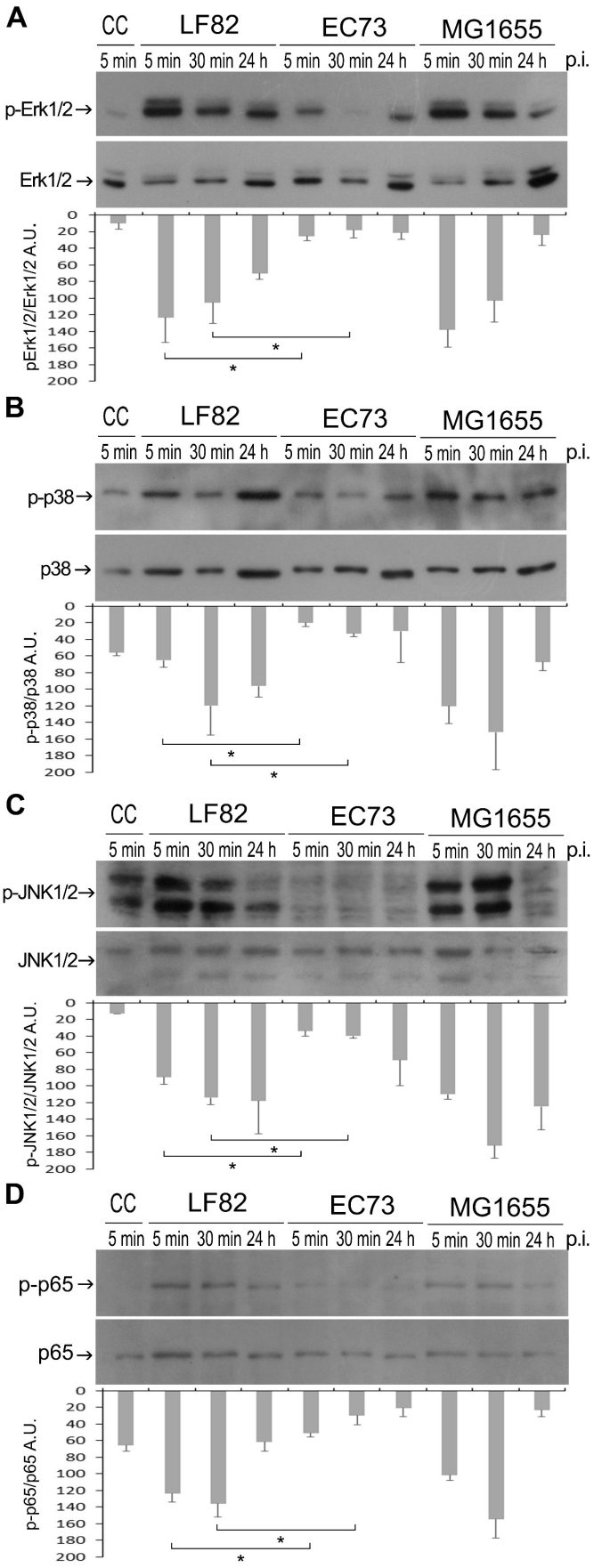

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