EspJ of enteropathogenic and enterohaemorrhagic Escherichia coli inhibits opsono-phagocytosis

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Summary
A key strategy in microbial pathogenesis is the subversion of the first line of cellular immune defences presented by professional phagocytes. Enteropathogenic and enterohaemorrhagic Escherichia coli (EPEC and EHEC respectively) remain extracellular while colonizing the gut mucosa by attaching and effacing mechanism. EPEC use the type three secretion system effector protein EspF to prevent their own uptake into macrophages. EPEC can also block in trans the internalization of IgG-opsonized particles. In this study, we show that EspJ is the type three secretion system effector protein responsible for trans-inhibition of macrophage opsono-phagocytosis by both EPEC and EHEC. While EspF plays no role in trans-inhibition of opsonophagocytosis, espJ mutants of EPEC or EHEC are unable to block uptake of opsonized sheep red blood cells (RBC), a phenotype that is rescued upon complementation with the espJ gene. Importantly, ectopic expression of EspJ_EHEC in phagocytes is sufficient to inhibit internalization of both IgG- and C3bi-opsonized RBC. These results suggest that EspJ targets a basic mechanism common to these two unrelated phagocytic receptors. Moreover, EspF and EspJ target independent aspects of the phagocytic function of mammalian macrophages in vitro.

Introduction
Enteropathogenic and enterohaemorrhagic Escherichia coli (EPEC and EHEC respectively) belong to a family of medically important diarrhoeagenic pathogens, which colonize the gut mucosa by the attaching and effacing (A/E) mechanism (for review, see Kaper et al., 2004). The genes responsible for the A/E phenotype are carried on the locus of enterocyte effacement (LEE) pathogenicity island (McDaniel et al., 1995), which encodes transcriptional regulators, the adhesin intimin (Jerse et al., 1990), structural components of a type three secretion system (T3SS) (Jarvis et al., 1995), chaperones, as well as translocator and effector proteins (reviewed in Garmendia et al., 2005). A/E lesions are characterized by localized destruction of the brush border microvilli and intimate attachment of the bacteria to the apical membrane of enterocytes (Knutton et al., 1987).

EPEC and EHEC use the T3SS to inject into mammalian host cells dozens of effector proteins (Garmendia et al., 2005; Spears et al., 2006; Tomson et al., 2005), which target different subcellular compartments and affect diverse signalling pathways and physiological processes. Among the effector proteins are EspI/NleA which is targeted to the Golgi apparatus (Gruenheid et al., 2004; Mundy et al., 2004), EspG and EspG2 which disrupt the microtubule network (Matsuzawa et al., 2004; Hardwidge et al., 2005; Shaw et al., 2005a; Tomson et al., 2005); EspF which is targeted to the mitochondria and involved in disruption of the tight junction barrier, elongation of the intestinal brush border microvilli and cell death (Crane et al., 2001; McNamara et al., 2001; Nougayrède and Donnenberg, 2004; Nagai et al., 2005; Shaw et al., 2005b); Map which induces filopodia formation (Kenny et al., 2002); and Tir which downregulates Map-induced signals (Kenny et al., 2002) and is involved in extensive remodelling of the intermediate filament and the actin microfilament networks (Kenny et al., 1997; reviewed in Caron et al., 2006).

Avoidance of phagocytosis and the undermining of macrophage signalling are common strategies used by pathogenic bacteria to colonize the host while evading immune defences (Coombes et al., 2004). Phagocytosis is the process by which macrophages, neutrophils and dendritic cells internalize particulate material over 0.5 μm in diameter. Phagocytic uptake is a multistep, zipper-like
process, initiated by the ligation of surface receptors and driven by a local remodelling of the actin cytoskeleton. The two best-characterized phagocytic receptors, complement receptor 3 (CR3) and Fc gamma receptors (FcγR), bind to opsonins deposited onto their targets, respectively, complement fragment C3bi and IgG. These two receptors are thought to mediate most of the phagocytic events occurring during innate and adaptive immune responses. Despite a conservation in principles, the mechanisms of internalization through CR3 and FcγR are known to involve different mechanisms and signalling pathways (Allen and Aderem, 1996; Caron and Hall, 1998).

EPEC and EHEC colonize the gut epithelium while remaining extracellular. Interestingly, EPEC is able to block its own uptake by professional phagocytes, a process we refer to as cis-inhibition of phagocytosis (Goosney et al., 1999; Celli et al., 2001; Quitard et al., 2006). The mechanism involved depends on the T3SS effector EspF but is poorly understood, although subversion of a phosphatidyl inositol 3-kinase (PI3K)-controlled pathway has been invoked (Celli et al., 2001; Quitard et al., 2006). Importantly, EPEC were also reported to inhibit in trans the phagocytosis of IgG-opsonized zymosan particles via the FcγR upon infection of macrophages (Celli et al., 2001), although the mechanism involved is not known. The aim of this study was to investigate the basis for EPEC O127:H6 trans-antiphagocytic activity and to determine if the same mechanism is shared with EHEC O157:H7, which is the most common virulent EHEC serotype.

Results

EPEC inhibit opsono-phagocytosis in a T3SS-dependent mechanism

J774.A1 macrophages were infected for 1 h with wild-type EPEC O127:H6 (strain E2348/69) and in parallel with its isogenic T3SS-deficient EPECΔescN mutant (strain ICC192) (Garmendia et al., 2004). Uninfected macrophages were used as control. To maximize LEE gene expression EPEC strains were primed for 3 h in Dulbecco’s modified Eagle’s medium (DMEM) prior to infection (Collington et al., 1998). In order to assist detection of bacteria in infected macrophages, strains were transformed with a GFP-expressing plasmid (pFVp25.1). After infection, macrophages were washed then challenged with sheep red blood cells (RBC) pre-opsonized with either rabbit IgG or IgM and C5-deficient serum, to direct RBC for phagocytosis through FcγR and CR3 respectively (Caron and Hall, 1998). Macrophages were then fixed and RBC differentially labelled pre- and post-permeabilization in order to discriminate extracellular from phagocytosed RBC. Infection of J774.A1 macrophages with wild-type EPEC dramatically reduced uptake of both IgG- and C3bi-opsonized RBC (Fig. 1). In contrast, infection with the escN mutant resulted in phagocytosis of RBC at a comparable level to the non-infected control (Figs 1A and 3B).

Cos-7 cells transfected with phagocytic receptors provide an alternative model to study opsonophagocytosis in isolation from macrophage receptors and secreted products (Caron and Hall, 1998). In order to confirm the results observed in J774.A1, Cos-7 cells were transfected with a construct encoding human FcγRIIA, which confers strong IgG-dependent phagocytic ability; then infected with EPEC strains and challenged with IgG-opsonized RBC. Infection with wild-type EPEC abrogated phagocytosis of opsonized RBC, as seen in J774.A1 macrophages. In contrast, when infected with EPEC-ΔescN, efficient phagocytosis of RBC via the FcγR was observed (Fig. 1B).

EspJ mediates EPEC trans-antiphagocytic activity

In order to identify the EPEC T3SS effector responsible for the trans-inhibition of opsono-phagocytosis, we tested a collection of T3SS effector mutants (listed in Table 1) for their ability to inhibit FcγR-dependent phagocytosis. The striking difference in RBC phagocytosis distinguishing T3SS-competent (<4% of the bound RBC are internalized) and T3SS-deficient (~40% of the bound RBC are internalized) strains allowed us to make a rapid visual screen for mutants impaired in their ability to block FcγR-mediated uptake (Fig. 2A and B). Phagocytosis protects

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internalized RBC from antibody labelling in non-permeabilized cells, which makes them appear red under the microscope (Fig. 2A). None of the 17 effector mutant strains had any effect on the ability of IgG-opsonized RBC to bind macrophages (Fig. 2B, bottom and data not shown). The only mutant showing a significantly greater number of phagocytosed RBC than the parental strain was EPECΔespJ (strain ICC190) (Dahan et al., 2005).

Quantitative analysis revealed that infection of J774.A1 macrophages with E774.A1 macrophages expressing EspJ and EPEC

\[ \Delta \text{espJ} \]\n
resulted in a level of RBC phagocytosis equivalent to that seen in cells infected with EPECΔespJ or in uninfected controls (Fig. 2B). To verify the dependency of this inhibitory activity upon EspJ, we complemented the \( \Delta \text{espJ} \) mutant strain ICC190 with a plasmid encoding full-length EspJ (pICCC32). Expression of recombinant EspJ restored the ability of the \( \Delta \text{espJ} \) mutant to inhibit FcγR-dependent phagocytosis to a similar level as wild-type EPEC (\( P > 0.05 \)). These results demonstrate that EspJ is the main T3SS effector involved in the \( \text{trans}-\)inhibition of FcγR-mediated phagocytosis by EPEC. Interestingly, although EspF has recently been implicated in the inhibition of EPEC phagocytosis by macrophages (Quitard et al., 2006), we detected no statistically significant difference in the uptake of IgG-opsonized RBC by J774.A1 macrophages infected with either wild-type EPEC or the isogenic EPECΔespF mutant (Fig. 2B).

EspJ impairs CR3-mediated uptake

To study whether the anti-phagocytic function of EPEC EspJ is general or specific to the FcγR-dependent signalling pathway, we examined the impact of EspJ on CR3-mediated phagocytosis. Although phagocytosis mediated by FcγR or CR3 is opsonin-dependent and actin-driven, the signalling pathways responsible for actin polymerization downstream of these two receptors are different (Caron and Hall, 1998). J774.A1 macrophages were left uninfected or infected with wild-type EPEC, EPECΔescN, EPECΔespJ, and EPECΔespJ (pICCC32) (complemented), before challenge with C3bi-opsonized RBC. Wild-type EPEC inhibited phagocytosis of C3bi-opsonized RBC in a T3SS-dependent manner as no inhibition was seen after infection with EPECΔescN (Fig. 3B). Deletion of espJ also impaired the ability of EPEC to block CR3-dependent

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**Table 1.** Bacterial strains and plasmids used in this study.

| Strains/plasmids | Description | Reference |
|------------------|-------------|-----------|
| 85-170           | EHEC O157:H7 spontaneous stx1– stx2–, Nal\(^{R}\) | Stevens et al. (2004) |
| ICC217           | ΔescN::Kn in 85-170, Kn\(^{R}\) | This study |
| ICC188           | ΔespJ::Kn in 85-170, Kn\(^{R}\) | Dahan et al. (2005) |
| EDL933           | EHEC O157:H7 stx– | ATCC |
| ICC187           | ΔescN::Kn in EHEC O157:H7 strain EDL933 | Garmendia et al. (2004) |
| ICC184           | ΔespJ::Kn in EHEC O157:H7 strain EDL933 | Garmendia et al. (2004) |
| E2348/69         | EPEC O157:H6 | Levine et al. (1978) |
| ICC219           | ΔescN::Kn in E2348/69, Kn\(^{R}\) | Garmendia et al. (2004) |
| ICC211           | ΔespJ::Kn in E2348/69, Kn\(^{R}\) | Marchés et al. (2006) |
| ICC190           | ΔespJ::Kn in E2348/69, Kn\(^{R}\) | Marchés et al. (2005) |
| ICC193           | ΔnleC::Kn in E2348/69, Kn\(^{R}\) | Marchés et al. (2005) |
| ICC194           | ΔnleD::Kn in E2348/69, Kn\(^{R}\) | Marchés et al. (2005) |
| ICC225           | Δtir::Kn in E2348/69, Kn\(^{R}\) | This study |
| ICC257           | Δeae::Kn in E2348/69, Kn\(^{R}\) | This study |
| ICC243           | ΔespG1::Kn ΔespG2::Cm in E2348/69, Kn\(^{R}\) Cm\(^{R}\) | This study |
| ICC202           | Δmap::Kn in E2348/69, Kn\(^{R}\) | Simpson et al. (2006) |
| ICC246           | ΔespH::Kn in E2348/69, Kn\(^{R}\) | This study |
| MK41             | ΔespZ::AphT3 in E2348/69, Kn\(^{R}\) | Kanack et al. (2005) |
| ICC249           | Δmap::Kn ΔespF::Cm in E2348/69, Kn\(^{R}\) Cm\(^{R}\) | This study |
| ICC254           | ΔnleH1::Kn ΔnleH2::Cm in E2348/69, Kn\(^{R}\) Cm\(^{R}\) | This study |
| ICC248           | ΔespJ::Kn in E2348/69, Kn\(^{R}\) | This study |
| ICC256           | ΔnleI::Kn in E2348/69, Kn\(^{R}\) | This study |
| ICC252           | ΔnleF::Kn in E2348/69, Kn\(^{R}\) | This study |
| ICC250           | ΔnleB1::Cm in E2348/69, Cm\(^{R}\) | This study |
| ICC251           | ΔnleB2::Kn in E2348/69, Kn\(^{R}\) | This study |
| Plasmid pICCC32  | Derivative of pSA10 (Schlosser-Silverman et al., 2000) encoding EspJ::FLAG fusion protein | This study |
| pICCC31          | Derivative of pSA10 encoding EspJ::FLAG–FLAG fusion protein | This study |
| pRK5-EspJEHEC–FLAG | Derivative of pRK5 (BD Pharmingen) encoding EspJ::FLAG–FLAG fusion protein | This study |
| pFPV25.1         | Plasmid expressing gfpmut3a gene | Valdivia and Falkow (1996) |
| pSB315           | Source of aphT cassette | Dahan et al. (2005) |
| pKD3             | oriR\(^{F}\), blaM, Cm\(^{R}\) cassette flanked by FRT sites | Datsenko and Wanner (2000) |
| pKD4             | oriR\(^{F}\), blaM, Kmr\(^{R}\) cassette flanked by FRT sites | Datsenko and Wanner (2000) |
| pKD46            | ori101, repA 101 (ts), araBp-gam-bel-exo, blaM | Datsenko and Wanner (2000) |
uptake, reducing it to a level similar to EPECΔescN (Fig. 3A and B). Complementing the EPECΔespJ mutant restored the inhibition of C3bi-opsonized RBC uptake by infected macrophages close to wild-type EPEC levels (Fig. 3A and B). As seen for IgG-opsonized RBC, pre-infection with EPEC strains had no impact on the attachment of C3bi-opsonized RBC, suggesting that translocated EspJ does not interfere with surface expression of the phagocytic receptors (FcγR and CR3 respectively) but instead with a regulatory mechanism essential for both CR3- and FcγR-dependent uptake. These results show that EspJ inhibits opsono-phagocytosis via both the CR3 and FcγR receptors.

EspJ from EHEC O157:H7 inhibits opsono-phagocytosis

Whether or not EHEC O157:H7 have antiphagocytic activity is unknown. As espJ is conserved between EPEC and EHEC (Dahan et al., 2005), we examined whether EspJ mediates antiphagocytosis during EHEC infection of J774.1 macrophages. J774.1 were infected with the spontaneous stx minus EHEC O157:H7 strain 85-170 and its isogenic mutants EHECΔescN and EHECΔespJ (Table 1) before challenge with IgG- or C3bi-opsonized RBC. As shown in Fig. 4A and B, both FcγR- and CR3-dependent uptake of RBC were greatly diminished in J774.1 infected with the parental EHEC strain compared with the levels of uptake observed in uninfected macrophages (control) or in cells infected with the EHECΔescN or EHECΔespJ. Complementing the ΔespJ strain with a plasmid encoding either EspJEPEC or EspJEHEC restored the ability of the mutant strain to inhibit phagocytosis of IgG- and C3bi-opsonized RBC (Fig. 4A and B). As for EPEC (Fig. 3), impaired RBC phagocytosis was unrelated to changes in RBC adhesion, which indicates that antiphagocytosis is due to impaired phagocytic signalling.
EspF, not EspJ, is required for inhibition of bacterial phagocytosis

Several reports have involved EspF in bacterial-induced inhibition of EPEC phagocytosis by M cells and cultured macrophages (Quitard et al., 2006; Martinez-Argudo et al., 2007), a finding we have confirmed in this study (data not shown). However, no data exist on the potential inhibition of bacterial uptake by EHEC. To address this question, J774.A1 macrophages were infected for 4 h with wild-type (wt), ΔescN (escN), ΔespJ (espJ) and complemented ΔespJ plCC32 (espJ plCC32) EPEC strains, treated with 150 ng ml⁻¹ PMA to activate the C3b binding site on CR3 receptors, then challenged with C3b-opsonized RBC for 30 min. Extracellular RBC were stained in green using Alexa™488-conjugated anti-rabbit antibodies and, after permeabilization, all cell-associated RBC were stained in red using rhodamine-conjugated anti-rabbit antibodies; cell nuclei were stained with DAPI. All bacteria are transformed with a GFP-expressing plasmid.

EspJ expression is sufficient for antiphagocytosis in transfected Cos-7 cells

Given the striking phenotype exhibited by espJ mutant strains on the inhibition of FcyR- and CR3-dependent phagocytic pathways, we examined if ectopic expression of EspJ in phagocytes was sufficient to impair osono-
phagocytosis. espJ<sub>EHEC</sub> was cloned into the pRK5-FLAG eukaryotic expression vector and co-transfected with the FcγRIIA or CR3 phagocytic receptors. Cells were then challenged with IgG- or C3bi-opsonized RBC and scored for RBC binding and phagocytosis. EspJ expression had no effect on the binding of C3bi- or IgG-opsonized RBC to receptor transfected Cos-7 cells (data not shown). In contrast, a clear and statistically significant inhibition of both FcγR- and CR3-mediated uptake was observed in EspJ-expressing Cos-7 cells (Fig. 6A and B), showing that the expression of EspJ inside host cells is sufficient for inhibition of opsono-phagocytosis.

The presence of a FLAG tag on EspJ allowed us to examine the basic features of the inhibition in trans of opsono-phagocytosis. Overexpressed EspJ was excluded from the nucleus and localized throughout the cytosol of Cos-7 cells, both in a diffuse fashion and in large aggregates. RBC challenge of Cos-7 cells coexpressing FcγRIIA and EspJ did not affect the overall localization of EspJ (Fig. 7 and data not shown). Interestingly, ectopically expressed EspJ did not specifically accumulate to any significant extent at the plasma membrane or at sites of RBC attachment. Moreover, EspJ expression did not prevent actin polymerization underneath bound IgG-opsonized RBC (Fig. 7, top, arrowhead). Overall, these data suggest that EspJ blocks FcγR-mediated phagocytosis from a distance, rather than acting locally at the nascent phagocytic cup, possibly by interfering with the later stages of RBC uptake, after initial F-actin polymerization has taken place.

**Discussion**

In the past few years antiphagocytosis has been proposed as a pathogenic mechanism for EPEC (Celli et al., 2001). However the molecular basis of this phenomenon is poorly understood. Antiphagocytic activity is clearly dependent on the translocation of one or more effectors through the EPEC T3SS into the cytosol of macrophage-like cells and manifests itself both as an ability of EPEC to reduce their own uptake (cis-inhibition) and to block in trans the phagocytosis of IgG-opsonized zymosan par-

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articles through FcγR (trans-inhibition) (Goosney et al., 1999; Celli et al., 2001). Whether these two phenotypes correspond to a unique mechanism or reflects the existence of two independent antiphagocytic mechanisms was unknown. Other bacterial pathogens are known to modulate phagocytosis, either – like *Salmonella typhimurium* and *Shigella flexneri* – by stimulating their uptake by non-professional phagocytes or – like *Yersinia* and *Clostridium* spp. – by blocking phagocytic signalling in phagocytic cells. In all cases the underlying mechanisms involve the subversion of actin dynamics (reviewed in Rottner et al., 2005).

The mechanism by which EPEC block their own uptake has been attributed to the effector EspF (Quitard et al., 2006). We report in this article that EHEC O157:H7 also block their own uptake by macrophages in an EspF-dependent manner (Fig. 5), suggesting that antiphagocytosis is a general mechanism displayed by both EHEC and EPEC. In this report we demonstrate that (i) EHEC,

Fig. 5. EspF, but not EspJ, controls cis-inhibition of EHEC phagocytosis in macrophages. J774.A1 macrophages were infected for 4 h at 37°C with 1:100 dilutions of overnight cultures of GFP-expressing EHEC O157:H7 strains as indicated and processed for immunofluorescence. Extracellular bacteria were stained red, as described in Experimental procedures, and were therefore easily distinguishable from internalized bacteria, which are solely green. The percentage of bound EHEC internalized (% phagocytosis) and the total number of cell-associated bacteria (attachment index) were scored under the epifluorescence microscope. Results are expressed as mean ± SD from three independent experiments, with ≥ 100 macrophages scored per condition per experiment. Asterisks (*) denote a statistically significant difference with the wild-type strain.

Fig. 6. Intracellular expression of EspJ is sufficient for inhibition of FcγR- and CR3-mediated phagocytosis. Cos-7 cells were co-transfected by nucleofection with FcγRIIA (A) or CR3 receptor (B) and either with plasmid pRK5 or with pRK5-EspJ overexpressing EspJ from EHEC and were then challenged for 30 min with IgG- (A) or C3bi- (B) opsonized RBC. RBC phagocytosis was then quantified as described in Experimental procedures, the transfected cells being easily distinguishable from non-transfected cells by their unique ability to bind opsonized RBC. Results are the mean ± SD of at least two independent experiments.

Fig. 7. Intracellular distribution of ectopically expressed EspJ during FcγR-dependent uptake. Cos-7 cells were co-transfected with FcγRIIa and either with pRK5-EspJFLAG (top) or with empty pRK5 (bottom) and challenged with IgG-opsonized RBC for 30 min at 37°C, as described in the legend to Fig. 5. RBC-challenged cells were permeabilized and stained with an anti-flag mouse monoclonal followed by Cy2-conjugated anti-mouse antibodies (green), rhodamine phalloidin to visualize F-actin (red) and Cy5-conjugated anti-rabbit IgG-RBC (blue). Cells were observed by confocal microscopy; representative examples are shown. Scale bar, 10 μm.
like EPEC, are able to block the uptake of opsonized particles, (ii) EPEC and EHEC trans-inhibition of phagocytosis is T3SS-dependent, and (iii) EspJ (EPEC and EHEC) is the effector protein responsible for the inhibition of both FcγR- and CR3-mediated phagocytosis, suggesting that EspJ targets an essential host molecule or complex normally involved downstream of these two phagocytic receptors. Two clostridium toxins, the B toxins from Clostridium difficile strains 10463 and 1470, inhibit FcγR- and CR3-mediated phagocytosis (Caron and Hall, 1998; Caron et al., 2000). Both glucosylate and inactivate members of the Rho family of small GTP-binding proteins, known to regulate actin polymerization during a variety of eukaryotic processes, including phagocytosis (Just et al., 1995; Chaves-Olarte et al., 1999; Niedergang and Chavrier, 2005). However, to our knowledge, EspJ is the first example of a type three secretion effector that blocks both FcγR- and CR3-mediated phagocytosis.

Our results show that, as has been shown for EPEC, EHEC can inhibit opsono-phagocytosis. This may not be surprising, as EHEC are thought to have evolved from EPEC through the acquisition of phages encoding a Shiga-like toxin (Reid et al., 2000). It will be interesting to check whether other strains able to induce A/E lesions can also block phagocytosis. In order to find the effector protein conferring EPEC the ability to block phagocytosis in trans, we screened 17 candidate effectors mutants we had accumulated in the lab. Interestingly, neither EspF, involved in inhibition of EPEC phagocytosis by macrophages and M cells (Quitard et al., 2006; Martinez-Argudo et al., 2007); Tir, involved in redistribution of intermediate filament proteins and triggering of actin polymerization; Map, involved in filopodia formation; or EspG/EspG2, involved in disruption of the microtubule network (reviewed in Garmendia et al., 2005 and Caron et al., 2006) were involved. In contrast, deletion of espJ, which is carried upstream of tccP (Garmendia et al., 2004) on prophage CP-933U/Sp14 in EHEC, abolished phagocytosis of opsonized RBC. The phagocytosis defect was complemented by recombinant espJ. Interestingly EspJ is dispensable for cis-inhibition of EHEC or EPEC uptake (Fig. 5 and V. Covarelli, O. Marchès, G. Frankel and E. Caron, unpubl. results). Taken together our results show that inhibition of cis- and trans-phagocytosis are mediated by different effectors and are likely to involve different signalling pathways.

Sequence analysis reveals, as expected, that EspJ_{EPEC} shows a very strong (79%) sequence identity at the amino acid level with EspJ_{EHEC} and an open reading frame (75% identity) in Citrobacter rodentium, the mouse A/E pathogen (Dahan et al., 2005). Interestingly, database searches also show a 57% identity (74% similarity) with a putative protein from Salmonella bongori. S. bongori mainly infects cold-blooded animals but is also associated to rare cases of acute enteritis in humans (Giammanco et al., 2002). As S. bongori differs from Salmonella enterica by the absence of the SPI-2 (Salmonella pathogenicity island two), whose expression is induced intracellularly and which is essential for intracellular survival and replication within macrophages (Ochman and Groisman, 1996; Waterman and Holden, 2003), it is tempting to speculate that an antiphagocytic protein would allow S. bongori to survive its interaction with phagocytic cells in its hosts.

Our study establishes that EspJ is necessary and sufficient to block uptake of C3bi- and IgG-opsonized RBC. In a previous study EPEC were shown to be unable to block uptake of C3bi-opsonized zymosan (Celli et al., 2001). The reason for this discrepancy is unclear. While zymosan and RBC are distinct phagocytic targets, opsonization with IgG or C3bi fragments should ensure that when opsonized the two types of particles interact with identical surface receptors FcγR and CR3 respectively. The only other difference between the two studies is the source of phagocytic cells [bone marrow macrophage-derived cell line in Celli et al. (2001); J774.A1 and transfected Cos-7 cells in this study]. Because we obtained similar results in a murine macrophage cell line and in Cos-7 cells transfected with either FcγRIIA or CR3, and because EspJ expression is sufficient to block phagocytosis, we strongly believe that EspJ targets a critical regulatory pathway activated downstream of the two receptors; this pathway is furthermore conserved both in macrophages and in Cos-7 cells.

What is the mechanism by which EspJ blocks FcγR- and CR3-mediated opsono-phagocytosis of RBC? On the one hand, bioinformatic searches on EspJ did not yield any recognizable domain or sequence that could help us clarify its function. On the other hand, phagocytosis through these two receptors is ultrastructurally, pharmacologically and functionally different. In macrophages, FcγR-mediated uptake is constitutive and pro-inflammatory, involves the protrusion of actin-rich pseudopods and is controlled by tyrosine kinases, Cdc42 and Rac activity; in contrast, CR3-dependent internalization is not accompanied by major protrusions or production of pro-inflammatory signals, does not require tyrosine kinase, Rac or Cdc42 activity but is dependent on RhoA activity for actin polymerization at sites of particle binding (Allen and Aderem, 1996; Caron and Hall, 1998; Niedergang and Chavrier, 2005). Nonetheless, these two modes of engulfment share two requirements: local actin polymerization and membrane delivery at sites of particle binding (Allen and Aderem, 1996; Braun and Niedergang, 2006). We show that F-actin is still detected at sites of RBC binding in EspJ-expressing cells, suggesting that EspJ could interfere with the delivery of membrane at nascent phagosomes. In line with this observation, EspJ is found on intracellular structures, possibly endomem-
branes, when ectopically expressed in Cos-7 cells and is not recruited to forming phagosomes. Phagocytosis is known to involve the focal delivery of membrane from various intracellular sources at sites of particle binding (Gagnon et al., 2002; Braun et al., 2004). Identification of the putative EspJ-rich compartment would help us shed light on the EspJ-dependent antiphagocytic mechanism, which might involve the blocking either of membrane per se or of some unknown membrane-borne regulator(s) of uptake.

Current understanding of infections by A/E pathogens points towards an extracellular lifestyle, with bacteria adhering strongly to the surface of enterocytes. In EPEC and EHEC, type three secretion, which controls formation of A/E lesions, is important for host colonization (reviewed in Spears et al., 2006). It is now clear that the T3SS also controls antiphagocytosis (Goosney et al., 1999; Celli et al., 2001; this report). Importantly, ultrastructural studies showed that EHEC O157:H7 colonize Peyer’s patch mucosa in vivo (Phillips et al., 2000). C. rodentium first targets the caecal patch in vivo (Wiles et al., 2004) while RDEC-1 (rabbit EPEC) first targets ileal M cells, an intestinal phagocyte that binds but does not internalize these bacteria, before spreading to other intestinal sites (Inman and Cantey, 1983). It is conceivable that EspF-mediated, cis-inhibition of phagocytosis (Quitard et al., 2006; Martínez-Argudo et al., 2007) allows A/E bacteria to prevent their internalization early on during infection and thereby facilitates colonization of the intestine. EspJ-mediated trans-inhibition could be a mechanism to ensure that bacteria-associated host cells are not internalized by phagocytic cells that are recruited to the lumen of the gut as a result of inflammation once colonization of the epithelial is established (Inman and Cantey, 1983) or after an adaptive immune response has been mounted. Further studies are needed to unravel the molecular basis of EspJ and EspF antiphagocytic activities and their respective roles in colonization and infection.

**Experimental procedures**

**Bacterial strains**

The wild-type strains EPEC O127:H6 E2349/68 and EHEC O157:H7 85-170 used in this study and their mutants are listed in Table 1. Bacteria were grown in Luria–Bertani (LB) medium or in DMEM supplemented with kanamycin (50 μg ml⁻¹), chloramphenicol (5 μg ml⁻¹) and carbenicillin (100 μg ml⁻¹), when necessary. The mutant strains engineered during this study were constructed using the PCR one-step Red recombinase method (Datsenko and Wanner, 2000). Briefly, each mutation was obtained using a PCR product containing an antibiotic resistance gene flanked by the 5’ and 3’ ends of the target gene. Plasmids pKD4, pKK3 and pSB315 were used as PCR template. The PCR products were electroporated into the recipient strains carrying the Red system expression plasmid pKD46 and mutants were selected on LB plates with kanamycin or chloramphenicol. Recombinant clones were cured of pKD46 plasmid by growth at the non-permissive temperature (42°C) and mutation confirmed by different PCR reactions using primers flanking the targeted region and primers into the antibiotic resistance gene.

**Cell culture and transfection**

Cells from the murine macrophage J774.A1 and simian kidney fibroblast Cos-7 cell lines (ATCC) were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin. J774.A1 were seeded on glass coverslips (13 mm diameter) in 24-well plates at a density of 5 x 10⁴ cells per well 24 h before infection. Cos-7 cells were seeded on coverslips in 6 cm dishes (10⁵ cells per dish) and transfected either by nucleofection (Amamax, Cologne, Germany) or using the calcium/phosphate protocol (Cougoule et al., 2006). Briefly, DNA/calcium phosphate precipitates (10 μg of DNA/400 μl of calcium phosphate/6 cm dishes containing 3.6 ml of fresh medium) were added onto the cells for 16–18 h, washed and incubated in fresh medium for an additional 6 h.

**Plasmids**

pICC32 and pICC31 are derivatives of pSA10 (Schlosser-Silverman et al., 2000), a vector containing multiple cloning sites downstream of the tac promoter. Pair of primers EspJf1 5’-CGGAATTCATGCCAATCATAAAGAACTGC-3’ and EspJr1 5’-AAAAACTGCAGTTATTTATCATCATCATCTTTATACTTTTCTTTTTTTAGTGGGTTGATACAT-3’ and pair of primers EspJf2 5’-CGGAAATTCATGCAATTATAAAAAACTGCAGTTATTTATCATCATCATCTTTATACTTTTCTTTTTGAGGATTATATGCAAC-3’ and EspJr2 5’-AAAAACTGCAGTTATTTATCATCATCATCTTTATACTTTTCTTTTTCTTTTTAGGATTATATGCAAC-3’ were used to amplify espJ fused to a FLAG tag from the wild-type EPEC and the wild-type EHEC respectively. PCR products containing terminal EcoRI and PstI restriction sites were digested and cloned into pSA10, generating plasmids pICC32 and pICC31. Plasmid pRKs-EspJincpt-FLAG encoding EspJ from EHEC fused to a FLAG tag was obtained by EcoRI and PstI digestion of the PCR fragment obtained with primers EspJf2–EspJr2 and cloning into the eukaryotic expression vector pRKs (BD Pharmingen).

**Phagocytic assay and immunofluorescence**

Overnight EPEC cultures in LB were diluted 1:100 into DMEM containing 25 mM Hepes and 2 mM Glutamax (Invitrogen) and pre-activated by incubation for 3 h at 37°C in a 5% CO₂ atmosphere before infection. EHEC bacteria were grown overnight in DMEM supplemented with 5% FCS and directly added onto mammalian cells. J774.A1 or Cos-7 cells were incubated for at least 1 h in serum-free medium then infected either for 1 h with EPEC at a multiplicity of infection (moi) of 20:1 or for 4 h with EHEC at a moi of 200:1, conditions that lead a similar average number of bacteria (20–50) interacting with each macrophage at the end of the infection period. Conditions for optimal induction of LEE expression and optimal association with host cells have been described previously (Garmendia et al., 2004; Quitard et al., 2006).
**RBC phagocytosis.** Monolayers of infected cells were then washed with PBS and challenged for 30 min at 37°C in 5% CO₂ with 500 µl of IgG- or C3bi-opsonized sheep RBC (TCS) at a ratio of 30 RBC per cell. RBC were opsonized as previously described (Caron and Hall, 1998; Patel et al., 2002). Briefly, for FcγR-mediated phagocytosis, 0.5 µl of RBC per coverslip were opsonized for 30 min with a subagglutinating concentration of rabbit anti-RBC IgG (Cappel) in 1 ml of gelatin veronal buffer (GVB², Sigma), washed once with GVB² and re-suspended in 500 µl of DMEM/coverslip. For CR3-mediated phagocytosis, 0.5 µl of RBC per coverslip were opsonized with rabbit anti-RBC IgM (Cedarlane Laboratories) for 30 min at room temperature. IgG- or C3bi-opsonized RBC, cells were incubated for 30 min. For differential staining of external and internalized RBC, monolayers of infected cells were then washed twice with DMEM for optimal phagocytosis of opsonised particles in macrophages. EMBO J 23: 4166–4176.

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