Intracellular Antibody Neutralizes *Listeria* Growth

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Summary

We previously reported that treatment of mice with a neutralizing mAb against listeriolysin O (LLO), the pore-forming toxin of *Listeria monocytogenes*, provided resistance to this intracellular bacterium. We evaluated whether anti-LLO mAb would affect *Listeria* handling by macrophages, essential cells in *Listeria* resistance. Macrophages infected in the presence of anti-LLO mAb showed a marked reduction in intracellular *Listeria* growth, with a concomitant block in LLO-dependent *Listeria* passage from phagosome to cytosol. Anti-LLO mAb did not opsonize *Listeria* but, rather, acted within macrophages to neutralize LLO. Importantly, anti-LLO mAb effects on *Listeria* growth were independent of Fcγ receptor expression, IFNγ signaling, and production of nitric oxide and superoxide. These results identify a novel mechanism for antibody control of bacteria within macrophages.

Introduction

*Listeria monocytogenes* is a Gram-positive, facultative intracellular bacterium that has served as a model for understanding host resistance to infection. Early resistance is controlled by the innate immune response, such that neutrophils and macrophages largely control the initial phases of infection (Unanue, 1997). Antigen-specific T cells are then primed and activated over 4-6 days, resulting in the rapid clearance of all *Listeria* and the establishment of immunological memory (Busch et al., 1998, 1999). Antibody had long been thought to play no role in *Listeria* resistance, since early studies transferring serum from *Listeria*-immune mice to naive mice showed no ability to provide protection (Osebold and Sawyer, 1957; Mackaness, 1962; Miki and Mackaness, 1964). Recently, we reported the surprising finding that a neutralizing mAb to LLO, the secreted *Listeria* pore-forming toxin mediating bacterial escape to the host cell cytosol (Gaillard et al., 1987; Portnoy et al., 1988), revealed no reduction in *Listeria* growth. The neutralizing A4-8 anti-LLO mAb caused a marked reduction in *Listeria* number during the first 2 hr of infection (2- to 5-fold in four experiments), followed by the subsequent rapid replication of the organism within the cells (Figure 1A). A control mAb or E4-3, a nonneutralizing anti-LLO mAb, revealed no reduction in *Listeria* number during the first 2 hr of infection. At 6 hr postinfection, typically 5- to 10-fold fewer bacteria were present within the macrophages treated with A4-8, a reduction in *Listeria* number similar to that found in macrophages activated in vitro treatment with the cytokine IFNγ prior to infection (Figure 1B). Moreover, combined treatment with IFNγ and A4-8 was synergistic, causing a marked reduction in *Listeria* colony-forming units (CFU) greater than that provided by cytokine or antibody alone.

This pattern of *Listeria* growth in macrophages treated with neutralizing anti-LLO mAb is most consistent with an antibody-mediated interference in the passage of *Listeria* adhering to and being readily phagocytosed by macrophages in an opsonin-independent fashion (Pierce et al., 1996). Antibody opsonization of *Listeria* provides no increased host resistance (Mackaness, 1962; Miki and Mackaness, 1964). Therefore, we reasoned that anti-LLO antibody-mediated protection was occurring through a novel mechanism, distinct from the classical mechanism of enhanced macrophage phagocytosis of antibody-opsonized extracellular bacteria. One potential mechanism whereby antibody to LLO could provide protection against infection would be through the intracellular neutralization of LLO within phagocytic cells (a hypothesis first put forward by Casadevall [1998]). Neutralization of LLO would block *Listeria* escape to the host cell cytosol and thereby block the rapid bacterial replication which takes place at this site. Consistent with this hypothesis is the fact that mutant *Listeria* unable to secrete LLO are virtually avirulent in vivo, do not reach the host cell cytosol, and are rapidly killed within phagolysosomes of macrophages (Portnoy et al., 1998). Here, we describe experiments aimed at testing this hypothesis, utilizing resting murine peritoneal macrophages and mouse anti-LLO mAb. Our results indicate that indeed antibody can act intracellularly to neutralize a secreted *Listeria* virulence factor, having profound implications on the role of antibody to intracellular microbial pathogens in general. We provide evidence that this mechanism of protection also operates in vivo and may be responsible for our previous results.

Results

Antibody to LLO Limits *Listeria* Growth within Macrophages

To assess the effect of anti-LLO mAb on macrophage handling of *Listeria*, we measured intracellular *Listeria* growth in resident peritoneal macrophages. Cells were incubated ex vivo with the antibody, subsequently infected with *Listeria*, and then treated with gentamicin at a concentration sufficient to kill only extracellular organisms but having no effect on intracellular bacterial growth. The neutralizing A4-8 anti-LLO mAb caused a marked reduction in *Listeria* number within macrophages during the first 2 hr of infection (2- to 5-fold in four experiments), followed by the subsequent rapid replication of the organism within the cells (Figure 1A). A control mAb or E4-3, a nonneutralizing anti-LLO mAb, revealed no reduction in *Listeria* number during the first 2 hr of infection. At 6 hr postinfection, typically 5- to 10-fold fewer bacteria were present within the macrophages treated with A4-8, a reduction in *Listeria* number similar to that found in macrophages activated in vitro treatment with the cytokine IFNγ prior to infection (Figure 1B). Moreover, combined treatment with IFNγ and A4-8 was synergistic, causing a marked reduction in *Listeria* colony-forming units (CFU) greater than that provided by cytokine or antibody alone.
Figure 1. Neutralizing Anti-LLO Antibody Limits Intracellular Listeria Growth within Macrophages

(A) Resident peritoneal macrophages from SCID mice were treated in vitro with 10 μg/ml control mAb, E4-3 anti-LLO nonneutralizing mAb, or A4-8 anti-LLO neutralizing mAb and were infected with Listeria EGD. CFU per coverslip were evaluated at the indicated times (mean ± SEM).

(B) Resident peritoneal macrophages from SCID mice were treated in vitro with or without IFNγ (100 U/ml) and with control or A4-8 mAb (100 μg/ml) and were infected with Listeria EGD. CFU per coverslip were evaluated at 6 hr postinfection (mean ± SEM).

Listeria from phagosome to cytosol, allowing the macrophage to carry out listericidal mechanisms on those bacteria forced to remain in the phagosome. Those Listeria managing to reach the cytosol (a smaller number than in control cells) would still be able to divide in an uncontrolled manner within this host compartment, at a rate similar to the cytosolic bacteria in control cells. These ideas will be explicitly addressed in experiments below.

No reduction in Listeria growth took place when organisms were first incubated with the A4-8 mAb and washed prior to infection of the macrophages (Figure 2A), confirming that the A4-8 antibody was not acting as an opsonin. A4-8 treatment of macrophages from mice deficient in either the Fcγ activating receptors FcγRI and FcγRIII (Fcγ chain-deficient mice) or the inhibitory receptor FcγRII caused a reduction of Listeria growth indistinguishable from the treatment of control macrophages.

Figure 2. Anti-LLO Neither Opsonizes Listeria Nor Requires Fcγ Receptor Expression for Function in Macrophages

(A) Listeria EGD were incubated overnight with various concentrations of control mAb, A4-8 mAb, or buffer alone (0), washed, and then were used to infect resident peritoneal macrophages from SCID mice. CFU per coverslip were evaluated at 6 hr postinfection (mean ± SEM).

(B) Resident peritoneal macrophages from CB.17, Fcγ chain-deficient, or FcγRII-deficient mice were treated in vitro with control or A4-8 mAb (100 μg/ml) and were infected with Listeria EGD. CFU per coverslip were evaluated at 6 hr postinfection (mean ± SEM).
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smaller absolute number of organisms were present within A4-8-treated cells (for example, control cells: average of 5 bacteria/cell, 3 of 5 cytosolic; A4-8-treated: average of 2.6 bacteria/cell, 0.4 of 2.6 cytosolic). Treatment with neutralizing anti-LLO mAb, therefore, partially blocks Listeria escape to the cytosol and has a marked effect on limiting Listeria numbers within the macrophages.

Antibody Acts Intracellularly to Block LLO within Macrophages

We examined whether the A4-8 antibody could act within macrophages to neutralize LLO. Macrophages were incubated overnight with mAb prior to infection, extensively washed, and subsequently infected with Listeria organisms. Treatment with the A4-8 mAb in this protocol still resulted in a marked control of Listeria growth within macrophages (Figure 4A), although to a lesser degree than when antibody was present both before and during infection. To determine the effective life span of anti-LLO molecules within macrophages, cells were treated with mAb overnight, then washed extensively, and incubated for 0, 4, or 8 hr in the absence of mAb (so-called “chase”; Figure 4B). Cells were then infected with Listeria, and intracellular bacterial growth was measured. Anti-LLO mAb had significantly less effect on bacterial growth if chased for 4 or 8 hr, suggesting proteolytic breakdown of the antibody within the macrophages.

A4-8 molecules were seen in discrete vesicles throughout the macrophages and could be colocalized with Listeria-containing vacuoles by confocal microscopy (Figure 5). These Listeria were confirmed to be in phagosomes, as they did not stain with phalloidin. Antibody molecules could also be found in vesicles of macrophages treated with antibody prior to infection. In these cases, the macrophages were washed and infected for 2 hr in the absence of mAb. Control antibody-treated cells also showed intracellular antibody-containing vesicles that sometimes colocalized with Listeria-containing vacuoles, although many more bacteria in these cells were cytosolic.

To precisely determine the amount of A4-8 antibody taken up by the macrophages through fluid-phase endocytosis, we incubated macrophages overnight with 100 μg/ml A4-8, washed the cells extensively, and then made cell lysates. The amount of A4-8 contained within the lysates was quantitated through use of an ELISA assay with plate-bound, pure recombinant histidine-tagged LLO (see Experimental Procedures). The lysate of 3.88 × 10^5 cells contained a total of 4.95 ng A4-8, equivalent to ~5 × 10^5 functional antibody molecules/macrophage.

Requirements for IFNγ Signaling, RNI, and ROI during Anti-LLO Action

A4-8 mAb affected Listeria handling without activation of the macrophages. A4-8 treatment dramatically limited Listeria growth within peritoneal macrophages from mice deficient in either the IFNγ receptor or the STAT1 molecule, the transcription factor mediating IFNγ signaling (Figures 6A–6C). However, in these signaling-deficient macrophages, in vitro treatment with IFNγ or the
Immunity

Figure 4. Anti-LLO Functions Intracellularly within Macrophages to Limit Listeria Growth

(A) Resident peritoneal macrophages from SCID mice were treated in vitro with control or A4-8 mAb (100 μg/ml) and were infected with Listeria EGD. Antibody was present in the culture, either at all stages of the experiment (overnight preinfection, during infection, and during the time of gentamicin treatment) or just at specified times. CFU per coverslip were evaluated at 6 hr postinfection (mean ± SEM).

(B) Resident peritoneal macrophages from SCID mice were treated in vitro with control or A4-8 mAb (10 μg/ml) and were infected with Listeria EGD. Antibody was present in the culture, either at all stages of the experiment (throughout) or cells were washed and then chased in media in the absence of mAb for the indicated time period prior to infection. CFU per coverslip were evaluated at 6 hr postinfection (mean ± SEM).

combination of IFN-γ and TNF-α had no ability to induce listericidal activity. Thus, the effect of the A4-8 mAb in no way requires IFN-γ-mediated macrophage activation.

We performed experiments in which antibody-treated macrophages were also treated with the iNOS inhibitor aminoguanidine hemisulfate and/or the cell-permeable superoxide scavenging molecule MnTMPyP. Anti-LLO treatment in the presence of these chemicals still led to marked control of Listeria replication, indicating that macrophages utilize mechanisms other than RNI or ROI to control Listeria growth (Figure 6D). To confirm our data with MnTMPyP, we also utilized macrophages from mice deficient in the gp91 subunit of the NADPH oxidase and again confirmed the presence of RNI- and ROI-independent killing mechanisms (Figure 6E).

To further explore this issue of RNI- and ROI-independent Listeria killing, we compared Listeria growth in macrophages infected with wild-type Listeria (10403S) or a mutant strain deleted for LLO (EJL1) (Figure 6F). This strain of Listeria is completely retained within macrophage phagosomes (data not shown). Therefore, use of EJL1 provided a second method to confirm our results regarding radical-independent killing of phagosome-retained organisms, which we demonstrated above through the use of anti-LLO mAb-mediated blockade of Listeria escape to the cytosol. Macrophages efficiently controlled the growth of the EJL1 strain, independent of cytokine activation, RNI, and ROI.

Anti-LLO Action within Macrophages Occurs In Vivo

To determine whether anti-LLO-mediated Listeria resistance in vivo (Edelson et al., 1999) also occurs within macrophages, we performed the following experiment. SCID mice were treated with 1 mg mAb i.p. and 3 days later infected with 1 × 10⁸ Listeria i.p. Infected peritoneal macrophages were collected 1 hr postinfection, and the intracellular replication of bacteria in these cells was followed. The growth of Listeria in cells from mice treated with the anti-LLO mAb was markedly less than in cells from mice treated with control mAb (Figure 7). In fact, the pattern of growth was similar to that seen in our ex vivo infection system (Figure 1A), where a large number of bacteria were killed during the first 2 hr of infection, with subsequent growth of those organisms that had managed to escape to the cytosol.

Discussion

Antibody-Mediated Listeria Resistance

This report demonstrates that neutralization of LLO by a mAb occurred intracellularly within resting macrophages and that this neutralization resulted in the limitation of Listeria growth. This process was not mediated by Listeria opsonization nor required Fcγ receptor ligation. Antibody molecules were taken into macrophages through fluid phase endocytosis and could traffic within endosomal compartments of the cell to encounter Listeria-containing vacuoles. This encounter resulted in Listeria retention within phagosomes, akin to what occurs for Listeria organisms deficient in LLO production. Retention in the phagosome resulted in the demise of the organism through mechanisms that did not require the participation of RNI or ROI.

Macrophage uptake of soluble proteins from solution is known to occur at a rate proportional to their concentration in the culture medium and serves as one mechanism whereby macrophages acquire proteins to be presented by MHC class II molecules. Incubation in 0.7 μM mAb (100 μg/ml) resulted in ~5 × 10⁸ molecules/macrophage, an estimation very much consistent with that found for the fluid phase uptake of soluble HRP (Steinman and Cohn, 1972). This number of intracellular
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Figure 5. Anti-LLO-Containing Vesicles Co-localize with Listeria-Containing Vacuoles within Macrophages

Resident peritoneal macrophages from SCID mice were treated in vitro with A4-8 mAb (100 μg/ml) and were infected with Listeria EGD. At 2 hr postinfection, cells were stained with (A) rabbit anti-Listeria polyserum followed by Cy5 goat anti-rabbit IgG (blue), (B) Alexa 488 phalloidin (green), and (C) Cy3 goat anti-mouse IgG (red) to identify Listeria- and mAb-containing vesicles and were examined by confocal microscopy. (D) Represents the triple overlay of the images, with (E) and (F) being higher-power views of particular areas of the image in (D). Numbers in the left corner denote length in microns. Note that all Listeria (seven organisms in total) are contained within phagosomes, based on their negative staining with phalloidin. In (E) and (F), antibody-containing vesicles can be seen colocalizing with Listeria-containing vacuoles.

anti-LLO mAb molecules is sufficient to neutralize the relatively small amount of LLO thought to be produced by Listeria (measured to be one molecule per minute per bacterium; Villanueva et al., 1995). Based on our pulse-chase experiment (Figure 4B), we estimate that the half-life of functional A4-8 antibody within resting macrophages was 2–4 hr, consistent with a previous measurement of the half-life of 125I-IgG within murine macrophages (t1/2 = 2.6 hr; Mellman et al., 1983).

The concentration of antibody used in our ex vivo experiments is easily reached by the in vivo injection of A4-8, which 1 day after a 1 mg injection has a serum concentration of ~300 μg/ml (data not shown). Indeed, macrophages harvested as long as 3 days after anti-LLO mAb injection in vivo controlled Listeria infection ex vivo. This finding supports our contention that one locus of action of the antibody in the mouse is by a mechanism akin to what we have found ex vivo. In vivo, there was a significant reduction in organ Listeria burden in mice treated with anti-LLO mAb (Edelson et al., 1999).

This protection was evident very early (a 10-fold reduction in Listeria burden was seen as early as 6 hr postinfection) and by 2 days postinfection was very marked (2–3 log reduction in Listeria CFU in spleen and liver). During the first 2 days of Listeria infection in vivo, macrophages become activated via the action of cytokines, including IFN-γ. Our ex vivo results show a synergy between anti-LLO mAb and IFN-γ activation of macrophages, possibly explaining the significant reduction in Listeria CFU seen in vivo at 2 days postinfection after anti-LLO mAb treatment.

In addition to the intracellular neutralization of LLO within macrophages, anti-LLO mAb may also provide resistance in vivo through neutralization of the exotoxin activities of LLO (Guzman et al., 1996; Kayal et al., 1999; Merrick et al., 1997; Nishibori et al., 1996; Sibelius et al., 1996, 1999; Tang et al., 1996; Wadsworth and Goldfine, 1999). We have previously documented a role for extracellular LLO in the induction of lymphocyte apoptosis during early Listeria infection (Merrick et al., 1997), and
in preliminary experiments with anti-LLO mAb we have seen a significant decrease in the extent of lymphocyte apoptosis within the spleen at 48 hr postinfection (data not shown). This effect may be a direct one of neutralization of extracellular LLO or an indirect one as a result of the mechanism described here (i.e., intracellular neutralization limiting Listeria growth). Regardless, how the severe lymphocyte apoptosis plays a role in Listeria resistance is unclear at this time.

Our data with anti-LLO mAb represents a striking example of intracellular antibody neutralizing a secreted bacterial virulence factor, resulting in the inhibition of bacterial replication. However, there are precedents in viral resistance for the ability of antibody molecules to neutralize viral replication intracellularly and, in some cases, to also provide in vivo protection against viral infection (Bomsel et al., 1998; Burns et al., 1996; Gollins and Porterfield, 1986; Levine et al., 1991; Mazanec et al., 1992, 1995; Virgin et al., 1994). IgA and IgM molecules were shown to neutralize viral replication within or viral transcytosis across epithelial cells. In these studies, antibody applied to the basolateral surface was transported through cells via the polymeric Ig receptor, and virus was applied on the apical surface. Antibody and virus were seen to colocalize within endosomes, resulting in a restriction in viral titer and a recycling of antibody-virus complexes to the apical surface. Other mechanisms have been shown to exist for IgG-mediated intracellular viral neutralization, involving either antibody inhibition of intraendosomal proteolytic viral uncoating or inhibition of intraendosomal fusion of the viral envelope with the endosomal membrane.
Figure 7. Anti-LLO Antibody Acts In Vivo within Macrophages

CB.17-SCID mice were treated with 1 mg control or anti-LLO mAb and 3 days later were infected with \(1 \times 10^6\) Listeria EGD i.p. At 1 hr postinfection, peritoneal macrophages were collected, and the intracellular growth of Listeria was followed. CFU per coverslip were evaluated at 0, 2, 4, and 6 hr. At time 0, control CFU per coverslip were 1.42 \(\times\) \(10^3\) \(\pm\) 2.39 \(\times\) \(10^3\), and anti-LLO CFU per coverslip were 2.05 \(\times\) \(10^3\) \(\pm\) 4.08 \(\times\) \(10^3\). Listeria growth is represented as the percent relative to time 0. Within control macrophages, Listeria number remained constant during the first 2 hr of infection, with bacteria completing almost three cell divisions between times 2 and 6 hr of infection. Within macrophages from mice with anti-LLO, approximately half of all bacteria were killed during the first 2 hr of infection, at which time the bacterial replication rate was similar to that in control cells.

Macrophage Control of Listeria Growth

Over the past decade, several studies examined the mechanisms of Listeria killing by macrophages (Alvarez-Dominguez and Stahl, 1999; Beckerman et al., 1993; de Chastellier and Berche, 1994; Fehr et al., 1997; Fleming and Campbell, 1997; Frei et al., 1993; Higginbotham et al., 1992; Inoue et al., 1995; Leenen et al., 1994; Muller et al., 1999; Ohy et al., 1998a, 1998b; Portnoy et al., 1989; Shihol et al., 1999; Tanaka et al., 1995). Experimental protocols have varied considerably and have resulted in different conclusions on how macrophages kill Listeria, particularly with regard to the role of RNI and ROI in killing.

Here, we attempt to consolidate these disparate findings into a coherent picture of how macrophages handle Listeria, in light of our results here with anti-LLO mAb. Upon initial infection, Listeria organisms are contained within primary phagosomes. Some organisms escape from this compartment to the cytosol via the action of LLO, prior to phagosome-lysosome fusion. After this fusion event, organisms which have not yet reached the cytosol will be killed. Evidence for this model comes from an electron microscopic study of Listeria infection, which identified the lysosomal enzyme acid phosphatase within the compartment of Listeria killing (de Chastellier and Berche, 1994). This report suggested a "competition" between the efficiency of Listeria trafficking to this lysosomal compartment, where killing is believed to occur, and the speed and capacity of Listeria to escape from the primary phagosome. A second piece of evidence for this model comes from a study showing that blockade of phagolysosome fusion by antisense oligonucleotide depletion of the GTPase rab5a blocks killing of LLO-deficient Listeria (Alvarez-Dominguez and Stahl, 1999). Our results with anti-LLO antibody fit well with this model, such that antibody-mediated phagosome retention of Listeria allows for greater opportunity for phagosome-lysosome fusion, such that subsequent bacterial killing can take place.

We interpret our results on the role of IFN-γ as one regulating vesicular traffic and separate from the induction of RNI or ROI. Indeed, this model of Listeria trafficking may explain the paradoxical findings that macrophage activation with IFN-γ works to limit cytosolic escape and enhance the killing of wild-type Listeria yet induces little enhancement in the already efficient killing of LLO-deficient organisms (Figure 6F; Portnoy et al., 1989). For the case of wild-type Listeria, if IFN-γ treatment were to simply enhance the overall rate of phagosome-lysosome fusion, the time period of Listeria existence in the primary phagosome would be limited, and the number of organisms delivered to the phagolysosome for killing would be increased. For the case of LLO-deficient Listeria, all organisms are delivered to the phagolysosome, independent of the speed of trafficking, such that the cytokine would not enhance bacterial killing. Two studies have provided support for this idea of IFN-γ-mediated enhancement of phagosome-lysosome fusion in macrophages (Alvarez-Dominguez and Stahl, 1998; Via et al., 1998).

Regarding the role of RNI and ROI in Listeria killing by resident peritoneal macrophages, we found essentially no role for RNI and only a limited role for ROI. These results are largely consistent with the work of other investigators, with differences likely due to either the particular cell type used or the assessment of mechanisms of intracellular microbial killing. Two studies have provided support for this idea of IFN-γ-mediated enhancement of phagosome-lysosome fusion in macrophages (Alvarez-Dominguez and Stahl, 1998; Via et al., 1998).

Experimental Procedures

Mice

CB.17, CB.17-SCID, Fc receptor γ chain-deficient (Balb/c background), FcγRII-deficient (Balb/c background), 129 Sv/Ev, IFN-γ receptor-deficient (129 Sv/Ev background), STAT 1-deficient (129 Sv/Ev background), C57BL/6J, and NADPH oxidase-deficient (gp91phox−/−, C57BL/6J background) mice were maintained and bred under SPF conditions in the Washington University mouse facility (Huang et al., 1993; Meraz et al., 1996; Pollock et al., 1995; Takai et al., 1994; Takai et al., 1996). Fc receptor-deficient mice were a gift of Dr. Jeffrey Ravetch, Rockefeller University, New York, NY. 129 Sv/Ev, IFN-γ receptor-deficient, and STAT 1-deficient mice were a gift of Dr. Robert Schreiber, Washington University, St. Louis, MO. NADPH oxidase-deficient mice were a gift of Dr. Mary Dinauer, Indiana University School of Medicine, Indianapolis, IN. C57BL/6J mice were obtained from the Jackson Laboratory, Bar Harbor, ME. Macrophages were obtained from mice of either sex, between ages 8 and 20 weeks.
Bacteria
Listeria monocytogenes strains used in this study were the following: the wild-type strain EGD, the LLO deletion mutant EJL1, and the wild-type parent strain of EJL1, 10403S. EJL1 and 10403S were a gift of Dr. Hao Shen, University of Pennsylvania, Philadelphia, PA. Bacteria was stored as frozen glycerol stocks at −80°C. For ex vivo infection of macrophages, overnight cultures of Listeria were grown in BHIB (Becton Dickinson, Cockeysville, MD) at 37°C without agitation. Listeria concentration was estimated from a standard curve at OD600. Listeria was diluted into DMEM (GIBCO-BRL: Grand Island, NY) + 10% heat-inactivated FCS (HyClone; Logan, UT) (no antibiotics added) immediately prior to infection of macrophages.

Antibodies
mAbs utilized in this study were A4-8 (murine anti-LLO, IgG1), E4-3 (murine anti-LLO, IgG1), and the irrelevant control GIRL208 (murine anti-human IFNγ receptor, IgG1, a gift of Dr. Robert Schreiber, Washington University, St. Louis, MO). The generation and properties of anti-LLO mAbs A4-8 and E4-3 were described previously (Nato et al., 1991). Neutralizing A4-8 binds LLO in ELISA assays and blocks LLO-mediated lysis of RBC in vitro. Nonneutralizing E4-3 binds LLO in ELISA assays but does not block RBC lysis by LLO in vitro. mAbs were purified from ascites using standard methods on protein A-Sepharose (Sigma Chemical Company; St. Louis, MO) and contained less than ten endotoxin units per milligram protein by the QCL-1000 endotoxin quantitation kit (BioWhittaker, Walkersville, MD). Antibody concentration was determined by absorbance at 1 mg/ml equals 1.35 absorbance units.

Ex Vivo Listeria Infection of Macrophages
Resident peritoneal exudate cells (PEC) were obtained by peritoneal lavage and plated in DMEM + 10% heat-inactivated FCS supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin (complete media) on 12 mm glass coverslips (Fisher Scientific, Pittsburgh, PA) in 24-well plates (Costar, Corning, NY). PEC were plated at 5 × 10^5 cells per well in a volume of 1 ml and were incubated at 37°C for 2–4 hr. Coverslips were dipped five times in warm complete media to remove nonadherent cells and were placed in new 24-well plates. The remaining cells were typical adherent macrophages. Cells were cultured 24 hr in complete media and then washed three times in antibiotic-free complete media before the addition of mAbs.

For experiments in which macrophages were treated with mAbs, cells were cultured an additional 16 hr in the presence of 10 μg/ml or 100 μg/ml mAb in antibiotic-free media prior to infection. This media was removed from coverslips, and 1 × 10^5 Listeria, with or without fresh mAb, was added to each well. Plates were centrifuged at 600 × g for 10 min at room temperature to synchronize the infection of cells and then were incubated at 37°C for 15 min (time 0). After an additional 15 minutes at 37°C, media was again aspirated and changed to media containing fresh mAb and 5 μg/ml gentamicin (GIBCO-BRL), capable of killing any remaining extracellular Listeria but not capable of affecting the growth of intracellular bacteria. Plates were kept at 37°C during the course of the assay. For some experiments, macrophages were pulsed with mAb for 16 hr and then were washed three times prior to the infection and gentamycin steps, which were performed in the absence of mAb. In some experiments, after cells were washed, they were incubated in media for various time periods in the absence of mAb (chased) and were later infected and treated with gentamicin (both in the absence of mAb).

In some experiments, macrophages were pretreated for 2 days in vitro with murine IFNγ (100 U/ml or 100 U/ml) or murine TNFα (1000 U/ml; both gifts of Dr. Robert Schreiber, Washington University, St. Louis, MO). In other experiments, inducible nitric oxide synthase (iNOS) was inhibited with the specific chemical inhibitor aminoquinidine hemisulfate (Sigma), used at 1 mM, present at all times prior to and throughout the course of the infection (Beckerman et al., 1993; Corbett et al., 1992). Superoxide radicals were scavenged with the cell-permeable scavenger manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP; Alexis, San Diego, CA), used at 100 μM, present throughout the course of the infection (Gardner et al., 1996; Muller et al., 1999).

To test whether mAb acted as an opsonin for Listeria organisms, 2 × 10^8 bacteria were washed in PBS and were incubated in 1 ml mAb solution in PBS (mAb concentrations ranged from 1 μg/ml to 1 μg/ml). Bacteria and antibody were rotated overnight at 4°C, washed three times with PBS, and bacterial concentration was redetermined by OD650. This bacteria was then diluted and used for infection of macrophages.

At various time points postinfection, coverslips (each condition was assayed in triplicate) were taken to assess CFU/coverslip. Coverslips were dipped five times in warm PBS, then placed into 10 ml cold sterile deionized water, vortexed for 30 s to lyse cells, and serial dilutions of lysates were plated on BHI-agar plates. Bacterial colonies were counted after overnight growth at 37°C.

Quantitation of Antibody Uptake by Macrophages
Macrophages from CB.17-SCID mice were incubated with either control mAb or A4-8 (100 μg/ml), as described for the infection experiments. After 22 hr, total cell number per coverslip was determined by counting the cells in 12 high-power (400 ×) fields of view. The area per field of view was determined by use of a slide micrometer, allowing an assessment of the average cell number per field of view and therefore the cell number per coverslip. Coverslips were gently dipped in warm PBS a total of 20 times to remove extracellular antibody and then were placed into fresh media of a 24-well plate. Cells from 12 coverslips (4 × 10^5 cells total) were lysed in a total volume of 300 μl lysis buffer (PBS plus 40 mM N-methyl-N-octanoyl-D-glucamine, 40 mM N-methyl-N-nonanoyl-D-glucamine, 1 mM PMSF, 10 mM iodoacetamide, and 20 μg/ml leupeptin) by vigorous pipetting, and lysate was subsequently centrifuged at 12,000 × g for 5 min. Control coverslips without cells were treated in an identical fashion.

To determine the amount of A4-8 mAb in these lysates, an ELISA assay was performed with plate-bound histidine-tagged recombinant LLO (HisLLO; produced in E. coli, a gift of Dr. Daniel Portnoy, University of California, Berkeley, CA [Gedde et al., 2000]). This assay has been described previously and was only modified here by coating the plates with a greater amount of HisLLO (500 ng/well) to allow more sensitive detection of antibody (Edelson et al., 1999).

Cell lysis (100 μl) was used as primary antibody in the assay, washed and treated in identical fashion. Following secondary goat anti-mouse IgG-peroxidase (Boehringer-Mannheim, Indianapolis, IN), Plates were then treated with 1 mM ABTS in citrate buffer with 0.05% H2O2, and OD415 was measured. Absorbance values were compared to a standard curve made by performing the ELISA with dilutions of the A4-8 mAb in lysis buffer. The assay allowed the reliable detection of between 1 and 1000 nanograms of A4-8.

Immunofluorescent Localization of Listeria and mAb within Macrophages
Antibodies and reagents used for staining were as follows: rabbit anti-Listeria polysacrylam (serotypes 1 and 4, and used at 1/200 dilution; Difco, Detroit, MI), FITC goat anti-rabbit IgG (used at 1/200 dilution; Sigma), Cy5 goat anti-rabbit IgG (used at 1/400 dilution; Amersham Pharmacia, Piscataway, NJ), Cy3 goat anti-mouse IgG (used at 1/400 dilution; Amersham Pharmacia), Alexa 488 phalloidin (used at 1/20 dilution; Molecular Probes, Eugene, OR), and Alexa 594 phalloidin (used at 1/20 dilution; Molecular Probes).

Coverslips were taken after a 2 hr Listeria infection of macrophages for immunofluorescence staining. All steps were carried out at room temperature. Coverslips were washed in PBS, and cells were fixed for 30 min in 10% buffered formalin. Coverslips were washed again in PBS, and cells were permeabilized with Triton X-100 (150 mM NaCl, 20 mM Tris pH 7.4) 0.1% Triton X-100 (TBS-Tx) + 1% BSA (GIBCO-BRL) for 15 min. Cells were stained with rabbit anti-Listeria polysacrylam diluted in TBS-Tx + 1% BSA for 1 hr and then washed in TBS-Tx. Secondary staining was then performed with fluorochrome-conjugated goat anti-rabbit IgG (either FITC or Cy5) and/or Cy3 goat anti-mouse IgG (either Alexa 488 or Alexa 594) diluted in TBS-Tx + 1% BSA. Phalloidin staining identifies only those Listeria within the host cell cytosol, as Listeria within this compartment are known (1-methyl-(4-pyridyl) porphyrin pentachloride (MnTMPyP; Alexis, San Diego, CA), used at 100 μM, present throughout the course of the infection (Gardner et al., 1996; Muller et al., 1999).

To test whether mAb acted as an opsonin for Listeria organisms,
instructions. Cells were observed with either a standard Zeiss Axio- 
aphot fluorescence microscope (Zeiss, Germany) or with a Sarastro 2000 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, CA). Confocal images were within a single plane, with a thickness of ~0.5 µm.

For quantitative assessment of Listeria localization, organisms were scored as “phagosomal” if they stained positively with anti-Listeria antibody but not with phallolidin. Organisms were scored as “cytosolic” if they stained positively with both anti-Listeria antibody and phallolidin. Cells were observed at a magnification of 630×, counting the organisms contained within the central 12 cells per field of view. Six randomly selected fields of view were counted per slide.

In Vivo mAb Treatment and Listeria Infection
CB.17-SCID mice were infected with 1 mg mAb (p.A4-8 or GIR.208 control mAb) in saline. Three days later mice were infected with 1 × 10^8 Listeria EGD i.p. (diluted directly from a frozen stock into saline). One hour later, infected PECs were collected in complete media plus 5 µg/ml gentamicin (no penicillin or streptomycin). 5 × 10^6 cells per well were added to coverslips in a 24-well plate, and pelleted by centrifugation at 100 × g for 5 min at room temperature. After 30 min of adherence at 37°C, this time point was taken to be time 0, and CFU per coverslip were determined as described above, at times 0, 2, 4, and 6 hr.

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