

## Antibodies against Listerial Protein 60 Act as an Opsonin for Phagocytosis of *Listeria monocytogenes* by Human Dendritic Cells

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**Human-monocyte-derived dendritic cells (MoDC) are very efficient in the uptake of *Listeria monocytogenes*, a gram-positive bacterium which is an important pathogen in humans and animals causing systemic infections with symptoms such as septicemia and meningitis. In this work, we analyzed the influence of blood plasma on the internalization of *L. monocytogenes* into human MoDC and compared the uptake of *L. monocytogenes* with that of *Salmonella enterica* serovar Typhimurium and *Yersinia enterocolitica*. While human plasma did not significantly influence the uptake of serovar Typhimurium and *Y. enterocolitica* by human MoDC, the efficiency of the uptake of *L. monocytogenes* by these phagocytes was strongly enhanced by human plasma. In plasma-free medium the internalization of *L. monocytogenes* was very low, whereas the addition of pooled human immunoglobulins resulted in the internalization of these bacteria to a degree comparable to the highly efficient uptake observed with human plasma. All human plasma tested contained antibodies against the 60-kDa extracellular protein of *L. monocytogenes* (p60), and anti-p60 antibodies were also found in the commercially available pooled immunoglobulins. Strikingly, in contrast to *L. monocytogenes* wild type, an *iap* deletion mutant (totally deficient in p60) showed only a minor difference in the uptake by human MoDC in the presence or the absence of human plasma. These results support the assumption that antibodies against the listerial p60 protein may play an important role in Fc-receptor-mediated uptake of *L. monocytogenes* by human MoDC via opsonization of the bacteria. This process may have a major impact in preventing systemic infection in *L. monocytogenes* in immunocompetent humans.**

Dendritic cells (DC) are the critical antigen-presenting cells involved in an immune response against microbes (35, 36). DC exist in two functional stages. Immature DC develop from hematopoietic precursors and are scattered throughout the body in nonlymphoid organs, where they exert sentinel functions. Upon irritation of the tissue DC take up and process antigens. Subsequently, they migrate into lymphoid organs, where maturation of the DC occurs (20, 27). In lymphoid organs they present the antigen epitopes in the context with major histocompatibility complex (MHC) molecules I or II. DC thus play a crucial role in antigen presentation and the initiation of most T-cell-mediated immune responses (2, 7, 29, 32, 41, 42).

There are several identified mechanisms of how antigens are captured by DC. Macropinocytosis is constitutively active in DC (39) and has been shown for DC of mouse, rat, and human origins (26). In addition, immature DC are extremely well equipped with antigen-binding receptors, including FC $\gamma$  or Fc $\epsilon$ , macrophage mannose receptor, and complement receptors (2). Compared to macropinocytosis, receptor-mediated antigen uptake is more efficient for antigen presentation (2, 43) and results in DC activation (13, 33). We have previously

demonstrated that human MoDC are highly competent in the uptake of *L. monocytogenes* (23), but the mechanism of this uptake remained unclear.

*L. monocytogenes*, gram-positive bacterium, is an important pathogen of humans and animals due to its capability for invasion of nonphagocytic cells and its replication in the cytosol of these cells (4, 9, 11, 14, 18, 40). A number of virulence determinants involved in the induced processes have been characterized. InlA and InlB, members of the growing family of listerial internalins, trigger the uptake of listeriae by normally nonphagocytic cell types (8, 15). The PrfA-dependent gene cluster (6, 25) present in all *L. monocytogenes* isolates contains the genes essential for intracellular replication and cell-to-cell spread. Of these gene products, listeriolysin, a pore-forming cytolysin, is required, along with two phospholipases (PlcA and PlcB), for the lysis of the phagosomal membranes, while ActA is involved in the active polymerization process which mediates the mobility of *L. monocytogenes* within the host cells cytosol. The protein p60, encoded by the gene termed *iap*, is a major extracellular product secreted by all isolates of *L. monocytogenes*. This protein has peptidoglycan hydrolase activity but also influences the uptake of *L. monocytogenes* by nonphagocytic cells (24). Proteins related to p60 are also found in all other *Listeria* species (5). It has been shown that p60 protein is among the strongest antigens in listeriae for B- and T-cell responses (16, 17).

We show here that the uptake of *L. monocytogenes* EGD, in

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype	Plasmid <sup>a</sup>	Resistance marker	Source or reference
<i>L. monocytogenes</i> Sv 1/2a EGD	Wild type			Institute strain collection
<i>L. monocytogenes</i> Sv 1/2a EGD	$\Delta iap$			S. Pilgrim et al.
<i>L. monocytogenes</i> Sv 1/2a EGD	Wild type	<i>prfA*</i>	Erythromycin	This work
<i>L. monocytogenes</i> Sv 1/2a EGD	$\Delta iap$	<i>prfA*</i>	Erythromycin	This work
<i>L. monocytogenes</i> DP-L2161	$\Delta hly$			21
<i>L. innocua</i> Sv6a	Wild type	<i>PactA-gfp</i>	Tetracycline	Institute strain collection
<i>Y. enterocolitica</i>	Wild type			J. Heesemann
<i>S. enterica</i> serovar Typhimurium 14028S	Wild type			28
Plasmid pHPS9		<i>prfA*</i>	Erythromycin	J. A. Vazquez-Boland

<sup>a</sup> *prfA\**, Mutant *prfA* allele from P14-A.

contrast to *Salmonella enterica* serovar Typhimurium and *Yersinia enterocolitica* by human-monocyte-derived DC (MoDC), is strongly enhanced by human plasma and that Fc-receptor-mediated uptake of antibodies against p60 protein is crucial for this process.

#### MATERIALS AND METHODS

**Bacteria.** All bacterial strains used in this study are described in Table 1. The bacteria were grown in brain heart infusion medium at 30°C (*Y. enterocolitica*) or 37°C (*L. monocytogenes* and *Salmonella* sp.) until they reached the mid-log phase of growth.

**Isolation of human MoDC from peripheral blood.** Peripheral blood mononuclear cells (PBMC) were isolated from heparinized leucocyte-enriched buffy coats of healthy adult donors by Lymphoprep (1.077 g/ml; Nycomed, Oslo, Norway) density gradient centrifugation at 400 × *g* at room temperature. PBMC were plated on tissue culture dishes (3003; Falcon Labware, Oxnard, Calif.) at a density of 5 × 10<sup>6</sup> cells/ml in RPMI 1640 medium (Gibco), supplemented with L-glutamine (2 mM), 1% autologous human plasma, and 100 U of granulocyte-macrophage colony-stimulating factor (GM-CSF) per ml for 45 min at 37°C. Nonadherent cells were washed free with warm phosphate-buffered saline (PBS), and adherent cells were cultured for 7 days without antibiotics in RPMI 1640 medium, supplemented with 1% autologous human plasma, 2 mM L-glutamine, 1,000 U of recombinant human interleukin-4 (rhIL-4); (PBH, Hanover, Germany) and 800 U of rhGM-CSF (Leukomax; Sandoz, Basel, Switzerland) per ml. Cytokines were replenished every other day.

**Phenotypic characterization of human MoDC.** Flow cytometry was used to characterize the surface marker of MoDC. Indirect immunofluorescence was performed according to standard techniques, using murine monoclonal antibodies revealed by Phycoerythrin-conjugated anti-mouse immunoglobulin (Dianova, Hamburg, Germany). The primary antibodies used were: CD1a (OKT6, Rockville, Md.),  $\alpha$ -HLA class II DR/DO (9.3F10) (American Type Culture Collection, Manassas, Va.), CD16 (anti-Fc $\gamma$ RIII; clone 3G8), CD32 (anti-Fc $\gamma$ RII, clone FL18.26, 2003; Pharmingen, Hamburg, Germany) and CD64 (anti-Fc $\gamma$ RI; clone 10.1; Pharmingen). The stained cells were analyzed on an EPICS XL-MCL (Coulter Immunotech Diagnostics, Krefeld, Germany).

**Cellular uptake assay.** On day 7 nonadherent MoDC were collected prior to infection and transferred to new 24-well plates at a density of 5 × 10<sup>5</sup> cells/ml. MoDC were infected with logarithmically growing bacteria. After two washes with PBS, the bacteria were diluted in RPMI 1640 medium and added at the desired multiplicity of infection (MOI) to each well. The cultures were incubated in RPMI 1640 medium supplemented with different blood factors, including human autologous plasma, human heterologous AB-serum of healthy donors, human serum albumin (BRK), human immunoglobulins (complement-free) (Sandoglobin; Sandoz, Basel, Switzerland), or fetal calf serum (FCS) at 37°C for 1 h. The monolayers were washed twice with PBS. For selective removal of extracellular bacteria, 50  $\mu$ g (*L. monocytogenes*) or 100  $\mu$ g (*Salmonella* and *Yersinia* spp.) of gentamicin (Gibco) per ml was added to each well, and the plates were further incubated for 30 min at 37°C. To quantify the uptake of bacteria into MoDC, infected MoDC were centrifuged on cover slides and then visualized by Giemsa staining (see below).

For determination of CFU, the cells were washed for 30 min with PBS after

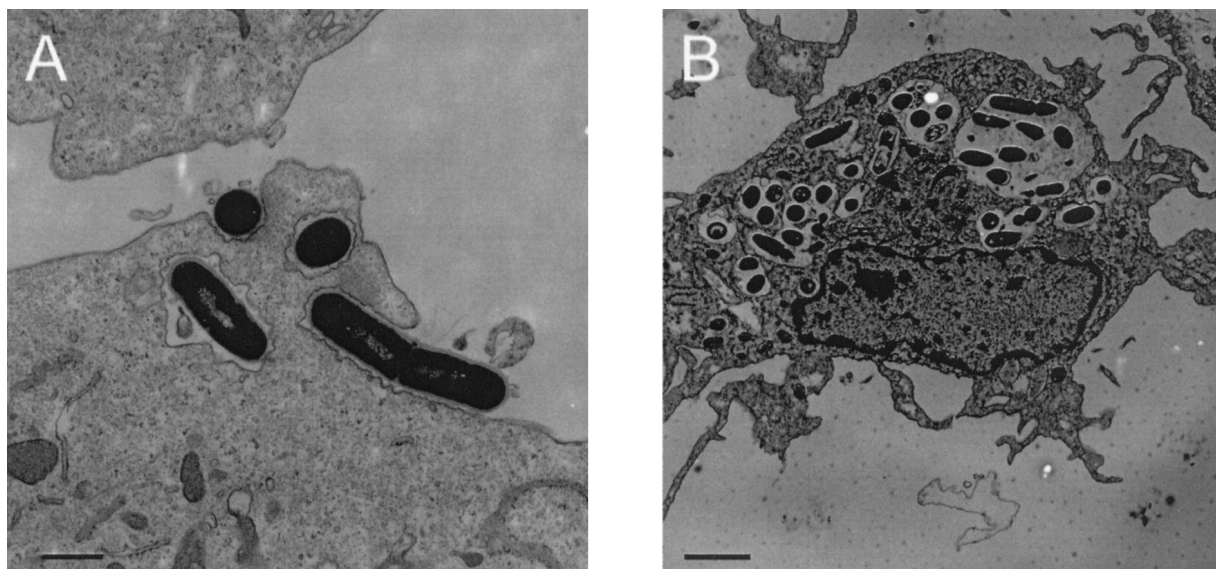


FIG. 1. (A) Human DC internalize *L. monocytogenes*. Transmission electron microscopy reveals the uptake process 1 h after incubation of DC with *L. monocytogenes* EGD in the presence of human plasma. *L. monocytogenes* are covered by thin folds of plasma membrane (MOI = 50; bar size, 1.1  $\mu$ m). (B) At 3 h postinfection, *L. monocytogenes* are located in the phagosome of human DC (MOI = 50; bar size, 2  $\mu$ m).

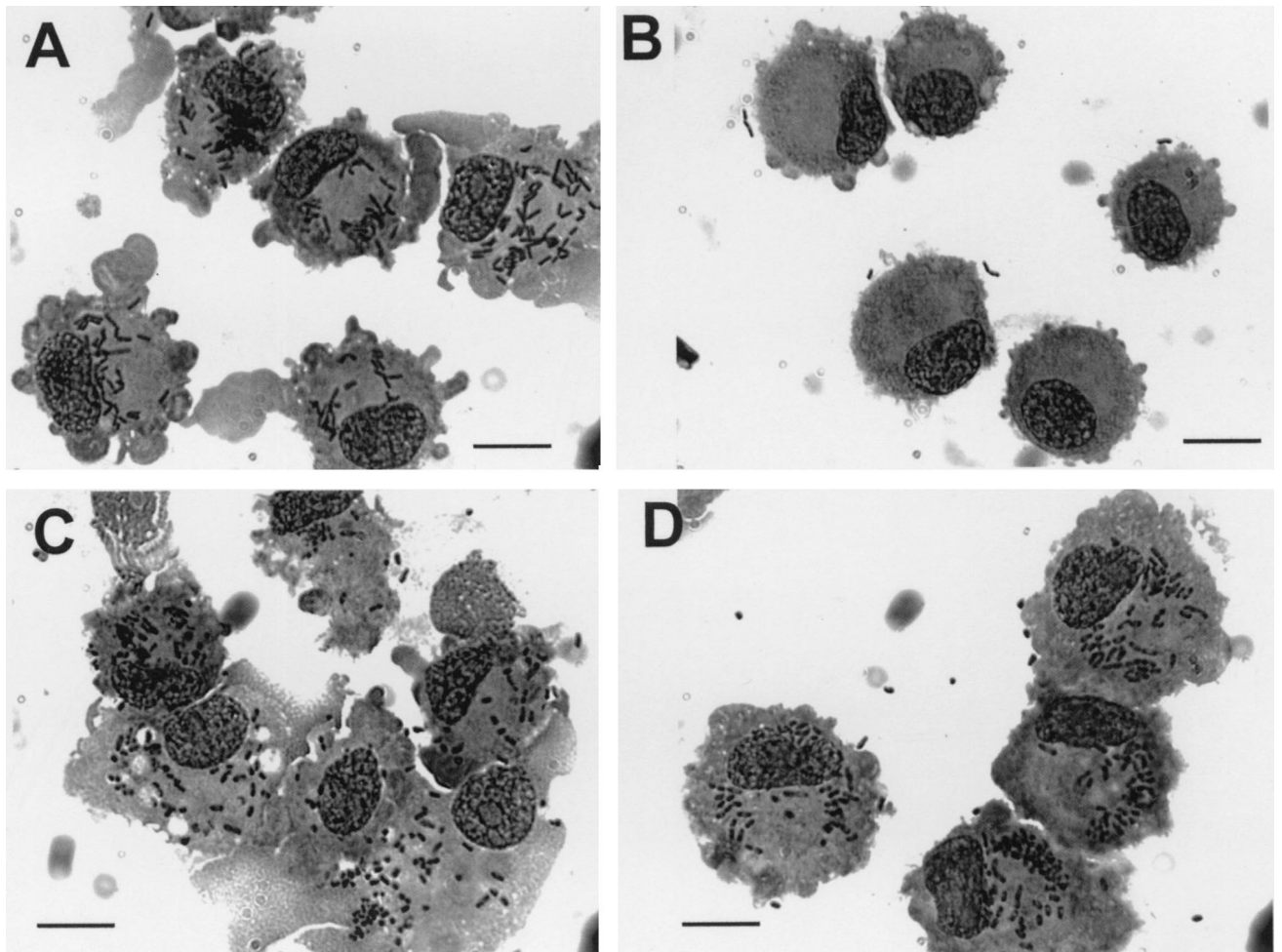


FIG. 2. Comparison of the uptake of *L. monocytogenes* EGD and *Y. enterocolitica* in DC with (A and C) and without (B and D) the presence of human plasma (MOI = 50; *L. monocytogenes* EGD, upper panel; *Y. enterocolitica*, lower panel; Giemsa staining was used 1.5 h after the addition of bacteria). Bars, 10  $\mu$ m.

the addition of gentamicin, lysed by the addition of ice-cold distilled water, and incubated for 20 min on ice.

To study the effect of human blood components on the adherence of *L. monocytogenes* to MoDC, bacteria were incubated with MoDC for 1 h in the presence of 2  $\mu$ g of cytochalasin D per ml. Cytochalasin D inhibits the bacterial uptake. After 1 h of incubation, the cells were washed six times. The number of adherent *L. monocytogenes* were determined by determining to CFU.

**Determination of bacteria in MoDC by light microscopy after Giemsa staining.** MoDC were infected with bacteria as described above. At different times (30 min, 1 h, 2 h, and 4 h), cells were washed, centrifuged on coverslips, fixed with methanol for 5 min, stained with Giemsa (1:20; Merck, Darmstadt, Germany) for 20 min, and then examined using a Leitz Dialux 20 microscope (oil immersion objective). The number of infected cells and the number of intracellular bacteria per 100 cells were counted in triplicate.

**Uptake inhibition studies.** The uptake assay was performed and analyzed as described above, except that the media contained either 5 mg of yeast mannan (Sigma), the competitor of the mannose receptor, per ml or 2  $\mu$ g of the inhibitor cytochalasin D per ml throughout the duration of the experiment.

In addition, blocking CD16 antibodies (anti-Fc $\gamma$ RIII; 3G8) were used to inhibit Fc $\gamma$ RIII-mediated phagocytosis in the presence of pooled immunoglobulins (5 mg/ml).

**Preadsorption of human immunoglobulins.** Pooled human immunoglobulins were adsorbed with  $10^{10}$  *L. monocytogenes* for 12 h at 4°C. The preadsorbed immunoglobulins were centrifuged at 6,000 rpm for 10 min at 4°C and added to the infection culture.

**Transmission electron microscopy.** MoDC were infected with *L. monocytogenes* EGD. At 1 h postinfection the cells were washed, fixed in 2.5% glutaraldehyde, postfixed in 2% osmium tetroxide, stained with 0.5% uranyl acetate, dehydrated in graded alcohols, and finally embedded in Lowicryl K4M.

**Immunoblotting.** Immunoblotting was performed to analyze *Listeria*, *Salmonella*, and *Yersinia* specific antibodies in human serum or plasma and pooled immunoglobulins. To analyze bacterium-specific antibodies, we used an overnight culture of *L. monocytogenes* EGD *prfA*\* and a p60 mutant of *L. monocytogenes prfA*\* strain, plus serovar Typhimurium (14028S) and *Y. enterocolitica*. The *Listeria* strains were characterized by high expression of the positive regulatory factor PrfA which controls the expression of most of listerial virulence factors.

Surface and supernatant proteins of all strains were prepared as described by Mollenkopf et al. (31) and then precipitated with 10% trichloroacetic acid (Roth) at 4°C for 1 h; proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being transferred to nitrocellulose (Immunoblot P Membrane; Millipore). For the isolation of surface proteins in the case of *L. monocytogenes*, the sodium dodecyl sulfate concentration was adjusted to 1%.

The nitrocellulose filters were then blocked with 2% bovine serum albumin in Tris-buffered saline (pH 7.5) and incubated with plasma or sera from healthy individuals (diluted 1:200) or the p60-specific monoclonal mouse antibody K3A7 (38) and pooled human immunoglobulins (15  $\mu$ g/ml) as controls.

Blots were developed with horseradish peroxidase-conjugated swine immunoglobulins to mouse immunoglobulins (Dako, Elstrup, Denmark) diluted 1:1,000



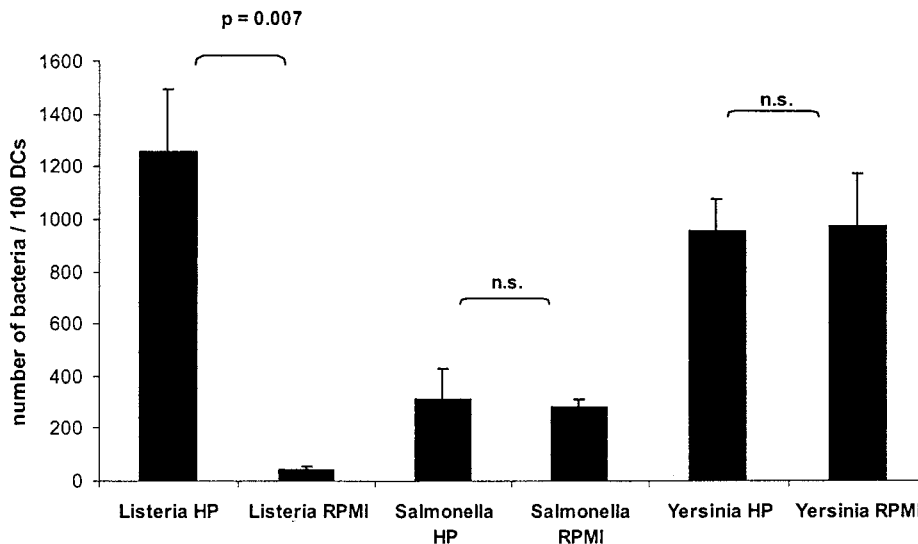


FIG. 3. Quantification of the uptake of *L. monocytogenes*, serovar Typhimurium, and *Y. enterocolitica* into DC with or without autologous human plasma by determination of the number of intracellular bacteria per 100 DC after Giemsa staining (1.5 h after the addition of bacteria; MOI = 20). Results are presented as mean values and the standard deviation (error bars) of three independent experiments. n.s., Not significant.

and horseradish peroxidase-conjugated goat anti-human immunoglobulins (IgA+IgG+IgM, H+L; Dianova, Hamburg, Germany) diluted 1:2,000. We used 4-chloro-1-naphthol (0.02% [wt/vol]) as a substrate.

**Statistical analysis.** The data are presented as the means and standard deviations (as indicated by error bars) of representative experiments run at least in triplicate. For statistical comparison, the Student *t* test and Mann-Whitney U test were performed when appropriate. *P* values of <0.01 were considered statistically significant.

## RESULTS

**Uptake of *L. monocytogenes* by human MoDC is enhanced by human plasma.** Human MoDC were cultured for 7 days in RPMI 1640 medium supplemented with 1% human plasma, rhGM-CSF, and rhIL-4. The cells exhibited the characteristic MoDC morphology and the pattern of surface markers typical of immature MoDC (3, 37). Flow cytometry analysis of cell surface markers showed that more than 80% of these cells expressed CD1a and MHC class II molecules. The 20% of the cells which did not express MoDC markers were predominantly T lymphocytes. Infection experiments with *L. monocytogenes* and purified human T cells showed that T lymphocytes were not infected with these bacteria (data not shown).

As shown previously, immature MoDC were capable of internalizing *L. monocytogenes* with high efficiency (23). Electron microscopy showed that most of the intracellular bacteria were located in phago(lyso)somal vacuoles (Fig. 1B). Only a few bacteria escaped into the cytosol. However, our earlier study did not address the mechanism of the uptake of *L. monocytogenes* by MoDC.

First, using transmission electron microscopy we found (Fig. 1A) that the entry of *L. monocytogenes* into these MoDC occurs by gliding of the plasma membrane over the bacterium. At the position where the bacterial cell is in contact with the host cell, the intimately apposed membrane was undulated. Major morphological changes of the MoDC surface such as membrane ruffles were not observed. Light microscopic visu-

alization of the intracellular bacteria by Giemsa staining showed that *L. monocytogenes* was very efficiently internalized by MoDC in the presence of 1% human plasma (Fig. 2A). At an MOI of 10 to 50, ca. 80% of the host cells were infected by one or more bacteria. Since the percentage of immature MoDC in the cell population is also about 80%, the data indicate that practically every immature MoDC has taken up *L. monocytogenes*. Like the *L. monocytogenes* EGD wild-type strain, the nonpathogenic species *L. innocua* and an *inlA-inlB* deletion mutant of *L. monocytogenes* (23), as well as heat- or gentamicin-killed *L. monocytogenes*, were effectively internalized in the presence of human plasma. No intracellular bacteria were found when MoDC were incubated at 4°C (data not shown).

In contrast to the highly efficient uptake of *L. monocytogenes* by MoDC in the presence of human plasma, infection carried out in RPMI 1640 alone or in RPMI 1640 supplemented with 10% FCS resulted in a strongly (20-fold) decreased internalization (Fig. 2B and 4). To test whether this difference in uptake was overcome by a longer incubation time, *L. monocytogenes* were incubated with MoDC for 15 min, 30 min, 60 min, 2 h, and 4 h. The difference in the efficiencies of listerial uptake in the presence or absence of human serum remained, however, the same (data not shown).

In contrast to the increased listerial uptake by MoDC in the presence of human plasma, adherence studies showed no effect of human plasma on the adherence of *L. monocytogenes* to MoDC (data not shown).

**Uptake of serovar Typhimurium and *Y. enterocolitica* by MoDC is not enhanced by human plasma.** To test whether the strong enhancement of listerial uptake in the presence of human plasma is specific for *Listeria* sp. or may also be observed for other bacteria, we performed experiments similar to those described above for serovar Typhimurium and *Y. enterocolitica*. As shown in Fig. 2 and 3, the uptake of both serovar Typhi-

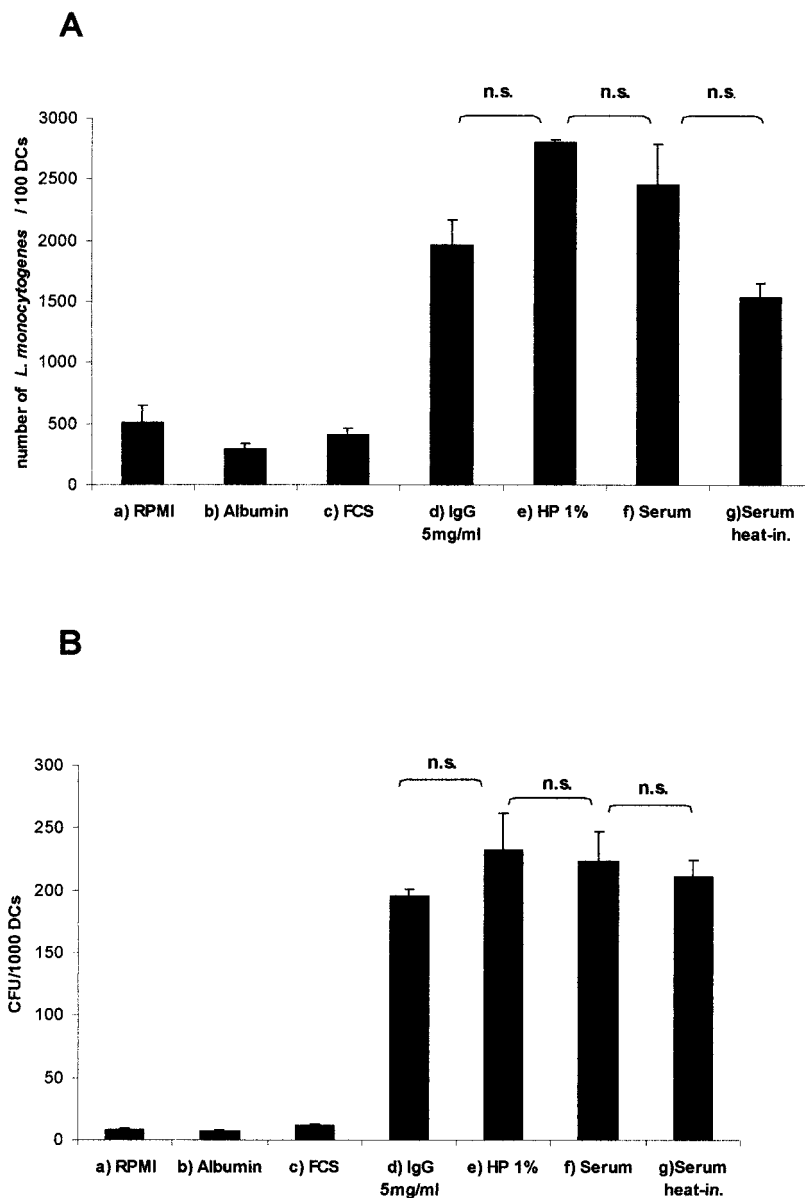


FIG. 4. Quantification of the uptake of *L. monocytogenes* into DC 1.5 h after the addition of bacteria with different infection media (*L. monocytogenes* EGD; MOI = 50). (A) Giemsa-staining. Determination of the number of intracellular bacteria was made per 100 DC. (B) Viable bacteria were determined by counting the CFU per 1,000 lysed DC. Columns: a, RPMI 1640; b, albumin (5%); c, FCS (10%); d, pooled human immunoglobulins (5 mg/ml); e, autologous human plasma (HP; 1%); f, heterologous AB-serum (5%); g, heat-inactivated (20 min, 56°C) heterologous AB-serum 5%. The results are presented as mean values with standard deviation (error bars) of three independent experiments. n.s., Not significant.

murium or *Y. enterocolitica* by MoDC was independent of human plasma. Interestingly, the uptake of serovar Typhimurium and, in particular, *Y. enterocolitica* by MoDC in RPMI 1640 medium in the absence of human plasma was much higher than that of *L. monocytogenes* under the same conditions.

**The enhancement in the listerial uptake by human plasma is due to opsonization of *Listeria* sp. by immunoglobulins.** We next examined which of the major factors present in human plasma may be responsible for the enhancement of listerial uptake. Supplementation of RPMI 1640

medium by serum albumin did not increase the low number of internalized *L. monocytogenes* obtained after incubation of MoDC with *L. monocytogenes* in RPMI 1640 alone. No difference in the uptake efficiency was observed between human serum and plasma, excluding an active role of fibrinogen in the internalization of *L. monocytogenes*. Similarly, heat inactivation of human serum had no significant influence on the uptake rate of *L. monocytogenes*, excluding a critical role of complement factors (Fig. 4). The large discrepancy between the number of internalized bacteria per DC, as determined by Giemsa staining assay (Fig. 4A) and plating of viable bacteria (Fig. 4B),

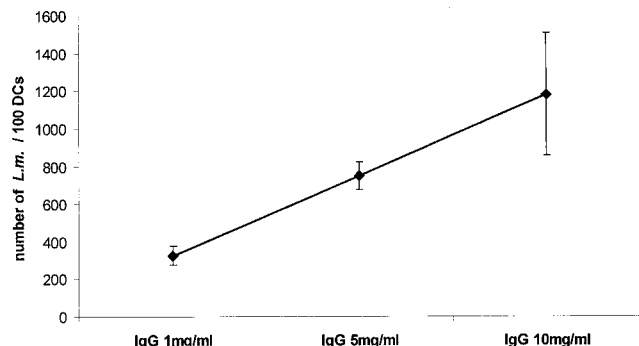


FIG. 5. Internalized *L. monocytogenes* EGD carrying out an infection with different concentrations of pooled human immunoglobulins IgG (*L. monocytogenes*, MOI = 20, 1.5 h after the addition of bacteria, light microscopy after Giemsa staining). The results are presented as the mean values and the standard deviation (error bars) of three independent experiments.

appears to be due to the efficient phagosomal killing of internalized listeriae.

Internalization of *L. monocytogenes* by MoDC, assayed in the presence or absence of yeast mannan, an established competitive inhibitor of the mannose-fucose receptor, also did not affect the efficiency of uptake, excluding a role for the mannose receptor in the internalization of *L. monocytogenes*.

Pretreatment of MoDC with cytochalasin D, which inhibits actin filament polymerization, inhibited uptake of *L. monocytogenes* by MoDC almost completely, indicating that the internalization process requires intact actin microfilament polymerization. The listerial uptake rate into human MoDC in the presence of human plasma without cytochalasin D was about 12 *L. monocytogenes* per MoDC compared to 0.5 listeria per MoDC after treatment with cytochalasin D (MOI = 20).

Kaplan (22) provided evidence that actin microfilaments are essential for phagocytosis via the Fc receptor but are much less important for phagocytosis via the C3 receptor. We therefore analyzed the influence of immunoglobulins in the internaliza-

tion of *L. monocytogenes* by human MoDC. Increasing concentrations (1, 5, and 10 mg/ml) of pooled human immunoglobulins were added to the RPMI 1640 medium, and internalization of *L. monocytogenes* was again measured by counting Giemsa-stained intracellular bacteria and viable bacterial cells. The results (Fig. 5) showed a dose-dependent increase of internalized bacteria. Interestingly, high immunoglobulin concentrations (10 mg/ml) resulted in a drop in the number of viable internalized bacteria (data not shown). These data strongly suggest that opsonization of *L. monocytogenes* with immunoglobulins is responsible for the enhanced uptake of *L. monocytogenes* by DC; high opsonization of the bacteria may then result in a more efficient elimination of viable bacteria through MoDC.

**CD16 monoclonal antibody reduces phagocytosis of *L. monocytogenes* by MoDC.** Our data suggest that the uptake of *L. monocytogenes* is Fc receptor mediated. Human immature MoDC express low amounts of CD16, CD32, and CD64 (data not shown). As shown in Fig. 6, the addition of blocking anti-CD16 antibodies reduces the uptake of *L. monocytogenes* significantly. These data support the idea that the uptake of *L. monocytogenes* is Fc $\gamma$ RIII receptor mediated.

**Antibodies against the listerial p60 protein act as a major opsonin in stimulating the uptake of *L. monocytogenes* by MoDC.** Western blot analysis showed that all used human sera and plasma samples of healthy individuals, as well as the commercially available pooled human immunoglobulins (only tested with *Listeria* sp.), contained antibodies directed against the three tested bacteria (Fig. 7). However, only *Listeria*-specific antibodies seemed to be critical for the uptake of *L. monocytogenes*, while the uptake of serovar Typhimurium and *Y. enterocolitica* by MoDC was apparently independent of the specific antibodies. The Western blots obtained by testing extracellular and cell-associated proteins of *L. monocytogenes* with the used human sera showed the p60 protein to be the major immune-reactive component with all serum and plasma samples (Fig. 7A). Human immunoglobulins preadsorbed against *L. monocytogenes* EGD and added (5 mg/ml) to the

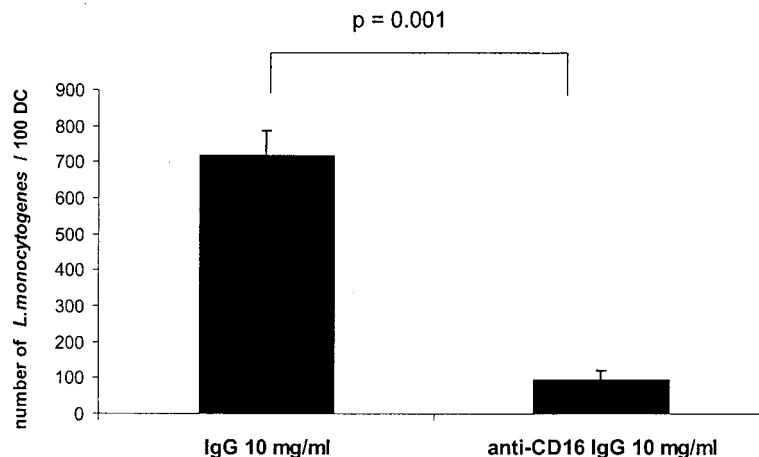


FIG. 6. Determination of internalized *L. monocytogenes* in human MoDC in the presence of pooled IgG (10 mg/ml) with or without blocking anti-CD16 antibodies (MOI = 20, 1.5 h after the addition of bacteria, light microscopy after Giemsa staining). The results are presented as the mean values and the standard deviation (error bars) of three independent experiments.

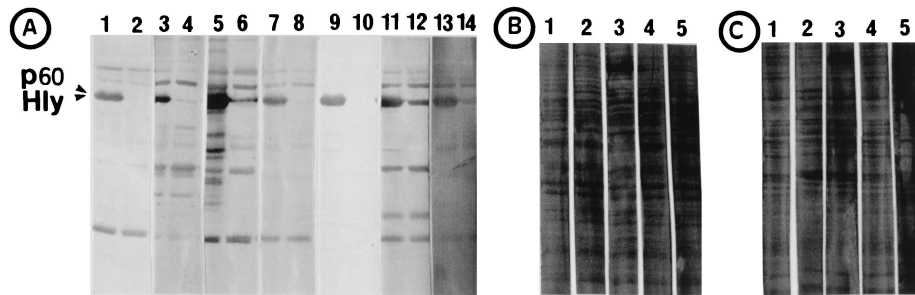


FIG. 7. Immunoblot analysis of plasma or sera from five healthy individuals by using supernatant and surface proteins of wild-type *L. monocytogenes* (A; odd-numbered lanes) or p60 mutant *L. monocytogenes* (A; even-numbered lanes), *Y. enterocolitica* 14028S (B; lane 1 to 5), and serovar Typhimurium (C; lanes 1 to 5) as antigens. *L. monocytogenes* EGD *prfA*\* and a p60 mutant of *L. monocytogenes prfA*\* strain are characterized by high expression of the positive regulatory factor PrfA, which controls the expression of most of listerial virulence factors. (A) Lanes 1 and 2 were treated with plasma 1; lanes 3 and 4 were treated with plasma 2, lanes 5 and 6 were treated with plasma 3, lanes 7 and 8 were treated with plasma 4, lanes 9 and 10 were treated with p60-specific monoclonal mouse antibody K3A7 (Rowan 2000) as a control, lanes 11 and 12 were treated with serum, and lanes 13 and 14 were treated with pooled human immunoglobulins (Sandoz). (B and C) In each lane, supernatant and surface proteins isolated from  $10^9$  cells were applied. The positions of p60 and listeriolysin (Hly) are indicated on the right side. Lane 1, plasma 1; lane 2, plasma 2; lane 3, plasma 3; lane 4, plasma 4; lane 5, serum.

RPMI 1640 medium resulted in a significantly ( $P = 0.002$ ) decreased uptake of *L. monocytogenes* compared to RPMI 1640 medium supplemented with nonadsorbed immunoglobulins (Fig. 8). Strong support for the assumption that p60 antibodies act as a major opsonin for the uptake of *L. monocytogenes* was obtained by using a recently constructed *iap* deletion mutant of *L. monocytogenes* (S. Pilgrim et al., unpublished data) which does not produce p60 at all (Fig. 7A). The addition of *iap*-preadsorbed immunoglobulins revealed a significant difference in the listerial uptake rate compared to *L. monocytogenes* wild-type-preadsorbed immunoglobulins (Fig. 8). In addition, internalization of this *iap* deletion mutant by MoDC showed only minor differences (only a 2-fold increase compared to a 20-fold increase using the *L. monocytogenes* wild type) in uptake efficiency when it was determined in RPMI 1640 medium with or without human plasma. In contrast, an *hly* deletion mutant of *L. monocytogenes* which is unable to produce listeriolysin still showed a very significant difference (10-fold) in the uptake when these two media were used in the

uptake assay (Fig. 9). Figure 9 also shows that the internalization of the *iap* mutant in the absence of human plasma was higher than the internalization of wild-type bacteria. We observed that under the same bacterial culture conditions of *L. monocytogenes* and its *iap* deletion mutant, the *iap* mutant culture contained more dead bacteria. The uptake of dead listeriae was comparable to that of living listeriae (data not shown). In our assay, both dead and living bacteria were counted as internalized by light microscopy after Giemsa staining.

## DISCUSSION

Immature human MoDC are highly efficient in taking up *L. monocytogenes* (23). Uptake by these important antigen-presenting cells occurs by normal phagocytosis since an *inlAB* deletion mutant, killed listeriae, and the noninvasive species *L. innocua* are internalized with an efficiency similar to that of virulent *L. monocytogenes*. The entry of *L. monocytogenes* into

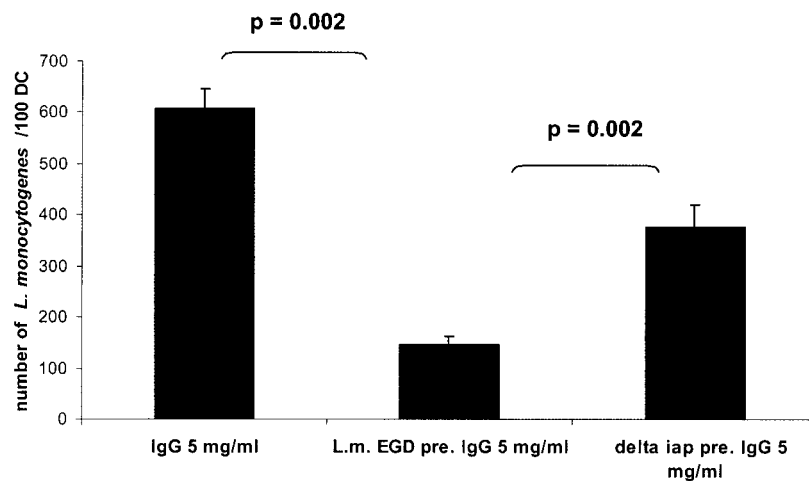


FIG. 8. Comparison of the uptake of *L. monocytogenes* into DC with IgG (5 mg/ml), *L. monocytogenes*-preadsorbed IgG (5 mg/ml), and  $\Delta iap$ -preadsorbed IgG (5 mg/ml) (MOI = 20; 1.5 h after the addition of bacteria, light microscopy after Giemsa staining).

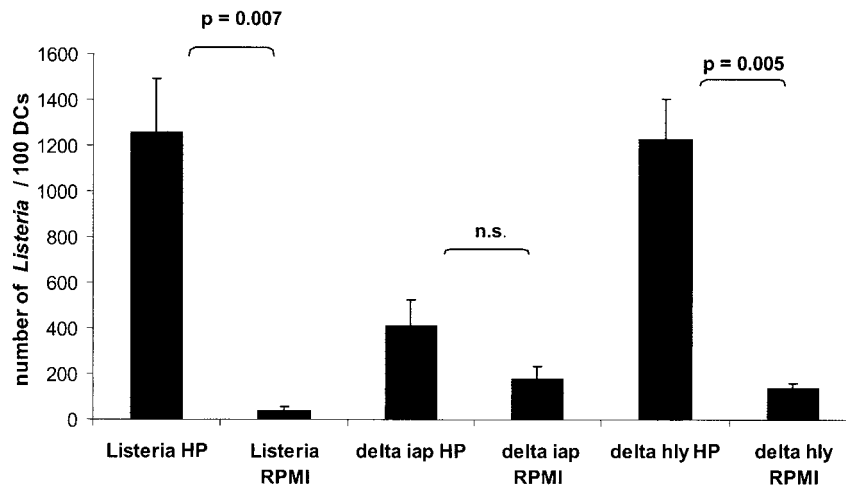


FIG. 9. Quantification of the uptake of *L. monocytogenes*  $\Delta iap$  and  $\Delta hly$  into DC with or without autologous human plasma by determination of the number of intracellular bacteria per 100 DC after Giemsa staining (1.5 h after the addition of bacteria; MOI = 20). The results are presented as the mean values and the standard deviation (error bars) of three independent experiments. n.s., Not significant.

MoDC, when analyzed by electron microscopy, shows that *L. monocytogenes* cells are engulfed by thin membrane extensions rising from the MoDC membrane enclosing the pathogen tightly, a phagocytosis process resembling a “zipper-like” mechanism (30) without visible morphological changes of the host cell surface.

We have found that the uptake of *L. monocytogenes* by MoDC is strongly dependent on the presence of human plasma which cannot be replaced by FCS or serum albumin. Internalization of *L. monocytogenes* in plasma-free medium is very inefficient. In contrast, the uptake of serovar Typhimurium and *Y. enterocolitica* by MoDC is independent of human plasma, suggesting a major difference in the uptake mechanism of listeriae compared to the two gram-negative bacteria by MoDC.

It has been suggested previously (39) that the mannose receptor present on DC may play a role in the internalization of foreign particles. However, the addition of soluble mannan did not inhibit the uptake of *L. monocytogenes*. Murine macrophages bind complement-opsonized *L. monocytogenes* via the complement C3b and C1q receptors (1, 10). DC carry also C3b receptors (2), and C3b generated through activation of the alternative complement pathway by *Listeria* cell wall fragments may act as an opsonin (19). However, it seems unlikely that complement receptors are essential for the uptake of listeriae by MoDC since heat inactivation of human serum did not significantly reduce phagocytosis. These results thus point to an immunoglobulin-induced uptake via Fc receptors. Indeed, uptake medium supplemented with pooled immunoglobulins resulted in an uptake rate which was comparable to that observed in the presence of human plasma, thus showing that immunoglobulins may be crucial as opsonins in an Fc-mediated uptake of *L. monocytogenes* by MoDC. DC express several receptors that bind to the Fc portion of immunoglobulins, mediating internalization of the formed antigen-immunoglobulin G (IgG)-complexes (12, 33). Immature MoDC express the Fc $\gamma$  receptors RI (CD64), RII (CD32), and RIII (CD16). An Fc $\gamma$ -mediated process was proven by an inhibition experiment

with CD16 antibodies. Binding of antibody-opsonized *L. monocytogenes* to Fc receptors on MoDC may also be responsible for the efficient maturation of MoDC and the upregulation of MHC class II molecules in these MoDC (23) by signaling via the gamma chain of the Fc receptor, which leads to DC activation and antigen presentation by MHC class II molecules (13, 33). Obviously, the uptake of serovar Typhimurium and *Y. enterocolitica* is not enhanced in the presence of human plasma, although antibodies reacting with surface components of these pathogens were also detected in the human plasma. These results suggest that the uptake of these gram-negative bacteria by MoDC may be different from that of *L. monocytogenes*.

Western blot analysis showed the presence of antibodies against the 60-kDa extracellular protein of *L. monocytogenes* (p60) in all human serum and plasma samples tested and in the commercially available pooled human immunoglobulins. Low titers of antilisteriolysin antibodies and other *Listeria*-specific antibodies were also detected in some human plasma. These antibodies may play a minor role as opsonins for Fc-mediated phagocytosis of *L. monocytogenes* by MoDC, as suggested by the slightly reduced internalization of the *hly* deletion mutant in the presence of human plasma. The important role of p60 antibodies for the uptake of *L. monocytogenes* into human MoDC could be shown by the use of an *iap* deletion mutant. This *iap* mutant is a complete null mutation of p60. It was reported that the *iap* gene encoding p60 is essential for bacterial viability (44). Possibly, our *iap* mutant strain expresses other enzymes, which may compensate for the lack of p60. Nevertheless, a significant number of nonviable cells are present in a culture of the *iap* mutant (Pilgrim et al., unpublished). However, preadsorption of the immunoglobulins with *L. monocytogenes* and, in particular, the use of the *iap* mutant, which does not produce any p60, indicated that anti-p60 antibodies act as the predominant listerial opsonin in the Fc-mediated phagocytosis. p60-like proteins containing the same prominent B-cell epitopes carried by *L. monocytogenes* p60 are produced by all *Listeria* species (5). The p60-specific antibodies



found in the plasma of most immune-competent humans seem to be derived from *L. innocua* and possibly other environmental nonpathogenic *Listeria* species to which humans are most likely permanently exposed and not necessarily from exposure to virulent *L. monocytogenes* (16). These cross-reacting serum p60 antibodies may, however, opsonize virulent *L. monocytogenes* and enhance the uptake of these pathogens by human MoDC, which are very active in killing *L. monocytogenes* (23), and thus this mechanism could provide an important barrier against the spreading of virulent *L. monocytogenes* into the system via the blood stream and may thus contribute to the rather low frequency of human infections by this intracellular pathogen.

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