

Phagocytosis mediated by *Yersinia* invasin induces collagenase-1 expression in rabbit synovial fibroblasts through a proinflammatory cascade

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SUMMARY

We show that the interaction of the *Yersinia* surface protein, invasin, with rabbit synovial fibroblasts mediates bead phagocytosis and induces expression of interleukin 1 α (IL-1 α), tumor necrosis factor- α (TNF- α) and MMP-1/collagenase-1 (CL-1). Presentation of invasin as a ligand on the surface of 4.5 μ m beads induced phagocytosis and increased CL-1 expression 20-fold after 24 hours. By contrast, presentation of invasin as a spreading substrate did not induce CL-1 expression. CL-1 induction following phagocytosis of invasin-coated beads was mediated by a mechanism dependent on high-affinity binding to β 1 integrins and the function of the small GTPase RhoA. Expression of a function-perturbing mutant, RhoAN19, abrogated bead-induced CL-1 expression. RhoA activation

coupled bead phagocytosis with signal transduction because expression of constitutively active mutant RhoV14 was sufficient to trigger CL-1 expression. The signal-transduction cascade elicited by bead phagocytosis triggered NF κ B activation, stimulating a proinflammatory cellular response with transient increases in TNF- α production that peaked at 2 hours and induction of IL-1 α that was sustained for at least 10 hours. Inhibition of IL-1 α function by blocking antibodies or IL-1 receptor antagonist showed that IL-1 α is the autocrine intermediary for subsequent CL-1 induction.

Key words: *Yersinia*, Invasin, MMP-1, Collagenase, RhoA, Integrin, Phagocytosis, Proinflammatory, Interleukin 1

INTRODUCTION

Several microorganisms use integrins to invade their target cells. The surface protein of the invading bacteria binds with high affinity to integrins, promoting their active uptake by non-phagocytic cells (Williams-Herman and Werb, 1999). The bacterial uptake is accompanied by a complex modification of cytoskeletal organization and by changes in cellular function and gene expression.

Reactive arthritis is associated with a number of infectious organisms, such as *Yersinia* species, that enter and pass through the intestinal epithelium and gain entry into Peyer's patches. Bacterium-like structures, antigens and RNA derived from these organisms can be found in the joints and synovial cells of patients with reactive arthritis (Gaston et al., 1999; Hammer et al., 1990). The molecular mechanism of cellular attachment and entry for *Yersinia* species has been well characterized (Heesemann et al., 1993). Attachment and entry of *Yersinia* species are primarily mediated by two outer membrane proteins – a dispensable collagen-binding factor Yad A (Roggenkamp et al., 1995) and invasin, a ligand for β 1 integrins (Isberg and Leong, 1990). Invasin, the surface protein of *Yersinia pseudotuberculosis*, mediates bacterial invasion by binding to α 1, α 2, α 4 and α 5 β 1 integrins with high affinity through a domain that does not contain an RGD sequence

(Hamburger et al., 1999; Isberg and Leong, 1990). In animal models of yersiniosis, synovial lining cells become hyperplastic, with excess breakdown of extracellular matrix (ECM) proteins and cellular exudates within the periarticular cavity; this hyperplasia is similar to that seen in human yersiniosis-associated joint inflammation (van der Heijden et al., 1997). Synovial fibroblast function can be switched to a pro-arthritis phenotype either by local invasion of microorganisms (Goldenberg, 1989), generating a septic arthritis, or by enteric infections, which distally can cause an aseptic reactive arthritis by a still-unknown mechanism (Sieper and Braun, 1999).

We have previously shown in rabbit synovial fibroblasts (RSFs) that integrin-mediated binding to different ECM proteins induces the expression of MMP-1/collagenase-1 (CL-1) through different mechanisms, depending on how the cells recognize the integrin ligands. When fibroblasts spread on the 120 kDa fibronectin fragment (120FN) or on an anti- α 5 integrin mAb, but not on intact fibronectin (FN), CL-1 expression is induced by a mechanism dependent on the direct activation of the transcription factor AP-1 (Tremble et al., 1995). The presence of additional ECM components can modify this response. For example, the addition of tenascin-C to a FN matrix induces CL-1 expression, whereas neither

tenascin-C nor FN are inductive when used alone as a spreading substrate (Tremble et al., 1994).

When these ECM components are recognized in a different context, for example as a soluble integrin ligand, CL-1 expression is induced by a distinct signal-transduction cascade that is associated with changes in cell shape and reorganization of the actin cytoskeleton. The addition of anti- $\alpha 5$ mAb (Kheradmand et al., 1998), SPARC (Tremble et al., 1993) or the fibronectin extra domain A (ED-A) fragment (Saito et al., 1999) to cell cultures induces cytoskeletal reorganization with a concurrent activation of nuclear factor κ B (NF κ B). The actin cytoskeleton rearrangement transduces a signal directly because cytochalasins can also induce the activation of the transcription factor NF κ B. The activation of NF κ B induces the establishment of an interleukin-1 (IL-1) autocrine loop, which is indispensable for the CL-1 induction that follows, as the effect of soluble integrin-ligands is abrogated by IL-1 receptor antagonist (Kheradmand et al., 1998).

Invasin is a high-affinity integrin ligand on the surface of a bacterium that may be recognized by the cells in an unusual context, that is, of inducing endocytosis. Therefore we asked whether invasin modifies CL-1 expression in primary cultures of RSF.

MATERIALS AND METHODS

Reagents

The mAb anti- $\beta 1$ (AIB2) and anti- $\alpha 5$ (BIIG2) integrins were gifts from Caroline Damsky, UCSF (Werb et al., 1989; Huhtala et al., 1995); mAb P4G6 (anti- $\alpha 4$ integrin), RGD peptides and 120 kDa FN were purchased from Gibco BRL (Grand Island, NY); anti-rabbit IL-1 α from Endogen (Woburn, MA); anti-mouse tumor necrosis factor- α (TNF- α) mAb from Genzyme (Cambridge, MA) and anti-p65 NF κ B polyclonal antibody from Santa Cruz (Santa Cruz, CA). HRP- and Alexa-conjugated secondary antibodies were from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ) and Molecular Probes (Eugene, OR), respectively. Human recombinant IL-1 α , mouse TNF- α , Protein A Sepharose and DEAE Dextran were from Sigma Chemical Co. (St Louis, MO). IL-1 receptor antagonist was from R&D Laboratories (Minneapolis, MN). Maltose binding protein (MBP) and anti-MBP antibodies were from New England BioLabs (Beverly, MA). Invasin (wild-type and mutant forms) were prepared as described previously (Leong et al., 1990). Fibronectin and anti-proteases (Complete) were purchased from Roche Laboratories (Indianapolis, IN).

Cells

Rabbit synovial fibroblasts were isolated as described previously (Aggeler et al., 1984) and cultured between passages 3 and 10 in Dulbecco's modified Eagle's medium H-21 (DME) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan UT) and 2 mM glutamine in 5% CO₂ at 37°C. Unless otherwise stated, RSFs were plated with DME 0.2% lactalbumin hydrolysate on dishes coated for 2 hours at ambient temperature with 20 μ g/ml fibronectin or 0.3 μ g/ml invasin in PBS. Magnetic beads of 4.5 μ m diameter (Dyna, Oslo, Norway) were coated by adsorption with the different integrin ligands following the manufacturer instructions and then added to cells at a ratio of five beads per cell 1 hour after plating.

Detection of IL-1 α , TNF- α and CL-1 expression

Interleukin-1 α , TNF- α and CL-1 expression was measured as described previously (Tremble et al., 1994) by slot blot analysis of serial dilutions of the treated cell culture supernatants. CL-1 expression was also detected by western blot analysis of the cell

culture supernatants using a mixture of mouse anti-rabbit CL-1 mAb and an HRP-conjugated anti-mouse secondary antibody; HRP activity was then developed by the enhanced chemiluminescence reaction. The bands were quantified and analyzed by densitometry using Image Quant (Molecular Dynamics).

Immunofluorescence

The monolayer was washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 minutes, permeabilized with 0.1% Triton X100 for 5 minutes and blocked for 1 hour with 15% FBS in PBS. Primary antibodies were diluted in 15% FBS and incubated for 1 hour at 37°C. After three washes with PBS 0.1% Triton X100, the samples were incubated with Alexa-labeled secondary antibodies for 45 minutes and embedded in Vectashield (Vector Labs) with DAPI. Samples were analyzed and photographed using a Leica DMR microscope with a long pass filter to visualize beads and immunostaining simultaneously.

Phagocytosis assay

To evaluate bead uptake, RSFs were incubated with beads for 3 hours. The bead coating was then visualized by immunostaining with an anti-MBP antibody (New England BioLabs) when invasin-coated beads were used or with anti-rat IgG when anti- $\alpha 5$ integrin mAb-coated beads were used. Afterwards, the same procedure as for immunofluorescence using an Alexa-green conjugated secondary antibody was followed, but without cell permeabilization. When a long band pass filter is used, this procedure allows the simultaneous distinction between adherent (green and red) or phagocytosed (red autofluorescence only) beads.

Competition of invasin binding with RGD

Cells plated in serum-free conditions were pre-incubated with 10 mg/ml RGD or RGE peptides or an equivalent volume of PBS for 15 minutes. Beads coated with INV497 or anti- $\alpha 5$ integrin mAb were added and the cells were incubated at 37°C for a further 30 or 120 minutes. The total number of beads per 100 cells from ten random fields was counted on an inverted microscope after three washes with PBS to remove nonadherent beads. Data are presented as means of three independent experiments.

Membrane protein extraction and western blotting

Rabbit synovial fibroblasts were washed in serum-free media and incubated with magnetic beads coated with wild-type invasin INV497, INV497D911A, FN or BSA, for 1 or 6 hours. Cells were then washed with PBS and lysed in isolation buffer (100 mM MES, pH 6.8, 1 mM EDTA, 0.5 mM MgCl₂ and anti-proteases) by three subsequent freeze/thaws in liquid N₂. Beads were extracted by magnetic field separation, washed in isolation buffer three times and centrifuged at 100,000 *g* to enrich for membrane proteins. This was followed by solubilization of the pellet in 1% Triton-X100, 50 mM Tris-buffered saline (pH 7.5) with anti-proteases. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose paper and blotted for adaptor protein-2 (AP-2, mAb AP.6, obtained from Francis Brodsky, UCSF).

Electromobility shift assay (EMSA)

Nuclear extracts were prepared from 5 \times 10⁶ cells by the method described previously (Finbloom et al., 1994). 5 μ g of nuclear extract were incubated with a [³²PO₄]-5' end-labeled NF κ B oligonucleotides (Promega, Madison, WI) and subjected to EMSA as described previously (Kheradmand et al., 1998).

Cell transfection

Marc Symon (Picower Institute) kindly provided the plasmids with RhoAV14 and RhoAN19, Rac1V12 and Rac1N17, Cdc42V12 and Cdc42 N17 (Qiu et al., 1995). To measure CL-1 induction, a minimal CL-1 promoter was used (Kheradmand et al., 1998). Control cells

were transfected with equal amounts of empty vector or a green fluorescent protein vector (pEGFPIC, Clontech, Palo Alto, CA) to monitor transfection efficiency. RSF transfection was performed using adenovirus (Forsayeth and Garcia, 1994), as described previously (Kheradmand et al., 1998). Briefly, the cells were trypsinized the day before transfection. For transfection, 3×10^6 cells were plated in a 3.5 cm dish with 2.5 ml of the following mixture: adenovirus stock diluted 1:20, 2 $\mu\text{g/ml}$ plasmid, 80 $\mu\text{g/ml}$ DEAE-dextran in serum and antibiotic-free DME. The cells were incubated with the transfection mixture for 2 hours at 37°C, washed for 1 minute with 10% DMSO in PBS and then incubated overnight with DME 10% FBS. After 24 hours, if the transfection efficiency was higher than 50%, the medium was changed to DME 0.1% LH, and 24 hours thereafter, equal numbers of cells were plated on dishes coated with fibronectin. The cells were analyzed 24 hours later by measuring luciferase activity in whole cell lysates using a commercial kit (Promega).

Statistical analysis

When indicated, data were analyzed using the software ESTAT to perform an unpaired, two-tailed student *t*-test.

RESULTS

Invasin mediates induction of CL-1 as a ligand on bead particles, but not as a spreading substrate

Spreading of RSFs on substrates that ligate $\alpha 5\beta 1$ integrin, but not $\alpha 4\beta 1$ integrin, induces expression of CL-1 (Huhtala et al., 1995). Because the interaction between invasin and $\beta 1$ integrins differs from that of ECM components and anti-integrin antibodies, we examined whether invasin can mediate cell spreading and CL-1 induction. We used a soluble form of invasin, INV497 which contains the extracellular region (including the integrin-binding domain) fused to MBP to coat dishes and beads. We evaluated CL-1 expression after plating or addition of beads, by western blot analysis of the cell culture supernatant. We observed that RSFs spread on INV497 expressed CL-1 at basal levels, comparable to that seen in cells plated on FN (Fig. 1). We next examined the effects of invasin INV497 adsorbed on 4.5 μm beads. Invasin was a powerful inducer when presented on beads, increasing the expression of CL-1 15.6 ± 8 -fold with FN as a substrate, compared with cells plated on FN. CL-1 was first detected at 10 hours after addition of beads, increasing through 24 hours. By contrast, fibronectin-coated beads were a poor inducer on cells spread on fibronectin, but induced more CL-1 in cells spreading on invasin, although not in a magnitude comparable to INV497-coated beads. These results show that induction of CL-1 expression by invasin depends on the context in which it is presented to the cells.

Invasin mediates phagocytosis of particles

By 24 hours after addition of INV497-coated particles, virtually all the beads added were inside RSFs. Therefore, we next asked whether the induction of CL-1 by invasin-coated particles was related to their phagocytosis. Because bacterial invasion is dependent on the binding affinity of invasin (Tran Van Nhieu and Isberg, 1993), we also coated beads with a mutant form of invasin – one that has a change in an essential aspartic residue (INV497D911E) – that binds integrins at low affinity (750 nM) and is unable to mediate phagocytosis (Leong et al., 1995). We compared beads coated with INV497, which exhibits high-affinity binding (5 nM), with INV497D911E-coated beads, and used MBP as a control for

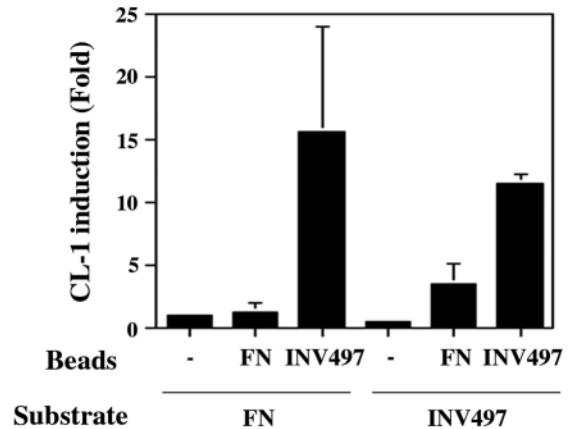


Fig. 1. Context-dependent induction of CL-1 expression by invasin. RSFs were plated on FN- or invasin-coated dishes. Beads coated with INV497 or fibronectin were added to cells spreading on FN or INV497. CL-1 expression was measured in the supernatant by slot blot 24 hours later. The error bars represent \pm s.e.m. of two or more independent experiments. INV497-coated beads produced a large increase in CL-1 secretion, whereas FN-coated beads did not in cells plated on FN. The difference in CL-1 produced in response to FN-coated beads in cells plated on FN, compared to cells plated on INV497 was small, but significant ($P=0.046$).

nonspecific binding. We exposed the cells to the beads coated with the different ligands and measured bead uptake at 3 hours and CL-1 expression at 24 hours (Fig. 1; Fig. 2). After 3 hours, 50% of the beads coated with INV497 were inside the cells (red). Beads coated with the low-affinity binding mutant INV497D911E (green) bound to RSF in a proportion comparable to INV497 beads, but few beads were ingested (Fig. 2). Beads coated with INV497 were significantly ($P<0.05$) more effective in increasing CL-1 expression than beads coated with INV497D11E. MBP-coated beads did not bind to the cells and were barely retained on the monolayer after the washes. These results show that induction of CL-1 correlates with the efficiency of bead phagocytosis.

$\beta 1$ Integrins mediate phagocytosis of invasin-coated beads

We next demonstrated that integrins serve as receptors for invasin in induction of CL-1 expression. Binding of INV497 beads was inhibited by the soluble GRGDSP peptide, but not by GRGESP peptide (Fig. 3A). A comparable number of anti- $\alpha 5$ coated beads bound and were displaced by RGD peptides, denoting the $\alpha 5\beta 1$ FN receptor as the main receptor for invasin-coated beads in RSF. This observation was confirmed by analysis of the cell-surface proteins that bound to beads after 60 minutes of incubation with surface-biotinylated RSF. We observed that integrin $\beta 1$, $\alpha 5$ and $\alpha 4$ chains were the major proteins associated with the beads (data not shown). Thus, invasin and peptides containing RGD compete for binding to $\alpha 5\beta 1$.

We next asked whether other ligands for $\alpha 5\beta 1$ integrin or cross-linking of other $\beta 1$ integrins mediate phagocytosis and CL-1 induction. We coated beads with the 120FN fragment, intact FN, mAb directed to $\alpha 4$ or $\alpha 5$ integrins, or a function-inhibiting anti- $\beta 1$ mAb (AIIB2). After 24 hours, CL-1 was highly induced by beads coated with anti- $\alpha 5$ mAb (BIIG2), anti- $\alpha 4$ mAb (P4G9) and 120FN (Fig. 3B), which were rapidly

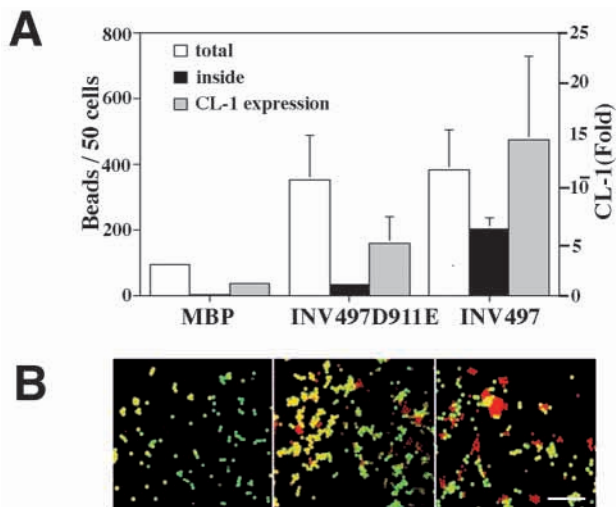


Fig. 2. Effect of affinity of invasin binding on bead phagocytosis and CL-1 induction. RSF were incubated for 2 or 24 hours with beads coated with MBP (nonspecific binding control), INV497D911E (low-affinity binding) or INV497 (high-affinity binding). Bead phagocytosis was analyzed after 3 hours and CL-1 expression was measured in the supernatant by slot blot after 24 hours. (A) Quantitative representation of bead binding and uptake after 3 hours and CL-1 expression after 24 hours for each type of coating. (B) Micrographs of the phagocytosis assay. The green beads are outside and the red beads are inside the cells. Error bars represent \pm s.e.m. of 3 independent experiments. Bar, 50 μ m.

ingested. By contrast, low induction was observed with beads coated with FN, AIIB2 or BSA (Fig. 3B). Interestingly, anti- α 4 mAb functioned like invasin: ligation of α 4 integrins by beads coated with anti- α 4 mAb induced CL-1 expression, even though ligation of α 4 integrins with mAb as a spreading substrate does not induce CL-1 expression (Huhtala et al., 1995). These experiments show that integrin-mediated phagocytosis is not restricted to one type of integrin and corroborate that this mechanism requires high-affinity binding to integrins because a low-affinity ligand, such as FN, does not induce. Furthermore, CL-1 induction is phagocytosis dependent because, unlike spreading, which induces CL-1 only when α 5 and not α 4 integrins are ligated, phagocytosis of beads coated with either anti- α 4 mAb or anti- α 5 mAb induces CL-1 expression. Significantly, beads coated with the anti- β 1 antibody, which binds to integrins and produces a nonfunctional state of β 1 integrins, were not inductive. The latter results suggest that high-affinity binding is not sufficient and proper function of the integrin β 1 chain is also required for bead ingestion and for the subsequent downstream signal leading to induction of CL-1 expression.

Invasin-mediated phagocytosis alters particle-associated proteins

We next examined whether there were any differences in membrane-associated proteins in cells ingesting various types of beads. After 1 hour of incubation with beads coated with FN, 120FN, INV497, INV497D911E, INV497D911A (a mutant that does not bind at all to cells (Leong et al., 1995)) or BSA, we detected a scant quantity of AP-2 in the bead membrane fraction of cells incubated with invasin-coated

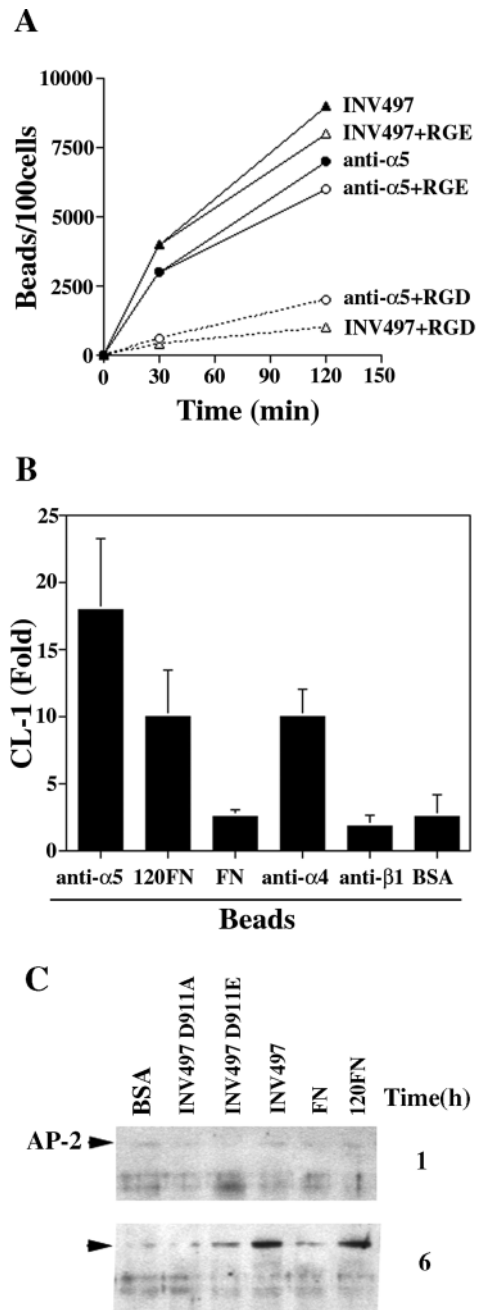


Fig. 3. Invasin induction of CL-1 expression is integrin dependent. (A) INV497 coated beads binding was inhibited by the addition of soluble GRGDSP peptide, but not by GRGESP peptide. As a control, GRGDSP peptides were used to compete for the binding of anti- α 5 integrin antibody. (B) Beads coated with other integrin ligands of high-affinity binding induce CL-1 expression. Beads coated with integrin antibodies: anti- α 5 integrin mAb (BIIG2), anti- α 4 integrin mAb (P4G6), function-blocking anti- β 1 integrin mAb (AIIB2) were compared with FN or 120FN in their potency to induce CL-1 expression. CL-1 expression was measured in the supernatant by slot blot after 24 hours of bead addition. Error bars represent \pm s.e.m. of three independent experiments. (C) High-affinity integrin ligands recruit AP-2 to the bead-associated membrane fraction. Membranes associated with beads coated with BSA, INV497D911A, INV497D911E, INV497, FN and 120FN were analyzed for the presence of AP-2 after 1 and 6 hours of binding.

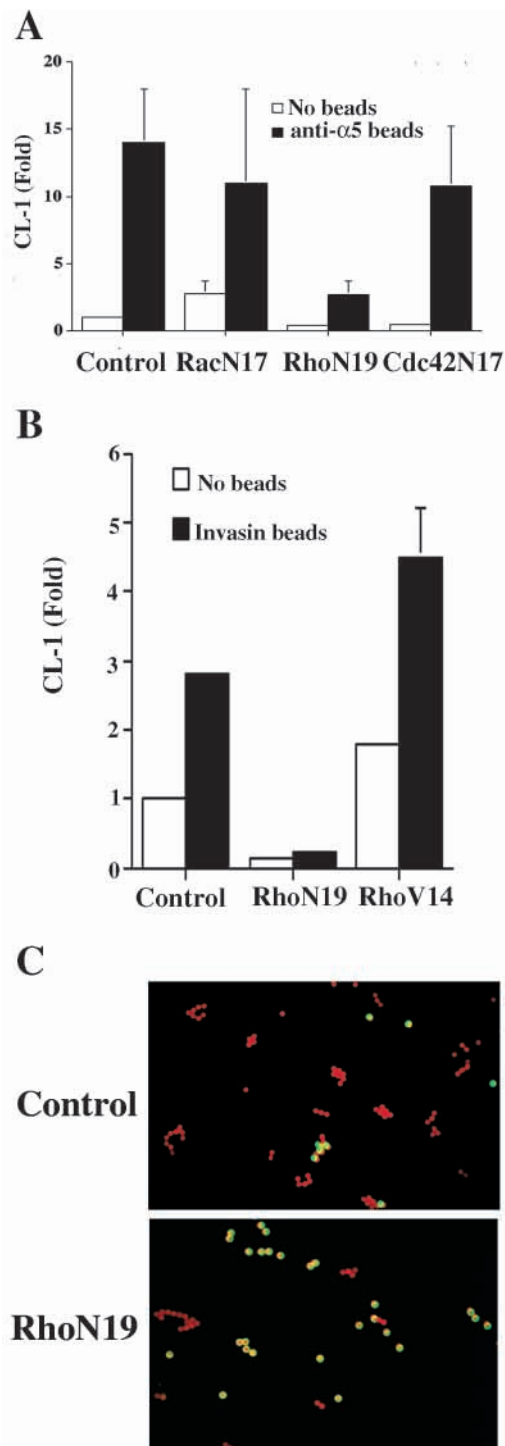


Fig. 4. RhoA is required for phagocytosis and bead-induced CL-1 expression. (A) Anti- $\alpha 5$ mAb-coated beads were added to cells transfected with dominant-negative mutants of Rac 1, RhoA or Cdc42 and a reporter construct of CL-1 promoter driving luciferase expression. Luciferase activity was measured 24 hours after bead addition. (B) CL-1 promoter driven luciferase activity was measured 24 hours after addition of invasin-coated beads to cells transfected with RhoAN19 or RhoAV14. Error bars represent \pm s.d. of triplicates in one of two experiments. (C) Micrographs of the phagocytosis assay after 2 hours of bead addition to dominant-negative RhoA-transfected cells.

beads. However, following 6 hours of incubation, we noted a more pronounced increase in AP-2 in the bead-associated membrane fraction of cells that had phagocytosed beads coated with INV497 (Fig. 3C) rather than lower-affinity binding ligands.

RhoA is required for bead phagocytosis and signaling for CL-1 induction

The function of members of the Rho GTPase family is associated with several bacterial invasion mechanisms (Fu and Galan, 1999; Watarai et al., 1997), as well as in phagocytosis in phagocytes (Caron and Hall, 1998) and in integrin-mediated CL-1 induction after cell spreading (Kheradmand et al., 1998). Therefore, we examined whether these proteins are involved in induction of CL-1 expression mediated by invasin-coated beads. We co-transfected RSF at high efficiency with dominant-negative or constitutively active mutants of Rac1, RhoA and Cdc42 and with a reporter construct containing the CL-1 minimal promoter coupled to luciferase; we then measured CL-1 expression induced by beads after 24 hours. We observed that the beads did not induce CL-1 expression in cells expressing the dominant-negative RhoAN19, implicating the activity of RhoA in the signal-transduction pathway associated to phagocytosis (Fig. 4A).

If RhoA activation is one of the elements in the signaling pathway, the activation of RhoA is expected to be sufficient to induce CL-1 expression. Hence, we analyzed the expression of CL-1 upon constitutive active RhoA expression. In cells transfected with the constitutively active mutant RhoAV14, we found that basal CL-1 expression increased twofold (Fig. 4B), and amplified the CL-1 expression induced by beads coated with integrin ligands, as would be expected for an intermediary molecule mediating signal transduction.

Because RhoA also has been implicated in early steps of phagocytosis during particle ingestion, we next examined whether RhoA function was required for bead uptake. The expression of RhoAN19 reduced phagocytosis to 50% after 3 hours without affecting bead binding significantly (Fig. 4C). Bead uptake was impaired not only in cells overexpressing the dominant-negative RhoA, but also in cells treated with C3 exotoxin (not shown). These experiments show that RhoA function is required both for bead ingestion and downstream activation.

Ingestion of invasin-coated particles by RSF activates the transcription factor NF κ B

Our initial observation that upregulation of CL-1 expression after ingestion of invasin-coated particles did not occur before 10 hours suggests an indirect mechanism of induction, as has been observed for SPARC treatment and integrin-mediated cell rounding (Tremble et al., 1993; Kheradmand et al., 1998). These integrin-induced alterations of cell shape induce activation of NF κ B expression, which is upstream of CL-1 expression. Interestingly, phagocytosis of bacteria activates NF κ B in macrophages, neutrophils and non-phagocytes (Aderem and Underhill, 1999; McDonald and Cassatella, 1997; Verma et al., 2000). Therefore, we tested whether phagocytosis of invasin-coated beads activates NF κ B in RSF. We detected nuclear translocation of NF κ B by immunofluorescence within the first hour after bead addition (arrows in Fig. 5A). EMSA analysis of nuclear extracts from

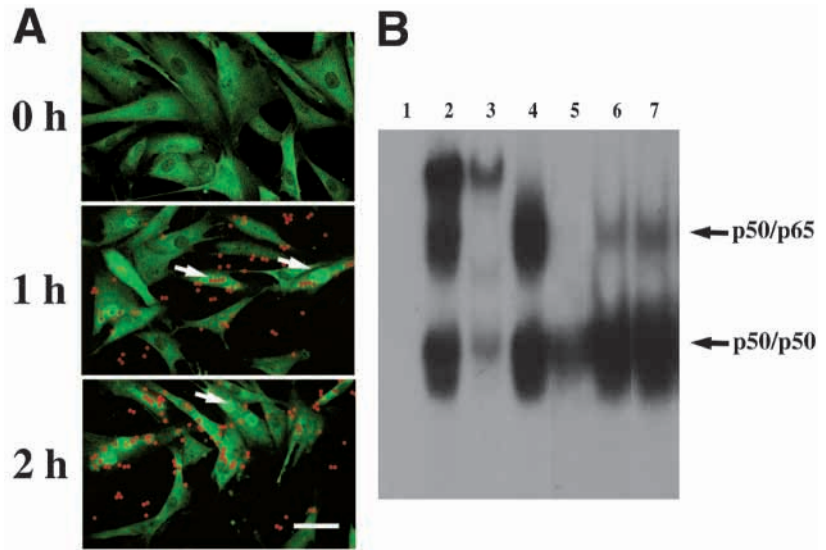


Fig. 5. Bead phagocytosis induces NF κ B activation. (A) NF κ B (green) nuclear translocation was visualized by immunofluorescence 1 or 2 hours after the addition of anti- α 5 mAb-coated beads (red autofluorescence). Arrows mark cells positive for NF κ B translocation and with beads inside. Bar, 50 μ m. (B) Activated NF κ B was detected by EMSA of nuclear extracts prepared from RSF incubated for 2 hours without beads (lane 5) or beads with anti- α 5 mAb (lane 6) or beads coated with INV497 (lane 7). Lane 1, no extract; lane 2, HeLa extract; lane 3, HeLa extract plus 1.75 pmol of unlabelled specific probe; lane 4, HeLa extract plus 1.75 pmol nonspecific competitor.

cells exposed for 2 hours to beads coated with INV497 or with anti- α 5 mAb also showed activation of NF κ B (Fig. 5B). These results indicate that integrin-mediated bead phagocytosis leads to the rapid activation of NF κ B.

Invasin-mediated ingestion of particles induces the proinflammatory cytokines TNF- α and IL-1 α , but only IL-1 α is required for CL-1 induction

We next asked whether NF κ B activation leads to a proinflammatory response. We detected an increase in the levels of TNF- α and IL-1 α within 2 hours after bead addition (Fig. 6A). TNF- α expression declined after 6 hours, whereas IL-1 α production continued at high levels even after 10 hours, when CL-1 protein starts to be detected in the supernatant. These results show that phagocytosis of invasin-coated beads induces a proinflammatory response in RSF that precedes CL-1 upregulation. Because IL-1 α and TNF- α can induce CL-1 expression in other cells (Siwik et al., 2000), we examined whether these cytokines play a role in bead-mediated CL-1 induction. We added cytokine-neutralizing antibodies together with beads and measured induced CL-1 expression after 24 hours. We found that IL-1 α and TNF- α induce CL-1 expression (Fig. 6B). When IL-1 receptor antagonist or anti-rabbit IL-1 α were added with the beads, upregulation of CL-1 was inhibited. Thus, although IL-1 acts as an autocrine or paracrine regulator of CL-1, TNF- α production was not required for phagocytosis-mediated CL-1 induction. When TNF- α -neutralizing antibodies were added, only a slight diminution of bead-induced CL-1 expression was seen. This suggests that, although TNF- α is produced and is a CL-1 inducer, it is not required for bead-mediated CL-1 induction. To verify the neutralizing activity of the antibodies, we tested them against their respective cytokines. Although both anti-IL-1 α and anti-TNF- α inhibited CL-1 induction mediated by their respective cytokine, we also found that TNF- α -mediated induction of CL-1 was inhibited by IL-1 α neutralizing antibodies or IL-1 receptor antagonist, indicating that TNF- α induction of CL-1 expression is mediated through IL-1 production. The anti-rabbit IL-1 α -neutralizing antibodies did not inhibit human IL-1 α . From these results we conclude that, although IL-1 α and TNF- α are produced upon bead

phagocytosis and both cytokines are able to induce CL-1 expression, only IL-1 α is indispensable for bead-mediated CL-1 induction.

The requirement for both IL-1 α synthesis and its release into the medium implicates this cytokine as an intermediary, acting both as an autocrine and paracrine factor. To determine whether IL-1 α has this dual function during phagocytosis, we added beads at low concentration and analyzed CL-1 expression by immunofluorescence. In these cultures there were both cells that had phagocytosed beads and cells without beads (Fig. 7B,D). Although the level of CL-1 expression correlated with the number of beads ingested (Fig. 7B), we found that, adjacent to the cells containing beads, there were some cells without beads that were also positive for CL-1 expression (arrows) suggesting that IL-1 α functions as a paracrine factor (Fig. 7D). The expression of CL-1 in all the cells was abrogated by the addition of anti-IL-1 α neutralizing antibody (Fig. 7C), demonstrating the role of this cytokine as an intermediary.

DISCUSSION

In the present work we show that phagocytosis of beads coated with the *Y. pseudotuberculosis* surface protein invasin induces inflammatory cytokines and CL-1, characteristic of a proarthritogenic response, in rabbit synovial fibroblasts. The uptake of invasin-coated particles is integrin-mediated, but is not integrin selective, and it is driven by the binding affinity of the coating ligand. RhoA function is required for bead phagocytosis and transduction of the signal for activation of NF κ B and production of TNF- α and IL-1 α . Subsequently, the endogenous IL-1 α acts by autocrine and paracrine mechanisms to induce CL-1 expression.

Invasin is an integrin ligand of high affinity that induces CL-1 expression in RSF depending on how it is presented. When invasin was presented as a spreading substrate, CL-1 expression was below the basal level seen with FN as a substrate and was not able to confer high CL-1 induction to fibronectin coated beads; this is consistent with our conclusion that signaling from the surface is not sufficient to induce CL-1 (see below). Compared with fibronectin as a substrate,

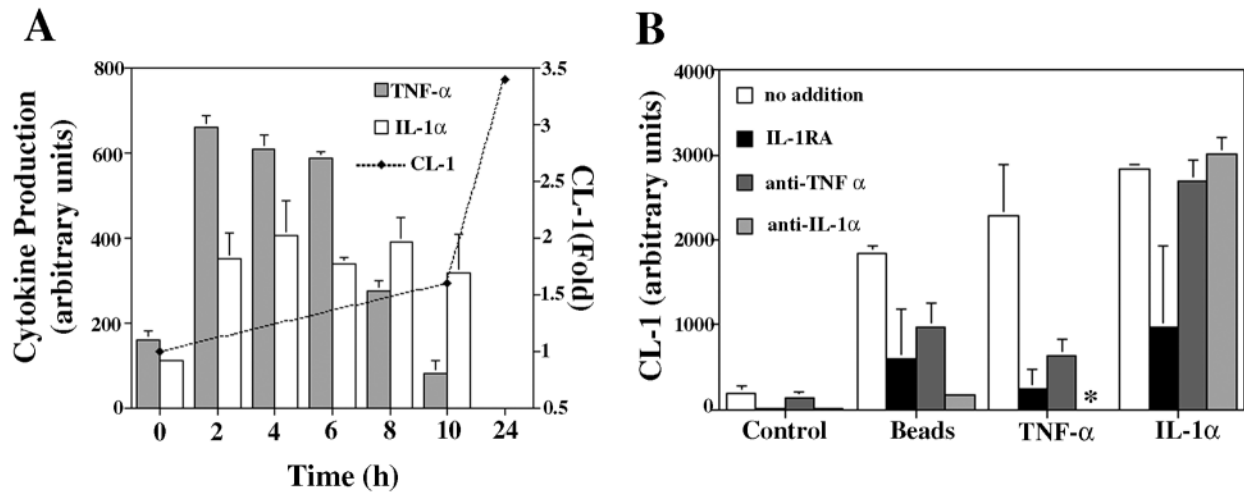


Fig. 6. Bead phagocytosis induces TNF- α and IL-1 α expression, however only IL-1 α production is required for bead-induced CL-1 expression. (A) Time course of TNF- α , IL-1 α and CL-1 production after addition of anti- $\alpha 5$ mAb-coated beads. The cytokines and CL-1 were measured in the culture supernatant by slot blot. Error bars represent \pm s.d. from one of three independent experiments. (B) CL-1 expression was measured in the supernatant after 24 hours after the addition of beads coated with anti- $\alpha 5$, IL-1 α (2 ng/ml) or TNF- α (1 μ g/ml) in the absence or presence of IL-1 receptor antagonist (400 ng/ml), anti TNF- α (8 μ g/ml) or anti IL-1 α (10 μ g/ml). Error bars represent \pm s.d. from one of three experiments. *, the effect of anti IL-1 α on TNF α -induced CL-1 expression was not assayed.

invasin changed the CL-1 levels induced by fibronectin- and invasin-coated beads; this was probably due to competition effect for binding to $\beta 1$ integrins, similar to the competition effect reported previously (Tran Van Nhieu and Isberg, 1993) for bacterial invasion, which is diminished in cells plated on high-affinity integrin-binding ligands. Thus, on cells spreading on invasin, INV497 beads are not very efficient because less $\beta 1$ integrins are available and fibronectin beads might be more inductive by binding to non- $\beta 1$ integrins ($\alpha v\beta 3$ or $\alpha v\beta 5$, for example). By contrast, when invasin was presented on the surface of beads, mimicking bacteria, CL-1 expression was strongly induced after phagocytosis. This dual function was also observed with anti- $\alpha 4$ integrin antibodies, which induced CL-1 expression when used as bead coating, but not when used as a spreading substrate (Huhtala et al., 1995). Therefore, the

discrimination between spreading and phagocytosis is not specific for invasin, but common to the context in which the integrin ligand is presented. In both forms of presentation, these integrin ligands are expected to induce similar occupancy and clustering of integrins, but the difference is that particles can be phagocytosed, whereas the spreading substrate is resistant to ingestion. Integrin-mediated ingestion could promote the assembly of a functionally distinct adhesion

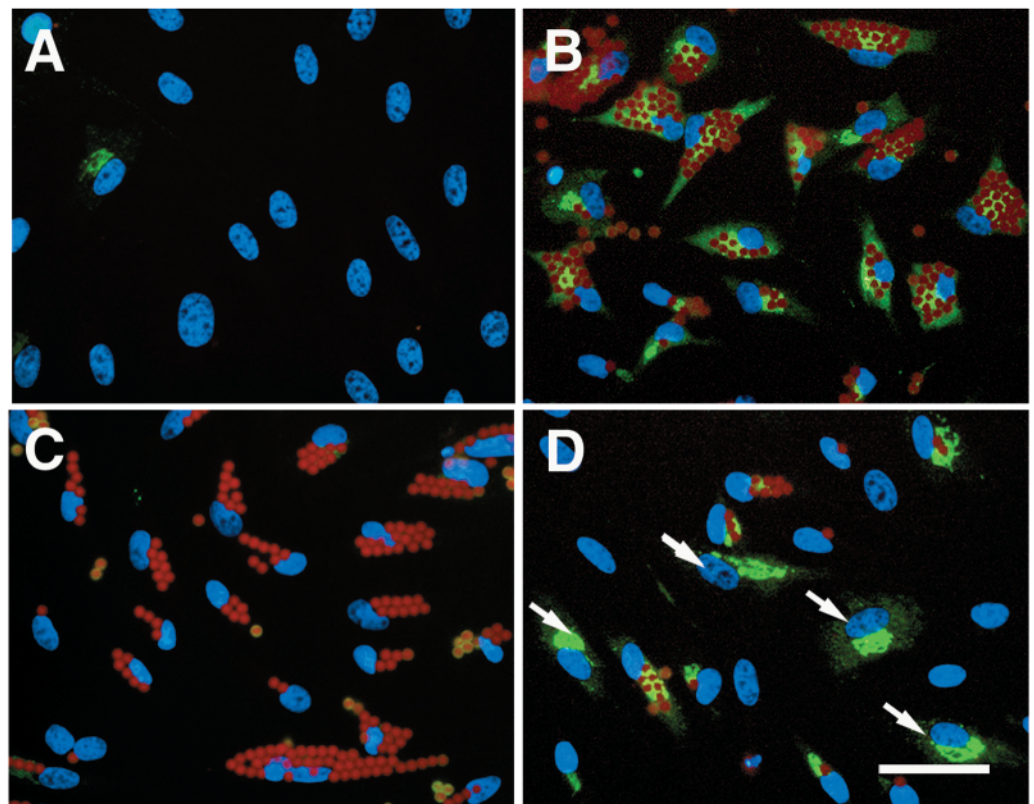


Fig. 7. IL-1 α activity in cis and in trans is required for bead induction of CL-1 expression. Micrographs of immunofluorescence staining for CL-1 expression (green) after 24 hours of anti- $\alpha 5$ mAb-coated beads (red) addition in the absence (A, B and D) or presence of 10 μ g/ml anti IL-1 α (C). Arrows point to cells expressing CL-1, but with no beads. Bar, 50 μ m.

complex committed to phagocytosis and to signaling. Integrins can not only recognize different types of ECM components, but also their rigidity. Katz and co-workers showed that fibrillar fibronectin drives the formation of fibrillar complexes rich in tensin, whereas cross-linked fibronectin induces the formation of focal complexes rich in paxillin (Katz et al., 2000). The recruitment of different protein complexes during phagocytosis would have a distinct functional outcome, inducing the expression of CL-1.

Presentation of an integrin ligand on a surface susceptible to be phagocytosed is not sufficient to induce phagocytosis, as it is demonstrated when low-affinity ligands are used to coat the beads. Invasin ($K_D=5$ nM) has no RGD sequence, but possesses an aspartyl residue (D) in the integrin-binding domain that, when mutated to E, retains binding capability to integrins but with reduced affinity (750 nM), equivalent to that of FN. Beads coated with the E mutant (INV497D911E) were still able to bind to the cells, but were poorly phagocytosed and induced little CL-1 expression, indicating that phagocytosis is driven by high-affinity binding of invasin. This attribute promotes bacterial uptake upon binding of *Yersinia* to several other cell types (Tran Van Nhieu and Isberg, 1993). CL-1 induction by phagocytosis driven by high-affinity binding is not specific to invasin, because it was also observed to be proportional to the binding affinity of other $\alpha 5\beta 1$ -integrin ligands. When used to coat beads, FN (800 nM) was a poor CL-1 inducer, whereas the 120 kDa fibronectin fragment (370 nM) (Akiyama et al., 1985) was of intermediate potency and invasin or antibodies directed against the integrin $\alpha 5$ or $\alpha 4$ chain were good inducers. Furthermore, this mechanism is dependent on the proper activation state of the integrin $\beta 1$ chain, because beads coated with a function-inhibiting anti- $\beta 1$ antibody that converts the $\beta 1$ to an inactive state (Takada and Puzon, 1993) did not induce CL-1 expression. Taken together, these results suggest that clustering of functional integrins by high-affinity ligands is required to assemble a phagocytosis-competent complex. There is a similar requirement for Fc-mediated phagocytosis, which is triggered by nonselective high-affinity ligands. Aggregation of the receptors induces the recruitment and clustering of Syk tyrosine kinase. This latter step is necessary and sufficient to induce phagocytosis and proinflammatory signaling (Greenberg et al., 1996; Hutchinson et al., 1995).

In our case, we found that beads coated with high-affinity ligands recruited AP-2, an adaptor involved in clathrin-mediated endocytosis from the plasma membrane. Because $\alpha 5\beta 1$ integrins can mediate forms of endocytosis that are dependent or independent of formation of clathrin-coated pits (Altankov and Grinnell, 1995), this result suggests that phagocytosis-promoting ligands are directing the endocytic mechanism. This result supports our notion that high-affinity integrin-binding ligands promote the formation of a distinct protein complex involved in phagocytosis. Interestingly, it has been shown that on the contact surface with the extracellular matrix, a spread cell assembles adhesion sites that differ from focal adhesions and that are characterized by coated pits and vesicles (Maupin and Pollard, 1983). These structures also form during macrophage spreading on immune complexes (Takemura et al., 1986). However, under the conditions of spreading, these complexes do not complete endocytosis/phagocytosis. Therefore, this result also implies that fulfillment of phagocytosis is a requirement for signaling.

Several of our observations support the hypothesis that signaling does not come from integrin cross-linking at the cell surface, but from the completion of the phagocytic process. This mechanism is not specific for the integrin α chain type or ligand; however, it is dependent on their phagocytosis-competent presentation. Moreover, it is not triggered by cell spreading, which can induce coated pit assembly without endocytosis. The signal could originate during the uptake of the bead or from the endosomal/lysosomal compartment. In support of this phagocytosis-dependent mechanism, we found that blocking phagocytosis specifically with RhoAN19 also blocked bead-induced signaling.

Our results are compatible with two alternative mechanisms for signaling and phagocytosis coupling. One possibility is that upon integrin clustering, RhoA could be recruited to assemble a complex that is going to generate a signal for NF κ B activation and lead to phagocytosis. A precedent for this situation has been described during the invasion of epithelial cells by *Shigella*, which induces the expression of inflammatory cytokines. The entry of *Shigella* into HeLa cells is through the binding of Ipa invasins to $\alpha 5\beta 1$ integrins and depends on RhoA function to recruit a multimolecular complex, because C3 toxin blocks FAK and paxillin phosphorylation and recruitment of F-actin, vinculin, talin and protein kinase C to the phagocytic cup (Watarai et al., 1997). Although, this study does not address how signaling to the proinflammatory cascade occurs, it suggests that RhoA might be involved in an early step of phagocytosis and signaling. Furthermore, this possible mechanism is supported by the observation that this GTPase is rapidly recruited beneath the plasma membrane, when integrins are clustered by antibody-coated beads (Burbelo et al., 1995).

Alternatively, upon particle binding, two parallel pathways may be triggered to convey signal transduction and phagocytosis, which progress independently, although both require RhoA function. There is a clear role for RhoA in phagocytosis (Chimini and Chavrier, 2000), although whether it can be generalized is not clear. C3 toxin, which has been used as a tool to inhibit the function of RhoA, also inhibits RhoB and RhoC (Wilde et al., 2000). However, different particles have been used in different assays. Particle size may induce different mechanisms for internalization (Koval et al., 1998). Bacteria may use more than one surface protein for internalization and the mechanism might be modified by the presence of virulence factors (Black and Bliska, 2000). Different roles have been assigned to the Rho GTPases family (RhoA, Rac and Cdc42) during different mechanisms of phagocytosis (Chimini and Chavrier, 2000). In professional phagocytes, RhoA is required for integrin $\alpha M\beta 2$ (complement receptor)-mediated phagocytosis, which is not associated with a proinflammatory response, whereas during Fc receptor-mediated phagocytosis, although all three GTPases are recruited, only Rac1 and Cdc42 are required for uptake of opsonized cells. In these cases the GTPases are required to recruit and activate the Arp2/3 complex, but not for activation of NF κ B (Caron and Hall, 1998; Lee et al., 2000; Massol et al., 1998; May et al., 2000), which does not exclude a possible role for RhoA in signaling. In nonprofessional phagocytic cells, the mechanisms might be different or include components of both types of phagocytosis. Several bacterial invasion mechanisms depend on activity of these GTPases, but

some clearly are dependent on RhoA function. *Shigella* invasion in epithelial cells (see above) and *Bartonella bacilliformis* invasion in endothelial cells are dependent specifically on RhoA activity and lead to a proinflammatory response (Mounier et al., 1999; Verma et al., 2000). However, in other models, ingestion of bacteria is not a requirement to induce a proinflammatory cascade: invasin-dependent adhesion of bacteria induces the expression of several inflammatory intermediaries in HeLa cells, however with a different cytokine profile and kinetics (Kampik et al., 2000).

On the other hand, RhoA function is required for several integrin-triggered signal-transduction pathways. RhoA function is required for the assembly of adhesion complexes induced by ECM (Hotchin and Hall, 1995), formation of actin stress fibers (Defilippi et al., 1997), FAK and paxillin phosphorylation in focal adhesion complexes during cell spreading (Clark et al., 1998; Flinn and Ridley, 1996) and fibronectin-induced progression through G1 (Danen et al., 2000). Our findings that RhoAN19 blocks bead phagocytosis without diminishing bead binding suggests that RhoA is indeed downstream of integrin engagement, making unlikely a role for RhoA upstream of integrin function (Wojciak-Stothard et al., 1999). This latter role of RhoA has been a source of confusion in the field, because some investigators found that C3 toxin inhibits Fc receptor clustering in macrophages and therefore particle binding (Hackam et al., 1997), whereas others did not (Caron and Hall, 1998).

Phagocytosis of invasin-coated beads in RSFs causes the activation of NF κ B, which then elicits expression of TNF- α and IL-1 α , which induces CL-1 by an autocrine mechanism. In nonprofessional phagocytes, such as fibroblasts and breast cancer cells, integrin-mediated phagocytosis is used for ingestion of different ECM components during ECM remodeling or tumor invasion (Coopman et al., 1998; Lee et al., 1996). No signal-transduction cascade has been associated with this form of phagocytosis. In the case of RSF, we showed that beads coated with high-affinity β 1-integrin ligands induce the activation of NF κ B. Within 1 hour after bead addition, some cells showed NF κ B translocation, correlating with bead uptake. Furthermore, after the first hours after bead addition, we detected the expression of NF κ B-activated genes, TNF- α and IL-1, which further supports phagocytosis-mediated activation of this transcription factor.

Both TNF- α and IL-1 α are good inducers of CL-1 expression by themselves, but our experiments with competing antibodies show that only IL-1 α is necessary for further CL-1 induction. The addition of neutralizing anti-TNF- α antibodies showed only a slight inhibition of bead-mediated CL-1 induction. This result confirms the central role of IL-1 α for CL-1 induction and suggests a secondary role for TNF- α , which could function as an amplifier of the response. The implication of a misbalance in the expression of these two cytokines and inhibitors has been shown previously for animal models of arthritis (van den Berg et al., 1999), where overexpression of IL-1 correlates with cartilage destruction. Mice genetically modified to overexpress TNF- α (Keffer et al., 1991) or IL-1 develop arthritis. Similarly, animals deficient in the expression of IL-1 RA, an endogenous inhibitor of IL-1 function, develop poly-articular arthritis spontaneously (Horai et al., 2000). The differential relevance of both cytokines for the development of arthritis has also been shown

in these models, as mice deficient in IL-1 β develop milder collagen-induced arthritis than TNF- α -deficient mice (van den Berg et al., 1999). When mice overexpressing TNF- α are injected regularly with neutralizing antibodies to IL-1 receptor (Probert et al., 1995), no arthritis develops, suggesting an essential role for IL-1, even in arthritis triggered by TNF- α . Thus, the observations made in these animal models support our mechanism, where TNF- α expression induces CL-1 indirectly, through IL-1 α as an intermediary. The analysis of IL-1 α function by immunofluorescence corroborated the role for this cytokine as both an autocrine and paracrine factor for CL-1 induction. In vivo, the paracrine mechanism could amplify the response within the synovium and extend it to the cartilage.

Finally, we propose that this model could be of relevance for the development of reactive arthritis, which in animal models can advance even in the absence of T-lymphocyte function (Heesemann et al., 1993). Thus, the invasin-mediated phagocytosis of bacteria or fragments would be sufficient as a stimuli to induce a cytokine misbalance and the production of metalloproteinases, as endocytosis of latex particles induces the expression of collagenase-1 and stromelysin-1 (Chin et al., 1985; Werb and Reynolds, 1974). The induction of these intermediates would in turn activate a cascade of tissue destruction and recruitment of inflammatory cells.

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