Phagosomes Induced by Cytokines Function as anti-*Listeria* Vaccines

**NOVEL ROLE FOR FUNCTIONAL COMPARTMENTALIZATION OF STAT-1 PROTEIN AND CATHEPSIN-D**

Eugenio Carrasco-Marín, Estela Rodríguez-Del Río, Elisabet Frande-Cabanes, Raquel Tobes, Eduardo Pareja, M. Jesús Lecea-Cuello, Marta Ruiz-Sáez, Fidel Madrazo-Toca, Christoph Hölscher, and Carmen Alvarez-Dominguez

From the Grupo de Genómica, Proteómica de Infecciones Bacterianas e Inflamación, Fundación Marqués de Valdecilla-IFIMAV and Hospital Santa Cruz de Liencres, 39120-Santander, Cantabria, Spain, the Bioinformatics Unit, Era7 Information Technologies SL, BIC Granada CEEI, Parque Tecnológico de Ciencias de la Salud-Armilla, 18100-Granada, Spain, and the Research Center Borstel, University of Lubeck, D-23845 Borstel, Germany

**Background:** The effectiveness of phagosomes as vaccines is unknown.

**Results:** Listericidal phagosomes contain a compartmentalized signaling pathway and a nontoxic listeriolysin form bound to immune molecules. As vaccines they activate effector T cells and recruit immune cells.

**Conclusion:** Protection with listericidal phagosomes requires recruitment of dendritic cells and T cell regulation.

**Significance:** Phagosomes are effective immunotherapies and are a new generation of vaccine tools.

Phagosomes are critical compartments for innate immunity. However, their role in the protection against murine listeriosis has not been examined. We describe here that listericidal phago-receptosomes are induced by the function of IFN-γ and IL-6 as centralized compartments for innate and adaptive immunity because they are able to confer protection against murine listeriosis. These phago-receptosomes elicited LLO(91–99)/CD8+ and LLO(189–201)/CD4+-specific immune responses and recruited mature dendritic cells to the vaccination sites controlled by T cells. Moreover, they present exceptional features as efficient vaccine vectors. First, they compartmentalize a novel listericidal STAT-1-mediated signaling pathway that confines multiple innate immune components to the same environment. Second, they show features of MHC class II antigen-loading competent compartments for cathepsin-D-mediated LLO processing. Third, murine cathepsin-D deficiencies fail to develop protective immunity after vaccination with listericidal phago-receptosomes induced by IFN-γ or IL-6. Therefore, it appears that the connection of STAT-1 and cathepsin-D in a single compartment is relevant for protection against listeriosis.

Macrophages (MØ) are critical cells for the innate and adaptive immune responses against *Listeria monocytogenes* (1–3). The phagosomal compartments in MØ regulate all of these immune processes by undergoing a profound transformation to mediate efficient listericidal functions, high levels of oxidative burst and lysosomal proteases, and increased antigen processing capacity (4–6). Several pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-6 enhance the microbialicidal mechanisms of MØs and restrict the intracellular growth of *L. monocytogenes* (7). It is unclear whether the microbialicidal signaling of these cytokines is connected with phagosomal trafficking or with protection against infections. Two main listericidal mechanisms the oxidative and nonoxidative pathways operate within the phagosomal compartments. However, degradation of *L. monocytogenes* requires the action of nonoxidative mechanisms (8–11) that are mediated by lysosomal proteins as cathepsin-D (CTSD). In this regard, CTSD participates in innate immunity and inactivates the main phagosomal *L. monocytogenes* cytolsin, listeriolysin O (LLO) (12–14). CTSD-mediated degradation of the immunodominant antigen LLO occurs through a unique cleavage site between 491/W/492 residues. This site also contains the phagosomal binding domain (15). Therefore, a connection might exist between listericidal components and *L. monocytogenes* immunity within the phagosomes. Here, we examine the hypothesis that a common listericidal route induced by pro-inflammatory cytokines may be compartmentalized in unique vesicles connecting STAT-1-mediated signaling, trafficking regulators, listericidal lysosomal enzymes such as CTSD, and immune phagosomal functions.

*This work was supported in part by Spanish Fondo de Investigaciones Sanitarias Grants 00/3073, PI01/3128, PI03-1009, PI07-0289, and PI10-0660, Fundación Marqués de Valdecilla-IFIMAV Grant API2011/PI10-0660 (to E.C.-M.), Spanish Ministerio de Ciencia, Investigación e Innovación Grants BIO2002-0628, SAF2006-08968, and SAF2009-08695, Fondo de Investigaciones Sanitarias Grant PI04-0324, and Fundación Marqués de Valdecilla-IFIMAV Grant API2010/03/SAF2009-08695 (to C. A.-D.). C. A.-D. dedicates this study to P. Stahl (Washington University, St. Louis) for introducing our group to the phagosomes field and to P. Saftig (Biochemisches Institute Albrechts-Ludwig-Universität of Kiel, Germany) for advising us with lysosomal proteins and providing personal encouragement and generously providing mice.

‡1 This article contains supplemental Figs. S1–S6, Tables S1–S4, and “Experimental Procedures.”

† To whom correspondence should be addressed. Tel.: 34-942-203584; Fax: 34-942-203847; E-mail: calvarez@hum.es.
We also examined the possibility that the compartmentalization of functions within phagosomes might be useful to confer protection against listeriosis. Our approach involved the use of differential gene expression methods combined with basic proteomic, functional analyses of L. monocytogenes phagosomes, and their use as vaccine vectors against listeriosis. All these studies were verified using MOs genetically deficient in putative upstream components of this signaling route such as STAT-1 and STAT-3 and the postulated downstream lysosomal component CTSD. Finally, we also evaluated the efficiency of phagosomes as vaccine vectors in wild type and experimental CTSDlow-deficient mice and explored the contribution of T cells in the potency of these vaccines using SCID mice.

In this study, we describe a novel phagosomal compartment, the listericidal phago-receptorsomes induced by IFN-γ or IL-6, which may be important Listeria-induced immune vesicles that regulate IL-6 production and constitute effective vaccines to confer protection against listeriosis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cytokine Treatment**—The following MO-like cell line was used throughout the study, J-774 cells cultured in DMEM, 10% FCS, 1 mM glutamine, 1 mM nonessential amino acids, 50 µg/ml gentamycin, 30 µg/ml vancomycin. Bone marrow-derived macrophages (BM-DM) were obtained as follows: from femurs of 8–12-week-old female CBA/J, C57BL/6, SCID, or 129/Sv mice (Taconic Farms, Denmark) (for CTSDlow experiments, Ctsdlow mice were previously reported, 15 or for SCID experiments); from 3-week-old CTSDlow and wild type littermate mice CTS+/- (for CTSD+/- and wild type littermates, mice (Taconic Farms, Denmark); or from femurs of 6–10-week-old Stat1−/− and wild type littermate mice (Taconic Farms, Denmark); or from femurs of 6–10-week-old macrophage/neutrophil-specific STAT3−/- (here called STAT3−/-) and wild type littermate mouse (named STAT3+/-). These mice were obtained by inter-crossing of FLOXSTAT3−/− mice from S. Akira with the macrophage/neutrophil-specific LysMcre mice and wild type littermate mice from L. Forster at Borstel animal facilities (Research Center Borstel, University of Lubeck, Borstel, Germany). Bone marrow-derived cells were cultured in DMEM, 20% FCS, 1 mM glutamine, 1 mM nonessential amino acids, 25 ng/ml M-CSF, 50 µg/ml gentamycin, 30 µg/ml vancomycin (D20) in bacteriological dishes for 7 days to differentiate into MO (BM-DM). Murine recombinant IFN-γ, TNF-α, IL-6, IL-10, or IL-12 cytokines were obtained from Sigma. Cells were treated 72 h with 10–20 ng/ml with the different murine cytokines before infection kinetics or phagosome isolation.

**Bacteria**—L. monocytogenes 10403S strain) was obtained from D. A. Portnoy (University of California, Berkeley), and GFP-Listeria monocytogenes variants of the L. monocytogenes strain DH-L1039 (GFP-L. monocytogenes) was kindly provided by D. E. Higgins (Harvard Medical School, Boston).

**Kinetic Infection Assays**—MO-like cell lines or BM-DM were cultured into 96-well plates at 1 × 10⁶ cells/ml in the presence or absence of the above-mentioned cytokines 72 h before infection. Cells were infected with L. monocytogenes at a ratio of 10:1 (bacteria/cell) as reported previously for different times (0, 4, 8, or 16 h). CFU ratios were performed as reported and represented the ratio of CFU at 8 h to CFU at 0 h ± S.D. of triplicates (4). Comparative kinetic infection assays were performed in J-774 cells and BM-DM from CBA/J cells pretreated or not with TNF-α, IL-6, or IFN-γ as reported previously (4, 10, 15).

**Measurements of H₂O₂ and Nitrite Production**—J-774 cells (2 × 10⁶ cells/ml) were cultured in microtiter plates. Cells were pretreated or not with cytokines for 72 h and next infected for 1 h with 2 × 10⁶ CFU/ml of L. monocytogenes. H₂O₂ and NO₂ production was measured as described previously (10). In brief, the H₂O₂ production was measured by the HRP-dependent conversion of phenol red by H₂O₂ into a compound with increased absorbance at 600 nm using a H₂O₂ standard curve. Results are expressed as nanomoles of H₂O₂ produced per cell. The nitrite production was determined with the Griess reagent using a sodium nitrite standard curve. Results were expressed as nanomoles of NO per cell. Samples were performed in triplicate, and results are the mean ± three independent experiments.

**Phagosome Isolation**—J-774 cells or BM-DM cells were cultured at 1 × 10⁶ cells in the presence or absence of cytokines 72 h before infection. Cells were infected with L. monocytogenes at a ratio of 10:1 (bacteria/cell) for 15 min at 37 °C, followed by a 45-min incubation in the presence of 20 µg/ml gentamycin followed by 4 h in D5 medium. Noninfected samples (NI) were cultured at same cell density and treated with the above cytokines. Basal level control corresponded with nontreated and noninfected samples (NT and NI samples). Later on, the total RNA was extracted from cells using the Qiagen kit (RNasey total RNA isolation kit, Qiagen, product number 74104). The amount of total RNA extracted per sample varied between 25 and 30 µg. RNA integrity was analyzed in 1% agarose gels (28 S and 18 S ribosomal RNA forms were observed as nondegraded in a 2:1 proportion). RNA quality was estimated by a close to 2.0 value of the ratio A₂₆₀/A₂₈₀ nm, and concentration was calculated with the assumption that 1 OD corresponds with 40 µg of RNA measured at A₂₆₀ nm. Differential microarrays were performed with the Affymetrix GeneChip MOE430A2.0 that evaluated 22626 mouse genes with GCOS 1.3 Affymetrix® software (Progenika S. A., Spain). The fold changes of gene expression values are expressed as the signal log ratio that corresponds to the log₂ fold change (FC) in a previous version of Affymetrix software. Therefore, signal log ratio values of ≥0.3 were induced genes as they corresponded to values ≥1.2 FC, and ≤−0.3 were depressed genes as they corresponded with values ≤−1.2 FC. All our final values were subtracted from the values of basal controls (NT and NI values). Other controls include L. monocytogenes infected versus NT and NI values and are shown in supplemental Table S1. Gene ontology information was derived from Progenika S.A. (Affymetrix NetAffx Analysis Center).
Listericidal Phagosomes Are Effective Vaccines

Recombinant Proteins and LLO Peptides—GST-PBDPAK (kindly provided by G. Bockoch, UCLA), the p21-activated kinase-derived binding domain for activated RAC2 protein, and the His-EEA1\textsubscript{218} (a gift from D. Lambricht, Texas University), the FYVE domain of the EEA1 protein containing the binding domain for RAB5A-GTP, were expressed in Escherichia coli BL-21 strain. Recombinant proteins were induced with 5 mM isopropyl-β-D-thiogalactoside for 5 h at 37 °C and purified with glutathione-Sepharose or TALON resins, respectively, according to instructions provided by the manufacturer (Clontech). LLO\textsubscript{1–99} and LLO\textsubscript{189–201} were synthesized by F. Roncal (Centro Nacional de Biotecnologia, Consejo Superior de Investigaciones Científicas, Madrid, Spain) with a purity higher than 95% after HPLC and mass spectrometry.

Westerns, Immunoprecipitations, and Overlay Assays—30 µg of isolated phagosomes were loaded per lane onto SDS-polyacrylamide gels. Gels were transferred onto nitrocellulose membranes. Primary antibodies were incubated overnight at 4 °C as follows: mouse anti-Stat1, mouse anti-inducible NOS (Pharmingen), rabbit anti-STAT1-YPhos, rabbit anti-STAT3-SPhos, rabbit anti-STAT3-YPhos (Vitro), rabbit polyclonal anti-LLO specific antibody (Diatheva), mouse anti-PLY5 monoclonal antibody that recognize LLO undecapeptide sequence (15), 4F11 (mouse monoclonal anti-Rab5a), rabbit anti-ASMase (a gift from O. Utermöhlen, Köln University, Köln, Germany), and rabbit anti-cathepsin-D and rabbit anti-Rac2 (kindly provided by G. Bockoch, UCLA). Thereafter, secondary antibodies were HRP-conjugated (The Jackson Laboratory) or CTSD (kindly provided by G. Bockoch, UCLA), the p21-activated kinase-derived binding domain for activated RAC2 protein, and the His-EEA1\textsubscript{218} (a gift from D. Lambright, Texas University). Immune complexes were detected with 100 ng/ml of His-EEA1\textsubscript{218} diluted into binding buffer. Immunoprecipitates were run onto SDS-PAGE and transferred to NC membranes. Primary antibodies were incubated overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibody. As internal control in all Western blots, we included the marker, RAB5C, and the expression level was not modified by the cytokine treatment (4). Western blots were developed by ECL.

Vaccination Protocols—Ctsd\textsuperscript{+/−} mice were treated (Ctsd\textsuperscript{low}) or not (Ctsd\textsuperscript{+/−}) (n = 5) for 3 days with 0.5 mg/ml pepstatin-A previous to vaccination and during the vaccination protocol as described previously (15). L. monocytogenes phagosomes containing 500 CFU/~30 µg of phagosomal proteins were obtained from BM-DM of Ctsd\textsuperscript{+/−} or CBA/J mice, previously treated for 72 h with 10 ng/ml mouse recombinant IFN-γ (P-IFN), IL-6 (P-IL-6), or left untreated (P-NT). These phagosomes were inoculated in the peritoneal cavity (i.p.) of Ctsd\textsuperscript{+/+} or Ctsd\textsuperscript{low} (n = 5) mice for 7 or 14 days or were nonvaccinated (NV). Next, all mice were inoculated with 10³ CFU of L. monocytogenes intraperitoneally for 3 additional days. The vaccination timing follows similar protocols previously reported for studies using L. monocytogenes as vaccine (16). Mice were bled before sacrifice and serum-stored at ~80 °C to measure cytokines by FACS analysis. Spleens and livers were homogenized, and CFUs were counted in homogenates. Similar protocols were performed in CBA/J, SCID, or 129/Sv mice using P-NT, P-IFN, or P-IL-6 phagosomes obtained from BM-DM from the bone marrow cells of these mouse strains and vaccinations each 7 days (SCID and 129/Sv mice) or 14 days (CBA/J mice).

FACS Analysis of BM-DM, Peritoneal Cells, and Cytokine Measurements—Peritoneal exudate cells (PEC) were obtained after Hank’s wash of the peritoneal cavity and cell surface-labeled with antibodies against the following markers: CD11b (marker for MØs), CD11c (marker for DC), Ly6C (marker for DCi), Ly6G (also known as Gr-1, marker for PMNs), Dx5 (marker for NK cells), CD3 and CD8 (markers for cytotoxic T cells) (Te), IA\textsuperscript{κ} (MHC-II for CBA/J mice), IA\textsuperscript{λ} (MHC-II for SCID and 129/Sv mice), or IA\textsuperscript{β} (MHC-II for Ctsdlow, CTSD\textsuperscript{+/−}, or CTSD\textsuperscript{low} mice) and analyzed by FACS.

We also used FACS analysis for cell surface labeling of BM-DM (100,000 cells) treated or not for 72 h with 10 ng/ml IFN-γ as activating stimulus using monoclonal antibodies FITC or phycoerythrin-labeled (BD Biosciences) against the above-mentioned cell surface markers. The analysis of BM-DM oxidative burst using L. monocytogenes as stimulus (10 min, 37 °C) and using the Phagoburst® kit (Orphgen Pharma, Heidelberg, Germany) was also performed by FACS analysis. TNF-α and IL-6 production by BM-DM infected with wild type L. monocytogenes was also measured in culture supernatants by FACS analysis as well as in sera from different mice (CBA kit from BD Biosciences). BM-DM were incubated in microtitre plates at a density of 2 × 10⁶ cells/ml with medium alone or with 2 × 10⁷ CFU/ml of L. monocytogenes for 1 h without antibiotics, followed by 24 h of incubation in D5 complete medium. Cells were centrifuged, and half of the supernatants volume was harvested and stored at ~80 °C until FACS analysis. Samples were performed in triplicate, and the results are the means ± S.D. of two separate experiments.

FACS Analysis of Spleens to Measure IFN-γ Intracellularly—We used FACS analysis for cell surface labeling of spleen cells (100,000 cells), using monoclonal antibodies FITC- or antigen-presenting cells labeled (BD Biosciences) against CD4 or CD8. Peptides used were LLO(1–99) or LLO(189–201). For in vitro culture, spleen cells were plated into 96-well round-bottom plates (5 × 10⁶ cells/ml) and stimulated with each of the LLO(91–99) or LLO(189–201) peptides independently (10⁻⁵ M each peptide) for 5 h in the presence of brefeldin A (intracellular cytokine staining) as described previously (16). Stimulated cells were surface-stained for CD4 and CD8 and then fixed and permeabilized using a cytofix/cytoperm kit (BD Biosciences). Cells were stained for IFN-γ with antibody anti-IFN-γ phycoeryth-
rin-labeled. Samples were acquired using a FACSCanto flow cytometer (BD Biosciences). Data were gated to include exclusively CD4<sup>+</sup>/H<sub>11001</sub> or CD8<sup>+</sup>/H<sub>11001</sub> events, and the percentages of these cells expressing IFN-γ/H<sub>9253</sub> were determined according to the manufacturer’s recommendations (BD Biosciences). Results of LLO peptide-stimulated splenocytes were corrected according to the percentages of total CD4<sup>+</sup>/H<sub>11001</sub> and CD8<sup>+</sup>/H<sub>11001</sub> cells, respectively. Data were analyzed using FlowJo software (Treestar, Ashland, OR).

**Statistical Analysis**—For statistical analysis, the Student’s t test was applied.

**Ethics Statement**—This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Spanish Ministry of Science, Research and Innovation. The Committee on the Ethics of Animal Experiments of the University of Cantabria approved this protocol (Permit Number 2009/12) that follows the Spanish legislation (RD 1201/2005). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

**RESULTS**

IFN-γ and IL-6 Trigger Similar Listericidal Mechanisms in MØ—J-774 MØ-like cells display basal levels of MØ-associated microbicidal abilities and can be transformed into highly bacterial cells upon cytokine treatment (4, 11, 17, 18). Therefore, we evaluated the activation of these cells when exposed to pro-inflammatory cytokines that are involved in *L. monocytogenes* immune responses (i.e. TNF-α, IFN-γ, IL-6, IL-10, and IL-12). IFN-γ, TNF-α, and IL-6 induced killing of intracellular *L. monocytogenes* in J-774 cells as observed by the reduced replication ability of *L. monocytogenes*, although IL-10 and IL-12 had no effect (Fig. 1A). We also confirmed that these cytokines promoted *L. monocytogenes* killing comparing the infection kinetics of J-774 cells and real MØ such as BM-DM. These cytokines clearly enhanced the microbicidal abilities of J-774 cells treated with IFN-γ or IL-6 for 8 h as observed by the reduced replication ability of *L. monocytogenes* (Fig. 1A). We also confirmed that these cytokines enhanced the microbicidal abilities of J-774 cells treated with IFN-γ or IL-6 for 8 h as observed by the reduced replication ability of *L. monocytogenes* (Fig. 1A).
Listericidal Phagosomes Are Effective Vaccines

BM-DM and also increased the listericial abilities of J-774 cells (supplemental Fig. S1, A and B). Therefore, J-774 cells and BM-DM were comparative models to study the listericial mechanisms induced by pro-inflammatory cytokines. Oxidative bactericidal mechanisms were examined by measuring hydrogen peroxide (H₂O₂) and nitric oxide (NO) production, both of which decrease intracellular L. monocytogenes viability. Only IFN-γ and IL-6 induced the production of both oxidative compounds in J-774 cells (Fig. 1B). We next determined the relevance of these cytokines at the phagosomal level by examining intraphagosomal L. monocytogenes viability as a measurement of L. monocytogenes degradation as reported previously (4, 13, 15). Only IFN-γ and IL-6 treatment of J-774 cells decreased bacterial viability, indicating their importance for L. monocytogenes degradation within the phagosomes (Fig. 1C, white bars). Lysosomal hydrolases are responsible for the non-oxidative listericial mechanisms that degrade L. monocytogenes within the phagosomal compartment (13–15). Chloroquine is a general inhibitor of lysosomal hydrolases that increase the phagosomal pH and block the transport of lysosomal proteases to L. monocytogenes phagosomes (19). Treatment of IFN-γ or IL-6-treated J-774 cells with chloroquine increased L. monocytogenes intraphagosomal viability to control levels, demonstrating that cytokine-induced L. monocytogenes degradation within the phagosomes required the activity of lysosomal hydrolases (Fig. 1C, black bars). Similar results were obtained using a cathespin-D inhibitor, pepstatin A (data in Fig. 1C, legend), further supporting the participation of CTSD in L. monocytogenes degradation (15). These results suggest that IFN-γ and IL-6 induce similar oxidative and nonoxidative listericial mechanisms in J-774 cells and that TNF-α does not mediate phagosomal L. monocytogenes degradation controlled by lysosomal proteases (Fig. 1C).

Listeria-specific Transcriptional Response Is Induced by IFN-γ or IL-6—To identify and select genes induced synergically by IFN-γ or IL-6, we performed a detailed transcriptional analysis. Therefore, we analyzed the differential expression of genes included on the Affymetrix GeneChip MOE430A2.0 (~14,000 mouse genes) in J-774 cells using three strategies (Fig. 2A). These three strategies were performed to differentiate the genes specifically induced or repressed by IL-6 or IFN-γ in activated J-774 cells in response to L. monocytogenes infection (strategy 1, IFN + LM or IL-6 + LM in Fig. 2A) from those genes induced or repressed by L. monocytogenes infection itself (strategy 2 in Fig. 2A). Strategy 3 was developed based on the observation that the IFN-γ-associated transcriptional signal was greater in J-774 cells than IL-6 associated signal (Fig. 2B, DE numbers). The data from five independent experiments examining differential gene expression (Fig. 2A) were analyzed using two different approaches. In the first approach detailed in the supplemental material, we analyzed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional term enrichment in the sets of differentially expressed genes without any aprioristic assumptions (supplemental Tables S1 and S2). In the second approach, we performed a hypothesis-driven analysis focused on genes specific for phagosomal destruction (cluster I) and those involved in L. monocytogenes immunity (cluster II) to determine the response localized within the phagosomes because they may function as destructive immune vesicles (20). Cluster I was focused on genes reported to encode phagosomal trafficking regulators and lysosomal proteins involved in phagocytic particles destruction (4, 12, 19, 21, 22). Cluster II was focused on genes reported to encode proteins important in the anti-L. monocytogenes innate immune response, mainly type I and II IFN-regulated genes (23). The housekeeping genes used as a reference for basal transcriptional expression were cytosolic β-actin and the pyruvate carboxylase. The results of all strategies analyzed indicated that, in the intracellular trafficking subfamily of the cluster I genes, the gene encoding RAB5A was induced, and IFN-γ induction of RAB5A was slightly higher than IL-6-induced expression (Fig. 2C, 1.55-fold change (FC) versus 0.98 FC, respectively). LAMP-2, the regulatory A subunit of the H⁺-ATPase (Atp6), and LIMP-2 were the lysosomal component-associated genes also induced by both cytokines in most strategies (Fig. 2C and Table 1). Genes belonging to the type I IFN pathway in the cluster II gene set, such as the secreted factors C3F6 and IL-6, were induced in all strategies. However, genes belonging to the type II IFN pathway in the cluster II gene set that are involved in L. monocytogenes-specific innate immunity such as the JAK-STAT cytosolic component, STAT-1 (5, 24, 25), appeared to be L. monocytogenes-specific; they were only induced by treatment with either cytokine plus L. monocytogenes. Because IFN-γ induced a stronger signal than IL-6 in J-774 cells (Fig. 2B), several genes from both clusters were only induced by IFN-γ. Examples of IFN-γ-induced genes are detailed in supplemental Table S2. We also observed no induction of the classical STAT-1-mediated genes after L. monocytogenes infection such as those encoding the MHC II IAb or Iaα chains or the transactivator CIITA. These results were not surprising given that strong signals such as IFN-γ or IL-6 would induce fewer additional genes with an L. monocytogenes infection. Moreover, these findings suggest that IFN-γ- or IL-6 induced genes share a single signaling pathway.

Phagosomal Components of the IFN-γ or IL-6 Listericidal Route—We confirmed the transcriptional results using a basic proteomic and functional analysis of isolated L. monocytogenes phagosomes from J-774 cells treated or not with IFN-γ or IL-6. Most of the induced genes, such as Rab5a and Rab5a-GTP forms, LAMP-2 and LIMP-2, belonged to cluster I and were also observed at high protein levels in L. monocytogenes phagosomes induced by IFN-γ or IL-6 (Fig. 3A). However, we also observed high RAC2-GTP and CTSD protein levels, although the corresponding genes were not induced or even repressed according to the differential expression analysis. Similarly, we observed high protein levels of P67bphox and inducible NOS in IFN-γ- or IL-6-induced phagosomes (Fig. 3B), both of which belonged to the phagosomally related type II IFN subfamily of genes, despite a lack of detectable induction of gene expression (Fig. 2C). Therefore, not all proteins involved in microbe inactivation need to be induced at the gene level to demonstrate increased protein levels in IFN-γ- or IL-6-induced L. monocytogenes phagosomes. The most surprising findings corresponded to the expression of the JAK-STAT-associated cytosolic specific genes belonging to the type II IFN subfamily, which are important for the anti-L. monocytogenes innate immune response.
response. We observed high protein levels of JAK1 and JAK2 and high total protein levels of STAT-1 (Stat1 lane in Fig. 3B) and active forms (P-Stat lane in Fig. 3B) in both IFN-γ/H9253- and IL-6/H9253-induced L. monocytogenes phagosomes. This is the first evidence demonstrating that the cytosolic components of the JAK-STAT signaling route associated with IFN-γ/H9253 and IL-6/IL-6 receptors can be found in phagosomes. The STAT-1-signaling route is connected to the induction of MHC II expression and transport to antigen-loading compartments (MIIC) (23). MIIC vesicles contain MHC II molecules loaded with peptides (26), and SDS-stable MHC II dimers provide a valid measurement of peptide-loaded MHC II molecules (27–29). We found that the phagosomes (P-IFN, P-IL-6, and P-NT lanes in Fig. 3C) demonstrated significant levels of SDS-stable MHC II dimers, especially the IFN-γ-induced L. monocytogenes phagosomes. However, endosomes only expressed low levels of SDS-
stable αβ MHC II dimers (Endo lane in Fig. 3C). These results suggest that phagosomes are MIIC-competent, and endosomes are not. However, higher or lower levels of SDS-stable αβ MHC II dimers are not necessarily a measurement of the antigen processing ability. Therefore, using LLO, the most common protein yields of CDA or CDA T cells are expressed as means ± S.D. (p < 0.05).

| Vaccine type | Percentages of cells in PEC after vaccination |
|--------------|---------------------------------------------|
|              | CD11c+LC (CD11c) | CD11c+DC (CD11c) | CD11c-DC (MHC) | NK | PMA |
| NY           | 7 ± 0.01         | 3 ± 0.01         | 75 ± 0.05      | 10 ± 0.04 | 70 ± 0.03 |
| P-NT         | 35 ± 0.02        | 35 ± 0.03        | 10 ± 0.03      | 10 ± 0.02 | 30 ± 0.02 |
| P-IFN        | 47 ± 0.08        | 83 ± 0.08        | 50 ± 0.02      | 10 ± 0.01 | 10 ± 0.07 |
| P-IL-6       | 46 ± 0.07        | 79 ± 0.05        | 50 ± 0.01      | 7.4 ± 0.01 | 7.4 ± 0.01 |
| BM-DM-IFN    | 47 ± 0.07        | 71 ± 0.05        | 50 ± 0.01      | 7.4 ± 0.01 | 7.4 ± 0.01 |
| BM-DM-IL-6   | 35 ± 0.05        | 71 ± 0.08        | 50 ± 0.01      | 7.4 ± 0.01 | 7.4 ± 0.01 |
| BM-DM-IL-6MN | 33 ± 0.04        | 65 ± 0.07        | 50 ± 0.01      | 7.4 ± 0.01 | 7.4 ± 0.01 |

| Vaccine type | Percentages of cells in PEC after vaccination |
|--------------|---------------------------------------------|
|              | CD14+T cells | CD8+T cells | CD10+T cells | CD14+T cells | CD10+T cells |
| NY           | 150 ± 15     | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  |
| P-NT         | 120 ± 0.01   | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  |
| P-IFN        | 120 ± 0.01   | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  |
| P-IL-6       | 120 ± 0.01   | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  |
| BM-DM-IFN    | 120 ± 0.01   | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  |
| BM-DM-IL-6   | 120 ± 0.01   | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  | 120 ± 0.01  |

* CBA/J mice were vaccinated i.p. or i.n (NV) with L. monocytogenes phagosomes (500 CFU/30 μg) (P-NT, P-IFN, or P-IL-6) or L. monocytogenes-infected BM-DM (10⁶ cells) (BM-DM/L. monocytogenes, BM-DM-IFN/L. monocytogenes, or BM-DM-IL-6/L. monocytogenes) for 14 days (n = 5/vaccination type) and challenged i.p. with 10⁵ CFU/mice of L. monocytogenes for 3 days.

** Phagocytes from vaccinated mice were LLO peptide-stimulated, and percent of total CDA or CDA T cells are expressed as means ± S.D. (p < 0.05).

| Vaccine type | Percentages of cells in PEC after vaccination |
|--------------|---------------------------------------------|
|              | CD11c+LC (CD11c) | CD11c+DC (CD11c) | CD11c-DC (MHC) | NK |
| NY           | 7 ± 0.01         | 3 ± 0.01         | 75 ± 0.05      | 10 ± 0.04 |
| P-NT         | 35 ± 0.02        | 35 ± 0.03        | 10 ± 0.03      | 10 ± 0.02 |
| P-IFN        | 47 ± 0.08        | 83 ± 0.08        | 50 ± 0.02      | 10 ± 0.01 |
| P-IL-6       | 46 ± 0.07        | 79 ± 0.05        | 50 ± 0.01      | 7.4 ± 0.01 |
| BM-DM-IFN    | 47 ± 0.07        | 71 ± 0.05        | 50 ± 0.01      | 7.4 ± 0.01 |
| BM-DM-IL-6   | 35 ± 0.05        | 71 ± 0.08        | 50 ± 0.01      | 7.4 ± 0.01 |
| BM-DM-IL-6MN | 33 ± 0.04        | 65 ± 0.07        | 50 ± 0.01      | 7.4 ± 0.01 |

** CBA/J mice were vaccinated i.p. or i.n (NV) with L. monocytogenes phagosomes (500 CFU/30 μg) (P-NT, P-IFN, or P-IL-6) or L. monocytogenes-infected BM-DM (10⁶ cells) (BM-DM/L. monocytogenes, BM-DM-IFN/L. monocytogenes, or BM-DM-IL-6/L. monocytogenes) for 14 days (n = 5/vaccination type) and challenged i.p. with 10⁵ CFU/mice of L. monocytogenes for 3 days.

* CBA/J mice were vaccinated i.p. or i.n (NV) with L. monocytogenes phagosomes (500 CFU/30 μg) (P-NT, P-IFN, or P-IL-6) or L. monocytogenes-infected BM-DM (10⁶ cells) (BM-DM/L. monocytogenes, BM-DM-IFN/L. monocytogenes, or BM-DM-IL-6/L. monocytogenes) for 14 days (n = 5/vaccination type) and challenged i.p. with 10⁵ CFU/mice of L. monocytogenes for 3 days.

* CBA/J mice were vaccinated i.p. or i.n (NV) with L. monocytogenes phagosomes (500 CFU/30 μg) (P-NT, P-IFN, or P-IL-6) or L. monocytogenes-infected BM-DM (10⁶ cells) (BM-DM/L. monocytogenes, BM-DM-IFN/L. monocytogenes, or BM-DM-IL-6/L. monocytogenes) for 14 days (n = 5/vaccination type) and challenged i.p. with 10⁵ CFU/mice of L. monocytogenes for 3 days.

* CBA/J mice were vaccinated i.p. or i.n (NV) with L. monocytogenes phagosomes (500 CFU/30 μg) (P-NT, P-IFN, or P-IL-6) or L. monocytogenes-infected BM-DM (10⁶ cells) (BM-DM/L. monocytogenes, BM-DM-IFN/L. monocytogenes, or BM-DM-IL-6/L. monocytogenes) for 14 days (n = 5/vaccination type) and challenged i.p. with 10⁵ CFU/mice of L. monocytogenes for 3 days.

* CBA/J mice were vaccinated i.p. or i.n (NV) with L. monocytogenes phagosomes (500 CFU/30 μg) (P-NT, P-IFN, or P-IL-6) or L. monocytogenes-infected BM-DM (10⁶ cells) (BM-DM/L. monocytogenes, BM-DM-IFN/L. monocytogenes, or BM-DM-IL-6/L. monocytogenes) for 14 days (n = 5/vaccination type) and challenged i.p. with 10⁵ CFU/mice of L. monocytogenes for 3 days.

* CBA/J mice were vaccinated i.p. or i.n (NV) with L. monocytogenes phagosomes (500 CFU/30 μg) (P-NT, P-IFN, or P-IL-6) or L. monocytogenes-infected BM-DM (10⁶ cells) (BM-DM/L. monocytogenes, BM-DM-IFN/L. monocytogenes, or BM-DM-IL-6/L. monocytogenes) for 14 days (n = 5/vaccination type) and challenged i.p. with 10⁵ CFU/mice of L. monocytogenes for 3 days.
from vaccinated mice examines the IFN-γ intracellularly of CD4+ or CD8+ T cells specific for the immunodominant LLO(91–99) or LLO(189–201) peptides and corrected for total CD4+ or CD8+, respectively (Table 1). The procedure is detailed in supplemental Fig. S4. Phagosomes from J-774 cells cannot be used for these studies because there is no available and genetically compatible murine model for in vivo experiments with these cells. The protection achieved after vaccination with IFN-γ or IL-6 L. monocytogenes phagosomes was in the same range of 85–95% than the protection obtained with L. monocytogenes-infected BM-DM pretreated or not with IFN-γ or IL-6 (data in legend of Fig. 3E). The protection achieved with L. monocytogenes phagosomes from nontreated BM-DM (P-NT) was lower in a range of 56%. These results indicated that P-IFN and P-IL-6 phagosomes were as good vaccine vectors as L. monocytogenes-infected BM-DM. Moreover, all vaccines showed good stability and integrity because lysis of phagosomal membranes previous to vaccination abolished the protection achieved with each vaccine type (supplemental Table S4). When we examined LLO-specific immunity elicited by these vaccina-
tion types, we observed higher percentages of spleen LLO-specific and IFN-γ producers LLO(189–201)/CD4+ and LLO(91–99)/CD8+ T cells after vaccination with P-IFN and P-IL-6 phagosomes than with P-NT phagosomes (Table 1, rows b and c). These values were even higher than the percentages of LLO-specific and IFN-γ producers LLO(189–201)/CD4+ and LLO(91–99)/CD8+ observed with BM-DM treated or not with IFN-γ or IL-6 and infected with L. monocytogenes (Table 1, BM-DM/LM, BM-DM-IFN/LM, or BM-DM-IL-6/LM rows). Therefore, the protection obtained with P-IFN or P-IL-6 against L. monocytogenes, correlates with LLO-specific immunity.

Analysis of Efficiency of the Vaccines Using Listericidal IFN-γ or IL-6 Phago-receptosomes—Recruitment of DC to the vaccination sites is related to vaccine efficiency (7, 30). Therefore, we explore the phenotypes of the PEC recovered after vaccination because we inoculated our vaccines intraperitoneally. We observed that P-NT recruited low amounts of mature and immature DC (DCm and DCi) in a range of 35% (Table 1, row d). In supplemental Fig. S5, we detailed the protocol followed to stain the double-positive CD11c+I-Ak+ (DCm) or CD11c+Ly6C+ (DCi) cells of PEC after vaccination with P-IFN. P-IFN and P-IL-6 recruited higher amounts of DCi in a range of 46–47% but interestingly very high amounts of DCm in a range of 79–83%. However, all vaccine types recruited MO, NK, or PMN to the vaccination sites at a low range of 1–10%. These results indicate that our vaccination protocol elicts a secondary effector immune response and not a primary response as L. monocytogenes alone induces, recruiting MO, NK, or PMN at high ranges of 10–75% (NV data in row d of Table 1).

Vaccination efficiency is also related to the production of several pro-inflammatory cytokines such as TNF-α (1, 3, 7, 25, 31) and down-regulation of IL-6 production (25). Therefore, we analyze the cytokine pattern after the vaccination of mice with the above-mentioned vaccine types. All vaccination types induced significant amounts of TNF-α. P-IFN or P-IL-6 showed low levels of IL-6 after 7 days of vaccination and 3 days with a second challenge of L. monocytogenes (10⁶ CFU/mice) (row e of Table 1). Therefore, the high protection obtained after P-IFN or P-IL-6 vaccination correlated to high peritoneal recruitment of matured dendritic cells (DCm).

Vaccine efficiency has also been related to the ability of DC to expand the immune response stimulating different T cells (30). To explore the role of T cells in the efficiency of L. monocytogenes phagosomal vaccines, we used a widely reported lymphocyte-deficient model, such as the SCID mice that show a normal innate immune response against L. monocytogenes (1). Nonvaccinated SCID and control 129/Sv mice showed similar numbers of bacteria in spleens (Fig. 3F, NV bars). However, SCID- and 129/Sv-vaccinated mice with L. monocytogenes phagosomal vaccines, P-NT, P-IFN, or P-IL-6 for 7 days, showed significant differences in protection (supplemental Fig. S6 and P-IFN and SCID bars in Fig. 3F). In fact, 129/Sv control mice showed 200% protection after vaccination with effective vaccines (Fig. 3F and supplemental Fig. S6) and SCID mice no protection but a 100-fold increase in L. monocytogenes numbers compared with NV mice (Fig. 3F). Moreover, the lack of protection of P-IFN vaccines in SCID mice correlated to low recruitment of mature and immature DC to the vaccination sites in ranges of 20% compared with 66% in 129/Sv control mice (Pie Chart in Fig. 3F). We conclude that T cells controlled the recruitment of DC cells to vaccination sites and seemed to improve the vaccine efficiency.

STAT-1 Participates Exclusively Upstream in the IFN-γ or IL-6 Listericidal Route—Next, we tempted to decipher the sequential action of the components of this listericidal IFN-γ or IL-6 signaling route. We envision upstream and downstream participants of this pathway. STAT-1 or STAT-3 could be participants of this pathway. STAT-1 or STAT-3 could be participants of this pathway. STAT-1 participates exclusively upstream in the IFN-γ or IL-6 listericidal route. BM-DM (1 × 10⁶ cells) from STAT-1−/−, STAT-1+/−, STAT3−/−, or STAT3+/− mice were treated or not with IFN-γ or IL-6 and infected with L. monocytogenes. A phagosomes were solubilized before CFU analysis. Results were expressed as CFU values × 10 ± S.D. of triplicates as under “Experimental Procedures.” Replication indices were expressed as CFU ± S.D. as follows: 10 × 10⁵ ± 120 for STAT-1−/−, 40 × 10⁵ ± 140 for STAT-1+/−, 11 × 10⁵ ± 112 for STAT3−/−, and 10 × 10⁵ ± 102 for STAT3+/− in nontreated cells; 3 × 10⁵ ± 12 for STAT1−/−, 43 × 10⁵ ± 10³ for STAT1+/−, 4 × 10⁴ ± 9 for STAT3−/−, and 3.5 × 10⁴ ± 13 for STAT3+/− in IFN-γ-treated cells; 4.1 × 10⁴ ± 11 for STAT1−/−, 45 × 10⁴ ± 112 for STAT1+/−, 3.2 × 10⁴ ± 12 for STAT3−/−, and 3.6 × 10⁴ ± 11 for STAT3+/− in IL-6-treated cells (p < 0.05). B, detection of RAB5A and Ctsd in L. monocytogenes phagosomes from to STAT-1−/−, STAT-1+/−, STAT3−/−, or STAT3+/− BM-DM, LLO binding to MHC-class II was detected as in Fig. 3D (IP: MoaMHC-II, WB: RhoLLO lane). Rab5c levels were used as controls.

FIGURE 4. STAT-1 participates exclusively upstream in the IFN-γ or IL-6 listericidal route. BM-DM (1 × 10⁶ cells) from STAT-1−/−, STAT-1+/−, STAT3−/−, or STAT3+/− mice were treated or not with IFN-γ or IL-6 and infected with L. monocytogenes. A, phagosomes were solubilized before CFU analysis. Results were expressed as CFU values × 10 ± S.D. of triplicates as under “Experimental Procedures.” Replication indices were expressed as CFU ± S.D. as follows: 10 × 10⁵ ± 120 for STAT-1−/−, 40 × 10⁵ ± 140 for STAT-1+/−, 11 × 10⁵ ± 112 for STAT3−/−, and 10 × 10⁵ ± 102 for STAT3+/− in nontreated cells; 3 × 10⁵ ± 12 for STAT1−/−, 43 × 10⁵ ± 10³ for STAT1+/−, 4 × 10⁴ ± 9 for STAT3−/−, and 3.5 × 10⁴ ± 13 for STAT3+/− in IFN-γ-treated cells; 4.1 × 10⁴ ± 11 for STAT1−/−, 45 × 10⁴ ± 112 for STAT1+/−, 3.2 × 10⁴ ± 12 for STAT3−/−, and 3.6 × 10⁴ ± 11 for STAT3+/− in IL-6-treated cells (p < 0.05). 8, detection of RAB5A and Ctsd in L. monocytogenes phagosomes from to STAT-1−/−, STAT-1+/−, STAT3−/−, or STAT3+/− BM-DM, LLO binding to MHC-class II was detected as in Fig. 3D (IP: MoaMHC-II, WB: RhoLLO lane). Rab5c levels were used as controls.
CTSD Participates Downstream in the IFN-γ or IL-6 Listericidal Route—Next, we examined if CTSD was the downstream component of the IFN-γ or IL-6 listericidal route using CTSD−/− BM-DM. *L. monocytogenes* phagosomes in CTSD−/− BM-DM displayed a 4-fold increase in *L. monocytogenes* intraphagosomal viability compared with Ctsd+/+ phagosomes (Fig. 5A, black bars). IFN-γ or IL-6 failed to induce any listericidal activity in the CTSD−/− phagosomes in contrast to CTSD+/+ BM-DM (Fig. 5A, gray and white bars). IFN-γ or IL-6 increased Rab5a levels in *L. monocytogenes* phagosomes; however, *L. monocytogenes* phagosomes from CTSD−/− BM-DM expressed only very low levels of Rab5A, and this was not modulated by IFN-γ or IL-6 treatment (Western blot in Fig. 5A). This confirms that CTSD and Rab5A are linked in this cytokine pathway. We verified the defect in cytokine signaling by visualizing GFP-*L. monocytogenes* infection using conventional fluorescence (Fig. 5B). The fluorescence signal observed in nontreated CTSD−/− BM-DM that corresponded to viable intracellular *L. monocytogenes* was clearly diminished in IFN-γ- and IL-6-treated BM-DM. However, the higher fluorescence signal observed in nontreated CTSD−/− BM-DM was barely unmodified in IFN-γ- or IL-6-treated BM-DM. The intracellular replication indices after 8 h of *L. monocytogenes* infection indicated similar *L. monocytogenes* replication in the BM-DM (Fig. 5B, legend). We also evaluated other functions of activated MØ that might have also been affected by a defect in cytokine signaling, such as the oxidative burst, using flow cytometry or the transformation of phagosomes into antigen loading competent compartments (MIIC) using confocal fluorescence images (29). Ctsd−/− BM-DM displayed a defect in the oxidative burst (Table 2, row b) and in the transformation of *L. monocytogenes* phagosomes into MIIC (Fig. 5B). MØ markers not involved in activation such as CD11b or CD11c were similar in CTSD−/− and Ctsd+/+ BM-DM (Table 2, row c). CTSD−/− BM-DM presented decreased co-localization of GFP-*L. monocytogenes* with anti-MHCII antibody.
ies after IFN-γ or IL-6 treatment, although GFP-L. monocytogenes phagosomes intracellular numbers were much higher (color images in Fig. 5B). These results suggested that CTSD−/− L. monocytogenes phagosomes might display impaired antigen processing abilities. We used the CTSD-mediated LLO phagosomal processing approach to confirm a defect of CTSD−/− BM-DM in antigen processing. CTSD−/− L. monocytogenes phagosomes contained only high levels of intact LLO that were detected with the anti-PLY-5 antibody in all conditions as follows: non-treated, IFN-γ-, or IL-6-treated BM-DM (Fig. 5C). We also confirmed that CTSD−/− L. monocytogenes phagosomes show no LLO form able to co-precipitate with MHC class II molecules (IP: Mo α MHC-II/WB, Rab α LLO lanes in Fig. 5C). RABSC was also used as loading control. These results verified that CTSD−/− L. monocytogenes phagosomes showed a clear impairment in LLO phagosomal processing and a severe defect in the phagosomal listerical mechanisms connected to STAT-1. Next, we confirmed that CTSD was the downstream component of the STAT-1 listerical pathway linked to L. monocytogenes-specific immunity. Following the same reasoning as in Fig. 3, we examined protection against listeriosis after i.p. inoculation of L. monocytogenes phagosomes (500 CFU/∼30 μg of phagosomal proteins) (P-NT, P-IFN, or P-IL-6 from BM-DM of CTSD+/+ mice) into CTSD−/− or CTSD+/+ mice. We used an inducible mouse model of CTSD deficiency previously used and described for in vivo experiments (CTSD−/−) (15) because CTSD−/−-deficient mice do not live long enough to allow vaccination protocols. We observed that 7 days of vaccination to CTSD−/− mice with P-NT phagosomes conferred a protection in a range of 20%. Vaccination with P-IFN or P-IL-6 phagosomes conferred protection in a higher range of 90−95% compared with nonvaccinated mice (Fig. 5D, black bars). The protection achieved with IFN-γ or IL-6 L. monocytogenes phagosomes was in the same range of 95% of protection obtained after vaccination for 14 days with L. monocytogenes-infected BM-DM pretreated with IFN-γ or IL-6, whereas no protection was obtained after vaccination for 7 days (data in legend of Fig. 5D). Protection requires L. monocytogenes phagosomal integrity because lysis of phagosomes before mouse inoculation causes no protection (Fig. 5D, gray bars). Vaccination of CTSD−/− mice with P-NT, P-IFN-γ, or P-IL-6 L. monocytogenes phagosomes confers no protection with exacerbated numbers of L. monocytogenes (Fig. 5E), high levels of IL-6 (Table 3, row a), no LLO-specific immune response in spleens (Table 3, row b), and an unusual peritoneal recruitment of PMN but a lack of recruitment of DC in (Table 3, row c). These results confirmed that CTSD was the downstream component of this STAT-1 listerical pathway connected with L. monocytogenes-specific immunity and protection.

**DISCUSSION**

Cell-free membrane vesicles are a good alternative as safer vaccine vectors against infectious diseases. Current efforts are focused to implement their efficiency. In the case of listeriosis, the search for live vaccine delivery vectors is mainly focused to refinements to enhance their potency or reduce their cytotoxicity to get the approval for their use in humans (7, 16, 32−33).

In this study, we show that MØ activated with IFN-γ or IL-6 to elicit a common listerical signal mediated by STAT-1. This signal mediates the degradation of L. monocytogenes within compartmentalized phago-receptosomes induced by IFN-γ or IL-6. This functional compartmentalization serves to...
confine all innate immune elements required for L. monocytogenes destruction within the same environment and to mediate the onset of L. monocytogenes-specific immunity. Here, we present that the phago-receptosomes are new vaccine vectors inducing protection against murine listeriosis. In fact, they postulate as safe vaccines showing no cytotoxicity at all and higher efficiencies with shorter vaccination protocols than other cell-based vaccine carriers such as MO infected with pathogens (34) or endosomes loaded with LLO (35). The induction of specific L. monocytogenes immunity is associated with their high competences as MIIC, binding of LLO-processed forms to MHC class II molecules, and down-regulation of IL-6. Protection appears linked to the recruitment of mature DC to the vaccination sites and induction of LLO-specific T cells in the spleens of vaccinated mice. What are the features of these listericidal compartments that make them unique as efficient vaccines against listeriosis (Fig. 6)?

The main characteristic of L. monocytogenes-specific immunity that makes this pathogen an attractive microbial vector is the ability to stimulate both arms of the immune system; it induces multiple innate immune pathways and an antigen-spe-

---

**Features 1-3:**
- Cytokine signalling
- Rab5 regulation
- Microbe killing
- Oxidative burst
- Ctsd protease activity
- LLO processing (LLO1-491)
- Antigen presentation-MHC-II

**Features 4-6:**
- Protection anti-LM
- Induction of LLOαβ/CD8+
- Induction of LLOαβ-2α/CD4+
- Recruitment of DC
- Production of TNF-α
- Down-regulation of IL-6

**FIGURE 6. Features of the L. monocytogenes phago-receptosomes induced by IFN-γ or IL-6.** IFN-γ or IL-6 binds to receptors and elicits a complete STAT-1 pathway, including phosphorylation of STAT-1 (P-STAT-1) bound to the receptor-associated kinases, JAK1 and JAK2 (feature 1). L. monocytogenes engulfed in phagosomes amplifies the STAT-1 signal and promotes Rab5a activation (RAB5A-GTP), triggering PHOX, inducible NOS, and CTSB-associated bactericidal mechanisms to assist L. monocytogenes killing and degradation, transforming the compartments in active innate immune vesicles with microbicidal abilities (feature 2). L. monocytogenes phago-receptosomes are transformed into competent MIIC immune vesicles by STAT-1-dependent mechanisms that stimulate CTSB-mediated antigen processing of LLO to its degraded LLO(1–491) form able to bind to MHC-II dimers. STAT-1-independent mechanisms as LIMP-2 also contribute transporting components of MHC as LAMP-2 and αβ MHC II stable dimers (feature 3). Features 1–3 of L. monocytogenes phago-receptosomes reflect that they are active innate immune vesicles. Validation of these vesicles as vaccine vectors confirmed their participation in specific immunity (features 4–6). First, they confer protection against listeriosis and induce specific LLO(91–99)/CD8α/129/Sv effector T cells (feature 4). Second, their vaccine efficiency is related to stimulation of innate elements expanding the signals to T cells through the recruitment of DC, to the vaccination sites and production of TNF-α with down-regulation of IL-6 (feature 5). Finally, protection requires intact innate and T cell-specific immune mechanisms in vaccine receptors (feature 6) because specific and protective immunity is achieved in CTSB+/− or 129/Sv control mice but CTSB-deficient (CTSB−/−) or lymphocyte-deficient (SCID) mice fail to acquire specific immunity and protection against listeriosis.
Listericidal Phagosomes Are Effective Vaccines

cific L. monocytogenes response that requires LLO to trigger a Th1 cytokine-based immune response (7, 32, 36). In this regard, IFN-γ or IL-6 initiates a complete listericidal pathway in MØ (Fig. 6, feature 1) via the activation of STAT-1 connected with the receptor-associated Janus tyrosine kinases, Jak1 and Jak2 (23). We obtained the first indication of this signaling pathway evaluating the global transcriptional response elicited in MØ by IFN-γ or IL-6 after infection with pathogenic L. monocytogenes. A general analysis of the enrichment of GO and KEGG terms indicated two profiles, cytokine and L. monocytogenes-specific patterns, and two main components, organelle regulation and L. monocytogenes immunity. The applied functional clustering based on L. monocytogenes phagosomal degradation (cluster I) and immunity (cluster II) restricted the common IFN-γ or IL-6 transcriptional responses to seven selected genes. Four genes corresponded to cluster I, and all of them played a role in phagocytosis, such as the trafficking regulator RAB5A (4, 19, 22, 37–39) and the lysosomal components LAMP-2 (21), LIMP-2 (4, 6, 29), and the regulatory subunit A of H+-ATPase (4, 12, 19, 21, 22). Three genes belong to cluster II and correspond to major components of L. monocytogenes-specific innate immunity, including the type I IFN-response genes C3F6 and IL-6 and the type II IFN response gene STAT-1 (1, 3, 5, 23). Therefore, it appears that this listericidal route connects signaling, trafficking regulators, listericidal lysosomal effectors, and cytokine production.

Next, L. monocytogenes internalized in phagosomes induces distinct phagosomal processes and amplifies the STAT-1-dependent listericidal signal to other innate immune elements (Fig. 6, feature 2). In this regard, this amplified signal promotes RAB5A translocation to phagosomes and activation of RAB5A to RAB5A-GTP (4) and the amplified signal promotes RAB5A translocation to phagosomes. The presence of high levels of JAK1, JAK2, and phosphorylated STAT-1 in these listericidal compartments appears to differentiate them from other reported phagosomal proteomes that contained latex beads but lacked these innate immune components (6). All together, these listericidal components, found in other phagosomal proteomes (6, 21, 22), contribute to the degradation of L. monocytogenes within the phagosomes. However, our functional validation of these platforms as powerful microbialic vesicles revealed their singularity as innate immune vesicles.

Their transformation into MIIC-competent immune vesicles (Fig. 6, feature 3) is dependent on the STAT-1-mediated stimulation of CTSD-induced antigen processing of LLO, degrading it to the LLO(1–491) form (29). Moreover, MHC-II dimers become loaded with these LLO(1–491) forms (this study). However, other STAT-1 independent elements such as LIMP-2 (29) might also contribute transporting MIIC markers such as LAMP-2 and SDS-stable αβ MHC-dimers and inducing TNF-α. This transformation into MIIC competent vesicles might trigger the onset of specific immunity. Collectively, the results from experiments using the STAT-1−/− and CTSD−/− BM-DM revealed the sequence of elements involved in this listericidal route. STAT-1 acts upstream of RAB5A and CTSD in this pathway. STAT-1 is linked to the loading of MHC-class II molecules with CTSD-processed LLO(1–491) forms lacking the phagosomal binding domain (15).

The validation of these immune vesicles as effective vaccine vectors provided the fourth indication that L. monocytogenes phago-receptosomes induced by IFN-γ or IL-6 participated in specific immunity (Fig. 6, feature 4). In fact, they show high protection capacities against listeriosis and good abilities to induce specific LLO(91–99)/CD8+ and LLO(189–201)/CD4+ T cells (40).

Their vaccine efficiency appears related to their ability to trigger a secondary LLO-specific T cell immune response that recruits mature DC cells to the vaccination sites and triggers the production of Th1 cytokines (i.e., TNF-α, MCP-1, and IFN-γ) (Fig. 6, feature 5). In fact, SCID mice lacking T lymphocytes after vaccination with listericidal phagosomal vaccines only induce primary responses and recruit high levels of MØs instead of DCm to the vaccination sites that causes a failure in protection against listeriosis.

Protection with L. monocytogenes phago-receptosomes also requires intact innate immune mechanisms in vaccine receptors (Fig. 6, feature 6). In fact, mice with severe deficiencies in innate immune responses (CTSD−/−) (13, 15, 29) displayed a clear MØ defect with poor bactericidal abilities, oxidative burst capacities, CTSD-mediated LLO phagosomal processing, and impaired transformation of L. monocytogenes phagosomes into MIIC vesicles due to the absence of MHC-II dimers loaded with LLO(1–491). Moreover, vaccination of experimental CTSD-deficient mice (CTSD−/−) previously described (15, 40) failed to confer protection against listeriosis because they do not recruit DC to the vaccination sites, showed uncontrolled neutrophilia and IL-6 exacerbated levels (25), and do not elicit LLO-specific T cells. Therefore, CTSD appears as the downstream component of the listericidal route that connects innate with specific immunity in listeriosis.

In brief, L. monocytogenes phago-receptosomes induced by IFN-γ or IL-6 present all the requirements to induce protective immunity, multiple innate immune elements, enhanced antigen presentation, stimulation of effector T cells, and recruitment of cells involved in the expansion of the signals (7, 30, 32, 34, 36, 41, 42). We envisage that these L. monocytogenes phago-receptosomes induced by IFN-γ or IL-6 appear to function as naturally occurring vaccine carriers that might be liberated after pyroposis of MØ at the inflammation sites (43, 44) and taken up by DC to confer protection (34, 45). Ongoing studies will evaluate their ability to induce long lasting T cell memory, the mechanisms of phago-receptosomes natural formation, and the refinements of doses and potency. Currently, they open up a field of possibilities for studies evaluating the relevance of natural vaccines that are similar to exosome release because of apoptosis (46). The lack of efficiency of this vaccination procedure in experimental deficiencies of CTSD or SCID mice lacking lymphocytes highlighted the convenience of testing bacterial vaccines in healthy donors as well as in immunodeficiencies.
with high susceptibility against certain bacterial groups (15, 40, 47).

Acknowledgments—We acknowledge the advice of A. Celada and group regarding bone marrow cells (Instituto de Research Biomédica, Barcelona, Spain); the critiques and antibody provided by R. Lindner (Hannover Medical School, Hannover, Germany); the peptide synthesis of F. Roncal (Proteomics Service, Centro Nacional de Biotecnología, Madrid, Spain); the suggestions of previous members of the laboratory, including J. Ramos-Vivas, L. Fernandez-Prieto, and C. Carranza-Cereceda; the Unidad de Microscopía Avanzada in the Instituto de Formacion e Investigacion Marques de Valdecilla (directed by M. Lopez-Fanarraga) for assistance with confocal images and the reagents; and bacterial strains from G. Bockoch, D. Lambricht, O. Utermöhlen, J. Schröder, D. Higgins, and D. A. Portnoy. We are also grateful for the approval from S. Akira for using the condition FloxLexStat3−/− mice and the approval from S. Akira for using the condition Stat3−/− mice.

REFERENCES

1. Unanue, E. R. (1997) Studies in listeriosis show the strong symbiosis between the innate cellular system and the T-cell response. Immunol. Rev. 158, 11–25
2. Portnoy, D. A., Auerbuch, V., and Glomski, I. J. (2002) The cell biology of Listeria monocytogenes infection. The intersection of bacterial pathogenesis and cell-mediated immunity. J. Cell Biol. 158, 409–414
3. Pamer, E. G. (2004) Immune responses to Listeria monocytogenes. Nat. Immunol. 5, 812–823
4. Prada-Delgado, A., Carrasco-Marín, E., Bokoch, G. M., and Alvarez-Dominguez, C. (2001) Interferon-γ-listericidal action is mediated by novel Rab5a functions at the phagosomal environment. J. Biol. Chem. 276, 19059–19065
5. Herskovits, A. A., Auerbuch, V., and Portnoy, D. A. (2007) Bacterial ligands generated in a phagosome are targets of the cytosolic innate immune system. PLoS Pathog. 3, 431–443
6. Jutras, I., Houde, M., Currier, N., Boulais, J., Duclos, S., LaBoissière, S., Bonneil, E., Kearney, P., Thibault, P., Paramithiotis, E., Hugo, P., and Desjardins, M. (2008) Modulation of the phagosome proteome by interferon-γ. Mol. Cell. Proteomics 7, 697–715
7. Zenewicz, L. A., and Shen, H. (2007) Innate and adaptive immune responses to Listeria monocytogenes. A short overview. Microbes Infect. 9, 1208–1215
8. Dinauer, M. C., Deck, M. B., and Unanue, E. R. (1997) Mice lacking recombination activating gene 5 (Rag-5) are also grateful for the approval from S. Akira for using the condition FloxLexStat3−/− mice and the approval from S. Akira for using the condition Stat3−/− mice.

Listericidal Phagosomes Are Effective Vaccines

C. (2006) Cutting edge. A novel nonoxidative phagosomal mechanism exerted by cathepsin-D controls Listeria monocytogenes intracellular growth. J. Immunol. 176, 1321–1325
14. Schramm, M., Herz, J., Haas, A., Krönke, M., and Utermöhlen, O. (2008) Acid sphingomyelinase is required for efficient phago-lysosomal fusion. Cell. Microbiol. 10, 1839–1853
15. Carrasco-Marín, E., Madrazo-Toca, F., de los Toyos, J. R., Cacho-Alonso, E., Tores, R., Pareja, E., Parada, A., Albar, J. P., Chen, W., Gomez-Lopez, M. T., and Alvarez-Dominguez, C. (2009) The innate immunity role of cathepsin-D is linked to Trp-491 and Trp-492 residues of listeriolysin O. Mol. Microbiol. 72, 668–682
16. Lauer, P., Hanson, B., Lemmens, E. E., Liu, W., Luckett, W. S., Leong, M. L., Allen, H. E., Skoble, J., Bahaj, K. S., Freitag, N. E., Brockstedt, D. G., and Dubensky, T. W., Jr. (2008) Constitutive activation of the PrfA regulon enhances the potency of vaccines based on live-attenuated and killed but metabolically active Listeria monocytogenes strains. Infect. Immun. 76, 3742–3753
17. Portnoy, D. A., Schreiber, R. D., Connelly, P., and Tilney, L. G. (1989) γ-Interferon limits access of Listeria monocytogenes to the macrophage cytoplasm. J. Exp. Med. 170, 2141–2146
18. Bhattacharya, M., Ojha, N., Solanki, S., Mukhopadhyay, C. K., Madan, R., Patel, N., Krishnamurthy, G., Kumar, S., Basu, S. K., and Mukhopadhyay, A. (2006) IL-6 and IL-12 specifically regulate the expression of Rab5 and Rab7 via distinct signaling pathways. EMBO J. 25, 2878–2888
19. Prada-Delgado, A., Carrasco-Marín, E., Peña-Macarro, C., Del Cerro-Vadillo, D., Fresno-Escudero, M., Leyva-Cobian, F., and Alvarez-Dominguez, C. (2005) Inhibition of Rab5a exchange activity is a key step for Listeria monocytogenes survival. Traffic 6, 252–265
20. Harding, C. V., and Geuze, H. J. (1992) Class II MHC molecules are present in macrophage lysosomes and phagolysosomes that function in the phagocytic processing of Listeria monocytogenes for presentation to T cells. J. Cell Biol. 119, 531–542
21. Huyhn, K. K., Eskelinen, E. L., Scott, C. C., Malevanets, A., Safitg, P., and Grinstein, S. (2007) LAMP proteins are required for fusion of lysosomes with phagosomes. EMBO J. 26, 313–324
22. Vieira, O. V., Bucci, C., Harrison, R. E., Trimble, W. S., Lanzetti, L., Gruenberg, J., Schreiber, A. D., Stahl, P. D., and Grinstein, S. (2003) Modulation of Rab5 and Rab7 recruitment to phagosomes by phosphorylinsitolositol 3-kinase. Mol. Cell. Biol. 23, 2501–2514
23. Ramana, C. V., Gil, M. P., Schreiber, R. D., and Stark, G. R. (2002) Stat1-dependent and -independent pathways in IFN-γ-dependent signaling. Trends Immunol. 23, 96–101
24. McCaffrey, R. L., Fawcett, P., Riordan, M., Lee, K. D., Havelv, E. A., Brown, P. O., and Portnoy, D. A. (2004) A specific gene expression program triggered by Gram-negative bacteria in the cytosol. Proc. Natl. Acad. Sci. U.S.A. 101, 11386–11391
25. Schwegmann, A., Guler, R., Cutler, A. J., Arendse, B., Horsnell, W. G., Flemming, A., Kottmann, A. H., Ryan, G., Hide, W., Leitges, M., Seogih, C., and Zorn, B. F. (2007) Protein kinase Cδ is essential for optimal macrophage-mediated phagosomal containment of Listeria monocytogenes. Proc. Natl. Acad. Sci. U.S.A. 104, 16251–16256
26. von Döwig, A., Musson, J. A., Shim, H. K., Lee, J. J., Walker, N., Harding, C. V., Williamson, E. D., and Robinson, J. H. (2005) Distribution of productive antigen-processing activity for MHC class II presentation in macrophages. Scand. J. Immunol. 62, 243–250
27. Carrasco-Marín, E., Shimizu, J., Kanagawa, O., and Unanue, E. R. (1996) The class II MHC I-Training molecules from nonobese diabetic mice are poor peptide binders. J. Immunol. 156, 450–458
28. Forestier, C., Deleuil, F., Lapaque, N., Moreno, E., and Gorvel, J. P. (2000) Brucella abortus lipopolysaccharide in murine peritoneal macrophages acts as a down-regulator of T cell activation. J. Immunol. 165, 5202–5210
29. Carrasco-Marín, E., Fernández-Prieto, L., Rodriguez-Del Rio, E., Madrazo-Toca, F., Reinebeck, T., Safitg, P., and Alvarez-Dominguez, C. (2011) LIMP-2 links late phagosomal trafficking with the onset of the innate immune response to Listeria monocytogenes. A role in macrophage activation. J. Biol. Chem. 286, 3332–3341
30. Westcott, M. M., Henry, C. J., Amis, J. E., and Hillbold, E. M. (2010) Dendritic cells inhibit the progression of Listeria monocytogenes intracellularly.
Lacticidal Phagosomes Are Effective Vaccines

lular infection by retaining bacteria in major histocompatibility complex class II-rich phagosomes and by limiting cytosolic growth. Infect. Immun. 78, 2956–2965

Tsukiyama, K., Kawamura, I., Takahashi, A., Nomura, T., Koda, C., and Mitsuyama, M. (2005) Listeriolysin O-induced membrane permeation mediates persistent interleukin-6 production in Caco-2 cells during Listeria monocytogenes infection in vitro. Infect. Immun. 73, 3869–3877

Tsuchiya, K., Kawamura, I., Takahashi, A., Nomura, T., Koda, C., and Mitsuyama, M. (2005) Listeriolysin O-induced membrane permeation mediates persistent interleukin-6 production in Caco-2 cells during Listeria monocytogenes infection in vitro. Infect. Immun. 73, 3869–3877

Hamilton, S. E., Badovinac, V. P., Khanolkar, A., and Harty, J. T. (2006) Listeriolysin O-deficient Listeria monocytogenes as a vaccine delivery vehicle. Antigen-specific CD8 T cell priming and protective immunity. J. Immunol. 177, 4012–4020

Singh, V., Jain, S., Gowthaman, U., Parihar, P., Gupta, P., Gupta, U. D., and Agrewala, J. N. (2011) Co-administration of IL-1β + IL-6 + TNF-α with Mycobacterium tuberculosis-infected macrophages vaccine induces better protective T cell memory than BCG. PLoS One 6, e16097

Rodriguez-Del Rio, E., Frande-Cabanes, E., Tobes, R., Pareja, E., Lecesa-Cuello, M. J., Ruiz-Saez, M., Carrasco-Marín, E., and Alvarez-Dominguez, C. (2011) The intact structural form of LLO in endosomes cannot protect against listeriosis. Int. J. Biochem. Mol. Biol. 2, 207–218

Corr, S. C., and O’Neill, L. A. (2009) Listeria monocytogenes infection in the face of innate immunity. Cell. Microbiol. 11, 703–709

Agaisse, H., Burrack, L. S., Philips, J. A., Rubin, E. J., Perrimon, N., and Higgins, D. E. (2005) Genome-wide RNAi screen for host factors required for intracellular bacterial infection. Science 309, 1248–1251

Cheng, L. W., Viala, J. P., Stuurman, N., Wiedemann, U., Vale, R. D., and Portnoy, D. A. (2005) Use of RNA interference in Drosophila S2 cells to identify host pathways controlling compartmentalization of an intracellular pathogen. Proc. Natl. Acad. Sci. U.S.A. 102, 13646–13651

Alvarez-Dominguez, C., Madrazo-Toca, F., Fernandez-Prieto, L., Vandeskerkhove, J., Pareja, E., Tobes, R., Gomez-Lopez, M. T., Del Cerro-Valdillo, E., Fresno, M., Leyva-Cobian, F., and Carrasco-Marin, E. (2008) Characterization of a Listeria monocytogenes protein interfering with Rab5a. Traffic 9, 325–337

Menzel, K., Hausmann, M., Obermeier, F., Schreiter, K., Dunger, N., Battaile, F., Falk, W., Scholmerich, J., Herfarth, H., and Rogler, G. (2006) Cathepsins B, L, and D in inflammatory bowel disease macrophages and potential therapeutic effects of cathepsin inhibition in vivo. Clin. Exp. Immunol. 146, 169–180

Skoberne, M., Schenk, S., Hof, H., and Geginat, G. (2002) Cross-presentation of Listeria monocytogenes-derived CD4 T cell epitopes. J. Immunol. 169, 1410–1418

Waite, J. C., Leiner, I., Lauer, P., Rae, C. S., Barbet, G., Zheng, H., Portnoy, D. A., Pamer, E. G., and Dustin, M. L. (2011) Dynamic imaging of the effector immune response to listeria infection in vivo. PLoS Pathog. 7, e1001326

Cervantes, J., Nagata, T., Uchijima, M., Shibata, K., and Koide, Y. (2008) Intracytosolic Listeria monocytogenes induces cell death through caspase-1 activation in murine macrophages. Cell. Microbiol. 10, 41–52

Sauer, J. D., Pereyre, S., Archer, K. A., Burke, T. P., Hanson, B., Lauer, P., and Portnoy, D. A. (2011) Listeria monocytogenes engineered to activate the Nlrc4 inflammasome are severely attenuated and are poor inducers of protective immunity. Proc. Natl. Acad. Sci. U.S.A. 108, 12419–12424

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F., Pamer, E. G., Littman, D. R., and Lang, R. A. (2002) In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity 17, 211–220

Beauvillain, C., Juste, M. O., Dion, S., Pierre, J., and Dimier-Poisson, I. (2009) Alphaviruses and Escherichia coli LPS: a combined host-pathogen approach. Mol. Microbiol. 72, 130–141

Steinfeld, R., Reinhardt, K., Schreiber, K., Hillebrand, M., Kraetzner, R., Bruck, W., Saftig, P., and Gartner, J. (2006) Cathepsin D deficiency is associated with a human neurodegenerative disorder. Am. J. Hum. Genet. 78, 988–998