Stress-Induced ClpP Serine Protease of *Listeria monocytogenes* Is Essential for Induction of Listeriolysin O-Dependent Protective Immunity

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The stress-induced protease ClpP is required for virulence of the facultative intracellular pathogen *Listeria monocytogenes*. We previously found that in the absence of ClpP, the virulence of this pathogen was strongly reduced, mainly due to the decreased production of functional listeriolysin O (LLO), a major immunodominant virulence factor promoting intracellular growth. In this work, a *clpP* deletion mutant of *L. monocytogenes* was used to study the generation of anti-*Listeria* protective immunity. We found that ClpP is required for the intracellular growth of *L. monocytogenes* in resident macrophages in vivo. Mice infected with doses as high as 10^6 *clpP* mutant bacteria were not protected against a lethal challenge of wild-type bacteria and did not develop any detectable LLO-specific cytolytic T cells or antibodies, suggesting that the amount of LLO produced in infected mice under these conditions was too low to induce a specific immune response. However, in contrast to the results obtained with a mutant with a disrupted *hly* gene, this lack of protection was overcome by inoculation of very high infecting doses of *clpP* mutant bacteria (5×10^8), thus producing sufficient amounts of LLO to stimulate anti-*Listeria* immunity. The role of ClpP was confirmed by showing that anti-*Listeria* immunity was restored in mice infected with a *clpP*-complemented mutant. These results indicate that the stress-induced serine protease ClpP is a potential target for modulating the presentation of protective antigens such as LLO and thereby the immune response against *L. monocytogenes*.

Listeria monocytogenes is a facultative intracellular gram-positive bacterium responsible for severe infections in humans and animals, including meningoencephalitis and abortion (19). It has been extensively used as a model to study host resistance against intracellular bacterial pathogens. The virulence of this ubiquitous microorganism is due to its capacity to invade and multiply within host cells, including macrophages (29). During its intracellular life cycle, *L. monocytogenes* produces several virulence factors involved at each step of the invasive process, including listeriolysin O (LLO), internalin, phospholipases, and ActA, all controlled by the pleiotropic transcriptional activator PrfA (11). LLO is a 58-kDa exotoxin allowing bacteria to escape phagosomes of macrophages and to multiply in the cytoplasm (1, 10, 12, 13, 40). LLO is presumably processed by the cytosolic pathway and plays a crucial role in the effective presentation of *Listeria* antigens to immune T cells (2, 3, 4, 21, 26, 33).

It was recently shown that stress proteins also play a role in the virulence of *L. monocytogenes*, especially during the early stages of intracellular growth (31, 36, 37). Uptake of *L. monocytogenes* by macrophages induces a set of bacterial proteins, including stress proteins (20). Direct evidence for the role of these proteins in virulence of *L. monocytogenes* includes the finding that a ClpC ATPase belonging to the Hsp-100 family promotes early escape of bacteria from the phagosomal com-

partment of macrophages (36, 37). ClpC acts synergistically with ClpE, another Hsp-100 family member also involved in the expression of virulence (31). Recently, a stress-induced 21.6-kDa protein, designated ClpP, required for *L. monocytogenes* growth under hostile conditions was identified (15). It was found that ClpP belongs to the large family of ClpP serine proteases highly conserved in prokaryotes and eukaryotes. ClpP of *L. monocytogenes* acts as a serine protease and prevents the accumulation of altered proteins that might be toxic for the bacteria under stress conditions (15). In the absence of ClpP, the secretion of functional LLO is reduced, thus explaining why a *clpP* deletion mutant demonstrated poor growth in cultured bone marrow macrophages in vitro (15). Since ClpP promotes the intracellular survival of *L. monocytogenes* and presumably the antigenic presentation of LLO and other protective antigens, ClpP may represent a potential vaccine target for *L. monocytogenes*.

In this work, we studied the role of the stress-induced serine protease ClpP in the generation of anti-*Listeria* protective immunity in vivo. We found that ClpP is essential for intracellular survival in macrophages and modulates LLO-dependent anti-*Listeria* protection. Following high infecting doses of a *clpP* deletion mutant, sufficient amounts of LLO were produced to induce specific immunity against *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains and culture media. We used *L. monocytogenes* reference strain LO28 (37), a *clpP* deletion mutant of LO28, and the same mutant *trans*-complemented with *clpP* (15). As a control, we used BOF 415, a previously described Tn917 insertion *hly* mutant of LO28 (10). Bacteria were grown in brain heart infusion (BHI) media. For virulence assays, bacteria were harvested while

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still in log phase (5×10^8 /ml), dispensed into vials (1-ml lots), and stored at -80°C until required. For each experiment, the contents of a vial were thawed and diluted appropriately in saline (0.15 M NaCl) before inoculation.

Infection of mice. Adult, 6- to 8-week-old pathogen-free female BALB/c mice were supplied by Janvier, Le Geneset St. Isle, France. Mice were maintained in a protected environment under filtered airflow in negative-pressure isolators (ESI, Cachan, France). Animals were fed with sterilized, vitamin-supplemented diet and sterile water (pH 3). Mice were inoculated intravenously (i.v.) with appropriate dilutions of *L. monocytogenes* in a volume of 0.5 ml via a lateral tail vein. Growth of bacteria in the spleen and the liver was monitored. At intervals, groups of five mice were killed; the organs were removed aseptically and homogenized separately in sterile saline. Then, 0.1-ml volumes of serial 10-fold dilutions were surface plated on BHI agar with a minimal detectable limit of 100 bacteria per organ. Protection was estimated after 30 days by inoculating i.v. a lethal dose of *L. monocytogenes* LO28 (10^6 bacteria). The 50% lethal dose of strain LO28 was estimated at 5×10^4 bacteria/mouse. Student's *t* test was used for statistical analysis.

Titration of hemolytic activity. The hemolytic activity of supernatants of mid-log-phase cultures incubated at 30 or 40°C was titrated with horse erythrocytes and expressed in arbitrary hemolytic units per 10^8 bacteria as previously described (15).

Western blot analysis. Proteins in culture supernatants were precipitated with 10% (vol/vol) trichloroacetic acid. Bacterial whole extracts were prepared by boiling the cells for 5 min in 100 mM Tris (pH 6.8)–200 mM dithiothreitol–4% (wt/vol) sodium dodecyl sulfate–0.2% (wt/vol) bromophenol blue–20% (vol/vol) glycerol. Proteins were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue or subjected to immunoblot analysis with a rabbit LLO-specific polyclonal antibody (18). Sheep anti-mouse immunoglobulin–horseradish peroxidase conjugate and an ECL kit (Amersham) were used for immunodetection. To determine the anti-LLO response, mice were bled 4 weeks after infection and the presence of anti-LLO antibodies (immunoglobulin G) was determined by Western blot analysis with purified LLO as previously described (39). Coating antibodies were detected with peroxidase-conjugated goat anti-mouse immunoglobulin G diluted 1/1,000 (Organon Teknika, West Chester, United Kingdom).

Anti-LLO cytolytic assay. Mice were sacrificed by day 6 postinfection. Their spleens were removed, and single-cell suspensions were prepared. Erythrocytes were lysed using ammonium chloride, and splenocytes were washed, counted, and adjusted to the desired cell numbers. LLO-transfected P815 cells, pHEM3.3 cells (33), were incubated with 50 mg of mitomycin C (Boehringer GmbH, Mannheim, Germany)/liter at 37°C . Prior to use in cultures, pHEM3.3 cells were washed extensively in culture media. Cultures containing 2.5×10^5 splenocytes and 2.5×10^4 mitomycin C-treated pHEM3.3 stimulator cells in a volume of 200 μl were established in round-bottom 96-well tissue culture plates (Falcon, Mountain View, Calif.) and incubated at 37°C for 6 days. Recombinant human interleukin-2 (5 U/ml; Peprotech, London, United Kingdom) was present throughout the culture period. Splenocytes were then harvested, washed, counted, and adjusted to the desired cell numbers. The anti-LLO cytotoxicity of the effector cells was determined by use of a 4-h release assay with 3,000 chromium (^{51}Cr)-labeled target cells, either pHEM 3.3 cells or nontransfected P815 cells, in cultures of 200 μl in round-bottom 96-well tissue culture plates. Effector cells were added to target cells at various ratios. Spontaneous release was measured by use of cultures with target cells incubated alone. Total release was measured by use of cultures containing target cells and 2% acetic acid. Spontaneous release was always less than 15% total release. The methods used for harvesting supernatants and the subsequent counting of radioactivity have been described previously (8). The specific cytotoxicity was calculated as follows: percent specific lysis = [(experimental release – spontaneous release)/(total release – spontaneous release)] $\times 100$.

IFN- γ production. Spleen cells (10^6 /ml) from infected mice and control mice were cultured in triplicate for 48 h in a volume of 200 μl in round-bottom 96-well tissue culture plates in the presence or absence of 10^7 heat-killed *L. monocytogenes* LO28. Subsequently, the supernatants were harvested, and the concentration of gamma interferon (IFN- γ) was measured using a sandwich enzyme-linked immunosorbent assay kit (Geneset, Cambridge, Mass.) according to the manufacturer's instructions.

Histologic analysis. Mice were challenged i.v. with 10^8 bacteria (in 0.5 ml) and killed by cervical dislocation 1 or 8 h after inoculation. Small pieces of liver were removed and processed for Gram staining and electron microscopy. For light microscopy, samples were fixed in 10% formalin and embedded in paraffin. Semithin sections were cut and stained with 1% toluidine blue. Samples to be processed for electron microscopy were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature, postfixed in 2% aqueous

osmium tetroxide (E. Merck AG, Darmstadt, Germany) for 1 h, dehydrated in graded ethanol solutions, and embedded in Epon 812 (TAA-Jamming). Ultrathin sections of the appropriate blocks were cut, stained with uranyl acetate and lead citrate, and examined with an electron microscope (model CX200; Jeol, Croissy-sur-Seine, France).

RESULTS

ClpP is required for the intracellular growth of *L. monocytogenes* in infected mice. It was previously demonstrated that ClpP of *L. monocytogenes* is required for intracellular growth in cultured bone marrow macrophages, as evidenced by a loss of virulence in a *clpP* deletion mutant (15). The relevance of this finding in vivo was studied by monitoring the fate of *L. monocytogenes* during the early phase of infection. Mice were inoculated i.v. with a high dose of either LO28 or its isogenic *clpP* deletion mutant (10^8 bacteria). After 1 and 8 h, mice were sacrificed and bacterial survival in the liver was monitored by light and electron microscopy. As illustrated in Fig. 1, bacteria from both strains were exclusively confined within Kpffer cells 1 h after infection. The electron microscopic study shows that most wild-type bacteria were intact inside vacuoles, whereas many of the mutant bacteria were already damaged (Fig. 1). After 8 h, wild-type bacteria were densely packed within Kpffer cells as a result of rapid intracellular growth. This dramatic multiplication of wild-type bacteria was associated with early invasion of adjacent hepatocytes (Fig. 1). As shown by electron microscopy, wild-type bacteria remained apparently undamaged inside the cytoplasm of infected cells. In contrast, rare *clpP* mutant bacteria were visible at 8 h in liver tissue, most of the bacteria being destroyed in Kpffer cells without any invasion of adjacent hepatocytes (Fig. 1). These results indicate that the ClpP serine protease is required for the intracellular growth of *L. monocytogenes* in vivo.

The ClpP protease favors the induction of anti-*Listeria* protective immunity. Mice were immunized i.v. with sublethal doses of wild-type bacteria (5×10^3) or *clpP* mutant bacteria (1×10^6 or 5×10^8). Controls included three groups of mice: (i) mice receiving phosphate-buffered saline (PBS), (ii) mice infected with a *clpP*-complemented mutant (5×10^3) expressing the same level of virulence as wild-type bacteria (15), and (iii) mice infected with an avirulent *hly* insertion mutant of LO28 (5×10^8) which does not produce LLO. All mice were challenged i.v. 42 days later with a lethal dose of wild-type LO28 (5×10^5). Mortality was then evaluated over a 10-day period, while bacterial survival in the spleen and the liver during the 3 days following the challenge was studied. As expected, most mice infected with wild-type bacteria or *clpP*-complemented mutant bacteria survived the lethal challenge (Fig. 2A). In contrast, mice infected with the lower dose of the *clpP* mutant (1×10^6) were not protected, with rapid death of all mice within 3 to 4 days ($P < 0.001$). As shown in Fig. 2B and C, bacterial growth was almost unrestricted in these mice, similar to that in uninfected control mice or mice inoculated with the *hly* mutant (5×10^8). However, mice inoculated with a very high dose of the *clpP* mutant (5×10^8) were fully protected against a lethal challenge, as demonstrated by their survival (Fig. 2A) and the rapid bacterial elimination in organs ($P < 0.01$) (Fig. 2B and C). These findings suggest that the lack of protection by the lower dose of the *clpP* mutant presumably results from insufficient stimulation of anti-*Listeria* protective T cells.

FIG. 1. In vivo survival and replication of *L. monocytogenes* in the livers of infected mice. Livers of mice infected with *L. monocytogenes* LO28 (A to D) and an isogenic *clpP* mutant (E to H) were examined by light microscopy of semithin sections stained with toluidine blue (left panel) and by electronic microscopy of ultrathin sections (right panel). After 1 h, wild-type bacteria (A and B) and mutant bacteria (E and F) were confined inside Kpfer cells of sinusoid capillaries and appeared intact. After 8 h, dense clusters of wild-type bacteria were visible throughout the parenchyma, with invasion of adjacent hepatocytes (C). Electron microscopy showed replicating bacteria in the cytoplasm of hepatocytes with actin polymerization (D). Conversely, *clpP* mutant bacteria were hardly visible after 8 h, suggesting that they had been cleared by resident phagocytes (G); ghosts of mutant bacteria were seen inside vacuoles of rare Kpfer cells (H).

The ClpP protease promotes the anti-LLO immune response. It was previously demonstrated (15) that under stress conditions, the hemolytic activity of LLO produced by a $\Delta clpP$ mutant was much lower than that of its wild-type parental strain or of a *clpP* trans-complemented mutant, although the amounts of secreted LLO were similar in the three strains, as shown by Western blot analysis with an anti-LLO antibody (Fig. 3). Since ClpP favors the production of functional LLO, we studied the production of anti-LLO antibodies in the sera of day 42 infected mice. Anti-LLO antibodies were detected in

mice having received low doses of wild-type bacteria (5×10^3) and in mice infected with a high challenge dose of *clpP* mutant bacteria (5×10^8), at titers of 1/400 and 1/20, respectively. In contrast, anti-LLO antibodies (titer of $>1/10$) were not found in mice inoculated with 1×10^6 *clpP* mutant bacteria or 5×10^8 *hly* mutant bacteria.

The generation of protective immunity against *L. monocytogenes* is dependent upon the activation of LLO-specific major histocompatibility complex (MHC) class I-restricted CD8⁺ cytotoxic T lymphocytes (CTLs), which recognize the immuno-

FIG. 2. Anti-Listeria protection in mice infected by isogenic mutants of *L. monocytogenes*. Groups of mice received i.v. PBS (○), 5×10^8 LO28Δhly (●), 1×10^6 LO28ΔclpP (△), 5×10^8 LO28ΔclpP (▲), 5×10^3 wild-type (■), or 5×10^3 trans-complemented LO28ΔclpP (□) bacteria. All mice were challenged i.v. 42 days later with a lethal dose of the wild-type strain (5×10^5 bacteria). (A) Mortality was monitored for 10 days with groups of 10 mice. Values are the percent survival after challenge. (B and C) Bacterial growth in the spleen (B) and liver (C) was monitored with groups of 16 mice for 4 days (means of bacterial counts; four organs per time point; standard deviations, ≤ 0.25). Mice immunized with the wild-type strain, the trans-complemented *clpP* mutant, or high doses of the *clpP* mutant (5×10^8 bacteria) survived the lethal challenge, with rapid bacterial elimination from the spleen and liver. In contrast, mice infected with 1×10^6 *clpP* mutant bacteria or LLO-deficient bacteria were not protected and died rapidly.

FIG. 3. ClpP modulates the expression of functional LLO under stress conditions. (A) Anti-LLO Western blot analysis of supernatants of bacteria grown to mid-log phase in BHI broth at 30 or 40°C. (B) Hemolytic activity was titrated with the same culture supernatants and is expressed in arbitrary units for 10^8 bacteria. LLO was overexpressed in all strains at 40°C, but hemolytic activity was strongly reduced in the absence of ClpP.

dominant *H2-K^d*-restricted epitope, 91-99, of LLO (21, 33). Therefore, we investigated the induction of LLO-specific CD8⁺ T cells in mice infected i.v. with LO28 (5×10^3), the *clpP* mutant (1×10^6 or 5×10^8), or the *hly* mutant (5×10^8). Spleen cells from infected mice and noninfected mice were collected by day 6 and restimulated with LLO-transfected P815 cells in vitro. The cytotoxic activity of activated splenocytes was subsequently tested against pHEM3.3 cells and nontransfected P815 cells. As expected, mice infected with 5×10^8 *clpP* mutant bacteria raised an anti-LLO CTL response at a level similar to that of mice infected with wild-type bacteria (5×10^3) (Fig. 4). In contrast, CTLs were not detected in uninfected mice, in mice infected with 1×10^6 *clpP* mutant bacteria, or in mice receiving the *hly* mutant (5×10^8), consistent with the absence of protective immunity.

The absence of a CTL response in mice infected with 1×10^6 *clpP* mutant bacteria could have been due to the rapid

FIG. 4. In vitro induction of LLO-specific CTL responses by the Δ*clpP* mutant. Mice were immunized i.v. with 5×10^3 wild-type strain LO28 (■), 5×10^8 LO28Δhly (●), 1×10^6 LO28ΔclpP (△), or 5×10^8 LO28ΔclpP (▲) bacteria. Six days after infection, splenocytes were recovered, restimulated, and subsequently tested for cytotoxic activity against LLO-expressing target cells at different effector/target (E:T) ratios. Results are expressed as the mean percent specific lysis of duplicate cultures; error bars show standard deviations. An LLO-specific CTL response was generated from mice infected with the wild-type strain. No CTL activity was detected with LLO-deficient bacteria or with 1×10^6 *clpP* mutant bacteria. In mice infected with 5×10^8 *clpP* mutant bacteria, the CTL response was similar to that in mice infected with wild-type bacteria. Lysis produced by effector cells from infected mice sensitized with PBS was $<2\%$ and is not represented.

FIG. 5. IFN- γ response to total listerial antigens. Mice were immunized as described in the legend to Fig. 4. After 6 days, splenocytes were recovered and stimulated in vitro with heat-killed *L. monocytogenes*; IFN- γ production was measured by an enzyme-linked immunosorbent assay. Data represent the mean and standard deviation for groups of six to eight mice. Splenocytes from mice infected with 10^6 *clpP* mutant bacteria produced IFN- γ at levels comparable to those produced by splenocytes from mice infected with wild-type bacteria.

elimination of bacteria and therefore to the lack of in vivo T-cell activation. This idea was tested by titrating IFN- γ production by spleen cells collected from mice and incubated in the presence or absence of heat-killed bacteria (LO28) for 48 h. When restimulated with heat-killed *Listeria* bacteria in vitro, splenocytes from mice infected with 1×10^6 *clpP* mutant bacteria produced an amount of IFN- γ similar to that produced by splenocytes from mice infected with wild-type LO28 (Fig. 5). Moreover, no difference was observed between mice infected with 1×10^6 and 5×10^8 *clpP* mutant bacteria, suggesting that T cells can be stimulated by both regimens.

DISCUSSION

We show in this work that the ClpP serine protease is essential for the in vivo intracellular growth of *L. monocytogenes* and plays a major role in the induction of anti-*Listeria* protective immunity. By using a *clpP* deletion mutant of *L. monocytogenes*, we first demonstrated that mutant bacteria failed to grow in macrophages during the early phase of infection (8 h) in vivo. After i.v. inoculation, most mutant bacteria were destroyed by Kpffer cells of the liver without invasion of hepatocytes (Fig. 1). In contrast, wild-type bacteria multiplied rapidly and invaded adjacent hepatocytes, as previously reported (14, 32). Indeed, the kinetics of elimination of the mutant in host tissues closely resemble those for LLO-deficient mutants (10, 12, 27, 30, 35). The role of ClpP was further confirmed by showing that intracellular growth in Kpffer cells was restored in a *clpP*-complemented mutant.

Our results are in agreement with the previous finding that the capacity of the *clpP* mutant to grow in cultured bone marrow-derived macrophages was strongly restricted in vitro (15). Under such conditions, only rare *clpP* mutant bacteria access the cytoplasm, where they multiply poorly and have very few actin comets (15). This behavior may be due to the reduction of functional LLO in the absence of ClpP, explaining the inability of the bacteria to escape the phagosomal compartment in macrophages. In addition, the reduction of intracytoplasmic multiplication and actin polymerization suggests that

ClpP may be required for the expression of other virulence factors, such as ActA (15).

LLO is an immunodominant antigen playing a crucial role in the development of anti-*Listeria* immunity (2, 4, 5, 9, 22, 26, 41). It has been demonstrated that the expression of LLO in heterologous bacterial hosts, such as *Bacillus anthracis* and *Salmonella enterica* serovar Typhimurium, is sufficient to specifically protect against *L. monocytogenes* (17, 39). Here, we demonstrate that low doses of viable *clpP* mutant bacteria failed to protect mice against a lethal challenge with wild-type LO28. Neither antibodies nor CTLs against LLO were detectable in these mice. This absence was not due to an inability to stimulate T cells in vivo, since splenocytes from these mice produced the same level of IFN- γ after in vitro exposure to *Listeria* antigens, thus confirming the major role of LLO in anti-*Listeria* protection. However, this lack of protection was overcome by use of high infecting doses of mutant bacteria (5×10^8). Under these conditions, a significant level of protection was induced in infected mice, as confirmed by the presence of anti-LLO CTLs and antibodies. This result indicates that the antigenic threshold necessary to stimulate significant anti-*Listeria* protective immunity is probably very low. Indeed, even with a high challenge dose, the very rapid destruction of bacteria in host tissues visualized in Fig. 1 suggests that minute amounts of LLO are sufficient to protect mice against listeriosis. In contrast, we found that an *hly*-disrupted mutant producing a truncated LLO completely failed to protect against listeriosis, even with a high infecting dose of 1×10^8 , as previously reported for other LLO-deficient mutants (3, 30). These observations demonstrate that LLO must be intact to adequately induce protective immunity.

Escape from the phagosome and intracytosolic multiplication of *L. monocytogenes* are important steps for the effective presentation of *Listeria* antigens to T cells, including CD4⁺ or CD8⁺ $\alpha\beta$ T cells and $\gamma\delta$ T cells, a step which is necessary for the development of specific immunity against listeriosis (24, 25). Although an alternative presentation pathway may also play a role (28, 34), *Listeria* antigens are mainly processed by the cytosolic pathway. Antigens are degraded to shorter peptides by the cytosolic proteasome machinery, transported through the endoplasmic reticulum, and presented as MHC class I-associated *Listeria* peptides to CD8⁺ cells (25, 42). The complexes presented by *Listeria*-infected cells are recognized and lysed by specific CD8⁺ cytolytic T cells. Among *L. monocytogenes*-specific antigens, LLO plays a major role through the activation of protective CD8⁺ MHC class I-restricted LLO-specific CTLs (22, 23, 33).

The LLO 91-99 peptide has been previously identified as the immunodominant epitope of *L. monocytogenes* (21, 33). However, CD8⁺ T cells specific for other *Listeria*-derived peptides (e.g., p60 227-225 and p60 449-457) have been shown to develop simultaneously with LLO 91-99-specific CD8⁺ T cells (6, 7, 16, 38, 42). Therefore, the lack of protection in the absence of ClpP may be due to a restriction of the availability of intracytoplasmic LLO-derived immunogenic peptides processed and presented to CD8⁺ T cells. This situation would result in a low cell surface density of MHC class I-presented epitopes, which would reduce the magnitude of the CTL response (6). In addition, the limited access to the cytoplasm for the *clpP* mutant may also alter the pathway of presentation of

peptides from other protective antigens, thus restricting the immune response against *L. monocytogenes*. In conclusion, our results show that the stress-induced serine protease ClpP modulates the production of LLO during the intracellular survival of *L. monocytogenes* in host tissues and thus represents a major determinant for immunity to *L. monocytogenes* by regulating the presentation of protective *Listeria* peptides by host macrophages.

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