α-Galactosylceramide Promotes Killing of *Listeria monocytogenes* within the Macrophage Phagosome through Invariant NKT-Cell Activation

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α-Galactosylceramide (α-GalCer) has been exploited for the treatment of microbial infections. Although amelioration of infection by α-GalCer involves invariant natural killer T (iNKT)-cell activation, it remains to be determined whether macrophages (Mφ) participate in the control of microbial pathogens. In the present study, we examined the participation of Mφ in immune intervention in infection by α-GalCer using a murine model of listeriosis. Phagocytic and bactericidal activities of peritoneal Mφ from C57BL/6 mice, but not iNKT cell-deficient mice, were enhanced after intraperitoneal injection of α-GalCer despite the absence of iNKT cells in the peritoneal cavity. High levels of gamma interferon (IFN-γ) and nitric oxide (NO) were detected in the peritoneal cavities of mice treated with α-GalCer and in culture supernatants of peritoneal Mφ from mice treated with α-GalCer, respectively. Although enhanced bactericidal activity of peritoneal Mφ by α-GalCer was abrogated by endogenous IFN-γ neutralization, this was only marginally affected by NO inhibition. Similar results were obtained by using a listeriolysin O-deficient strain of *Listeria monocytogenes*. Moreover, respiratory burst in Mφ was increased after α-GalCer treatment. Our results suggest that amelioration of listeriosis by α-GalCer is, in part, caused by enhanced killing of *L. monocytogenes* within phagosomes of Mφ activated by IFN-γ from iNKT cells residing in an organ(s) other than the peritoneal cavity.

*L. monocytogenes*, a Gram-positive facultative intracellular bacterium, is the causative agent of listeriosis, with an overall mortality rate of 30% (76). A major virulence factor of *L. monocytogenes* is listeriolysin O (LLO), a 58-kDa protein encoded by the *lly* gene (26, 42, 65). LLO promotes intracellular survival of *L. monocytogenes* in professional phagocytes such as macrophages (Mφ) by promoting listerial escape from the phagosome into the cytosol (10, 22, 26, 42, 62, 65). Cells of the innate immune system play a pivotal role as a first line of defense against *L. monocytogenes* infection and among these, mononuclear phagocytes are critical (56, 61). Activation of Mφ by gamma interferon (IFN-γ) is mandatory for elimination of *L. monocytogenes* (31, 35). Nitric oxide (NO) synthesized by inducible NO synthase, which is localized in the cytosol of professional phagocytes, participates in killing of *L. monocytogenes* (48, 52, 69, 71). Similarly, reactive oxygen intermediates (ROI) play a role in killing of *L. monocytogenes* within the phagosome (52, 53, 59).

Natural killer T (NKT) cells represent a unique T-lymphocyte subset that are capable of recognizing glycolipids presented by the nonpolymorphic antigen presentation molecule CD1d (6, 40). Activated iNKT cells activate both type 1 and type 2 effector responses, including the production of interferon-γ (IFN-γ), interleukin-4 (IL-4), tumor necrosis factor-α (TNF-α), and IL-12, and the promotion of cytotoxic T cells. The number of iNKT cells in peripheral blood is lower in SJL mice than in C57BL/6 mice, and this decrease is associated with increased susceptibility to bacterial infection (32, 51). IL-15-deficient (IL-15−/−) mice are protected against murine peritonitis by iNKT cells (52, 53), and IL-15 increases the expression of CD1d on Mφ, which promotes iNKT cell activation (59). In vivo administration of α-GalCer causes prompt release of various cytokines by iNKT cells, which are involved in the control of various diseases, e.g., tumor rejection and prevention of autoimmune diseases (33, 41, 67, 70). Although α-GalCer has been reported to enhance host resistance to some microbial pathogens (27–29, 37, 39, 44, 55, 64), its potential role in protection against intracellular bacterial infections remains enigmatic.
We have recently described that α-GalCer ameliorates murine listeriosis, which is, in part, caused by accelerated infiltration of inflammatory cells into the liver (18), although iNKT cells themselves exacerbate disease (19). Because Mφ play a central role in the elimination of L. monocytogenes, we considered the possibility that Mφ participate in enhanced resistance to L. monocytogenes infection caused by α-GalCer treatment. In the present study, we examined the influence of α-GalCer on listicericidal activities of Mφ using a virulent and an avirulent strain of L. monocytogenes.

MATERIALS AND METHODS

Mice. C57BL/6, IL-15-/-, SJL, and recombination-activating gene 1/- (RAG-1/-) mice were purchased from Japan SLC (Hamamatsu, Japan), the Jackson Laboratory (Bar Harbor, ME), Charles River Laboratories Japan (Yokohama, Japan), and the Jackson Laboratory, respectively, and maintained under specific-pathogen-free conditions at our animal facilities. Weight-matched female mice were used at 8 to 12 weeks of age in accordance with institutional guidelines of Gunma University, Max Planck Institute for Infection Biology and Nara Medical University. Unless otherwise stated, C57BL/6 mice were used.

Bacteria and infection. The parental wild-type strain of L. monocytogenes used in the present study was EGD. The Δhly mutant strain was constructed from the wild-type strain of L. monocytogenes by homologous recombination as described previously (30). L. monocytogenes EGD and Δhly strains recovered from infected liver were grown in tryptic soy broth (Difco Laboratories, Detroit, MI) at 37°C for 18 h, and aliquots were frozen at –80°C for later use. The final concentration of viable bacteria was enumerated by plate counts on tryptic soy agar (Difco Laboratories). Mice were infected intraperitoneally (i.p.) with 2 × 10⁹ L. monocytogenes bacteria. Unless otherwise stated, strain EGD was used.

Antibodies. Monoclonal antibodies (MAbs) against Fcγ receptor (FcγR) and IFN-γ were used (145-2C11, XMG1.2, respectively) were purified from hybridoma culture supernatants by ammonium sulfate precipitation and affinity chromatography on Protein A- or G-Sepharose (Amersham Biosciences, Freiburg, Germany). Alexa 594-conjugated anti-rabbit antibody (Ab) at room temperature for 2 h, followed by Alexa 594-conjugated anti-rabbit IgG Ab at room temperature for a further 2 h. Specimens were stained with Alexa 488-conjugated phalloidin (Molecular Probes) and/or rabbit anti-TNFα Ab at room temperature for 2 h.

Flow cytometry. Cells were incubated with anti-FcγR MAb and then stained with conjugated MAb at 4°C for 15 min. Stained cells were washed with phosphate-buffered saline containing 0.1% bovine serum albumin (Thermo, Hamilton, New Zealand) and 0.1% sodium azide (Wako Pure Chemical Industries, fixed with 1% paraformaldehyde (Merek, Darmstadt, Germany), acquired by FACSCalibur (BD Biosciences, Mountain View, CA), and analyzed with CellQuest software (BD Biosciences). Cells were stained with phycoerythrin (PE)-labeled α-GalCer/CD1d tetramer for 15 min at room temperature after blocking.

Enzyme-linked immunosorbent assay. IFN-γ levels were determined by Quanti-kinie (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer’s instructions. The detection limit was 2 pg/ml.

In vivo measurement. NO was measured using the Griess reagent system (Promega, Madison, WI) according to the manufacturer’s instructions. The detection limit was 2.5 μM.

Immunohistochemistry. Mφ (10⁶/well) incubated in a Lab-Tek Chamber Slides (Nalge Nunci International, Napeville, Ill.) were infected with L. monocytogenes (10⁶ bacteria/well; MOI = 10) for 1, 2, or 4 h. After a wash with prewarmed RPMI 1640 containing 10% FCS and gentamicin (5 μg/ml), the cells were stained with Alexa 488-conjugated phalloidin (Molecular Probes) and/or rabbit anti-TNFα Ab at room temperature for 2 h followed by Alexa 594-conjugated anti-rabbit IgG Ab at room temperature for a further 2 h.

Reverse transcription-PCR. TCRVγ14 mRNA expression was analyzed as described previously (20), with slight modifications. Briefly, total RNA extracted with an RNAgent total RNA isolation system (Promega) was primed for reverse transcription with random hexamers. To normalize cDNA content for further comparative analysis, β-actin-specific PCR was performed with serial dilutions of cDNA. The β-actin-specific primers were 5’-TGGAACTCTGCTGGCATTCACTGACAC-3’ (forward) and 5’-TAAAAACGACGCTGATACAGTCTC-3’ (reverse).

After a denaturation step at 94°C for 3 min, PCR cycles were run at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, followed by an extension step at 72°C for 7 min. β-Actin PCR products were analyzed by 1.2% agarose gel electrophoresis and ethidium bromide staining. For analysis of TCRVγ14 mRNAs, a normalized amount of cDNA yielding equivalent amounts of β-actin-specific PCR products was applied. The cDNA was denatured at 94°C for 2 min, and PCR cycles were run at 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s for 30 cycles. The PCR products were subjected to electrophoresis on a 1.8% agarose gel. The 3’ and 5’ primers for the TCRVγ14 PCR were (3’): (5’-AAGCTTGCTGTTGTCAGCAGGTTGTCAG)-3’ and (5’): TCCAGAGGAAAAAGCGAGGATCCCGCATCA-3’ (reverse). The statistical significance was determined by using a Mann-Whitney U test, and a P value of <0.05 was regarded as significant.

Luminol-dependent chemiluminescence. The activity of respiratory burst in peritoneal Mφ was evaluated by using luminol-dependent chemiluminescence (CL) as described previously (23, 46). In brief, a peritoneal Mφ suspension (1.2 × 10⁶ to 10 × 10⁹ cells in 400 μl of Hanks balanced salt solution [HBSS] containing 1% gelatin [HGBSS]) was mixed with 30 μl of luminol solution (10–6 M; Laboscience Co., Tokyo, Japan) in a counting tube, which was kept at 37°C for 5 min with gentle agitation. L. monocytogenes grown in tryptic soy broth at 37°C for 18 h was sedimented (1,200 rpm) for 20 min and washed twice in HBSS before being adjusted to a concentration of 6 × 10⁸ CFU/ml. The bacteria were osmopsonized for 60 min at 37°C with 10% normal pooled mouse serum in HBSS before use for the CL assay. To generate CL, opsonized L. monocytogenes (10⁶ CFU in 150 μl of HBSS) was added to the cell mixture. Light release was measured by using a Lumiphotometer TD-4000 (Laboscience) over a 15-min period, and the peak response (in relative light units [RLU]) was noted.
**RESULTS**

α-GalCer treatment ameliorates murine listeriosis. C57BL/6 mice were left untreated or were treated with α-GalCer or vehicle and then infected with *L. monocytogenes*. CFU in peritoneal cavities, livers, and spleens were determined at different time points thereafter. Considerable numbers of viable bacteria were detected in peritoneal cavities of vehicle-treated and untreated mice 1 h postinfection (p.i.), and these were reduced by 1 order of magnitude after 4 h p.i. regardless of α-GalCer treatment (Fig. 1). Viable bacteria in peritoneal cavities became undetectable (<50 CFU) at 4 h p.i. regardless of α-GalCer treatment (data not shown). High numbers of viable bacteria were detected in the livers and spleens of vehicle-treated and untreated mice on day 4 p.i., and these were reduced up to 100-fold by α-GalCer treatment (Fig. 1). Thus, α-GalCer treatment ameliorated listeriosis.

**In vivo administration of α-GalCer enhances bacterial and phagocytic activities of Mφ in an iNKT-cell-dependent manner.** Mφ play a central role in elimination of *L. monocytogenes* (56, 61). We therefore examined the influence of α-GalCer on the bactericidal activity of Mφ. Peritoneal Mφ from mice left untreated or treated with α-GalCer or vehicle were infected with *L. monocytogenes*, and their bactericidal activities were compared. Since it has been shown that gentamicin has the potential to kill *L. monocytogenes* (13), we first examined whether *L. monocytogenes* in Mφ was killed by gentamicin. To address this issue, we compared numbers of viable bacteria in Mφ cultured in the presence or absence of gentamicin. In contrast to previous findings (13), the numbers of viable bacteria in Mφ were comparable between the two groups (data not shown). Consistent with this, many actin tails were found in TG-induced Mφ 4 h after *in vitro* culture in the presence of gentamicin (see Fig. 3D). Thus, the viability of *L. monocytogenes* in Mφ was apparently not affected by gentamicin in our experimental system. Because viable *L. monocytogenes* bacteria in resident Mφ from C57BL/6 mice were maximal at 60 min after phagocytosis and viable bacteria became virtually undetectable a further 60 min after incubation regardless of mouse strains, we determined phagocytic and bactericidal activities of Mφ at 60 and 30 min after infection, respectively. The data are expressed as a killing index and not as numbers of killed bacteria to standardize bactericidal activity, because it is possible that numbers of killed bacteria are higher in Mφ that phagocytose many bacteria than in those that phagocytose few bacteria. The bactericidal activity (for 30 min after 60 min of phagocytosis) of Mφ from α-GalCer-treated C57BL/6 mice was significantly higher than that from vehicle-treated and untreated C57BL/6 mice (Fig. 2A, upper panel). Accordingly, Mφ showed morphological changes after α-GalCer treatment (see Fig. 3A). In contrast to C57BL/6 mice, bactericidal activities of Mφ from IL-15−/− and SJL mice were virtually unchanged after α-GalCer treatment (Fig. 2A, upper panel). Bactericidal activities of Mφ from untreated C57BL/6, IL-15−/−, and SJL mice were comparable, suggesting that Mφ from these mouse strains have similar bactericidal potentials (data not shown). Hence, α-GalCer enhanced bactericidal activity of Mφ in an iNKT-cell-dependent fashion.

We next examined the influence of α-GalCer on phagocytic activity of Mφ by counting viable bacteria (colony count method) after 60 min of phagocytosis. Considerable numbers, but low efficacy of phagocytosis (ca. 0.1%), of viable bacteria were detected in Mφ from untreated and vehicle-treated C57BL/6 mice, and these numbers were significantly lower in Mφ from α-GalCer-treated C57BL/6 mice (Fig. 2B). To verify whether phagocytic activity of Mφ from C57BL/6 mice was indeed reduced by α-GalCer treatment, peritoneal Mφ from C57BL/6 mice that were left untreated or were treated with α-GalCer or vehicle were infected with *L. monocytogenes*, and the numbers of *L. monocytogenes* bacteria phagocytosed by Mφ were determined immunohistochemically using anti-*Listeria* Ab. In contrast to the colony count method, the numbers of *L. monocytogenes* bacteria ingested by Mφ from α-GalCer-treated C57BL/6 mice were markedly higher than those from vehicle-treated and untreated C57BL/6 mice (Fig. 3A and Table 1). No alterations were found in the numbers of *L. monocytogenes* bacteria ingested by Mφ from IL-15−/− and SJL mice after α-GalCer treatment (Fig. 2B). Hence, α-GalCer enhanced the phagocytic activity of Mφ in an iNKT-cell-dependent manner.

iNKT cells in organs other than peritoneal cavity participate in enhanced bactericidal activity of peritoneal Mφ by α-GalCer treatment. We wondered whether enhancement of bactericidal activity of peritoneal Mφ by α-GalCer involved iNKT cells present in the peritoneal cavity. Total PC from C57BL/6 mice were stimulated *in vitro* with α-GalCer and infected with *L. monocytogenes*, and bactericidal activity of Mφ was determined. In contrast to *in vivo* treatment, bactericidal activity of Mφ was unchanged by *in vitro* stimulation with α-GalCer (data not shown). We therefore rule out the pos-
sibility that enhancement of bactericidal activity of peritoneal Mφ by α-GalCer involved iNKT cells in the peritoneal cavity. We wondered whether the lack of enhanced bactericidal activity of peritoneal Mφ by α-GalCer was caused by the absence of iNKT cells in the peritoneal cavity. We therefore attempted to detect iNKT cells in PC using α-GalCer/CD1d tetramers. HL were used as a positive control, because iNKT cells are most abundant in the liver compared to other organs (16). α-GalCer/CD1d tetramer-reactive T cells were undetectable in peritoneal cavities not only of IL-15−/− and SJL mice (data not shown) but also of C57BL/6 mice regardless of α-GalCer treatment (Fig. 4A), although high numbers of α-GalCer/CD1d tetramer-reactive T cells were detected in the livers of untreated C57BL/6 mice, and these became undetectable after α-GalCer treatment (Fig. 4A) (18, 19). To verify whether iNKT cells were indeed absent in the peritoneal cavity, Vα14 mRNA expression in PC was analyzed. As positive and negative controls, Vα14 mRNA expression of HL from C57BL/6 mice and RAG-1−/− mice lacking T cells, including iNKT cells, was also analyzed. TCRVα14 mRNA was detected in HL from C57BL/6 mice but was undetectable in PC from C57BL/6 mice regardless of α-GalCer treatment, as well as in HL from RAG-1−/− mice (Fig. 4B). These results indicate that iNKT cells responsible for enhanced bactericidal activity of Mφ after α-GalCer treatment reside in organs other than the peritoneal cavity.

IFN-γ from iNKT cells participates in enhanced bactericidal activity of Mφ by α-GalCer treatment. We wondered whether cytokines secreted by iNKT cells participate in enhanced bactericidal activity of Mφ by α-GalCer. Resident peritoneal Mφ from C57BL/6 mice were incubated in the presence of peritoneal fluids from C57BL/6 mice left untreated or treated with α-GalCer or vehicle, and the bactericidal activities were determined. Bactericidal activities of Mφ were unchanged by peritoneal fluids from vehicle-treated or untreated mice, whereas those of Mφ were significantly enhanced by peritoneal fluids from α-GalCer-treated mice (Fig. 5A). These results suggest that cytokines enhance bactericidal activity of Mφ in the peritoneal cavities of α-GalCer-treated mice.
Experiments were performed at least twice with comparable results.

Ab, followed by Alexa 594-conjugated anti-rabbit IgG Ab (red) after a washing step. (C) Magnification /H9251

L. monocytogenes /H9004

D) or the /H9251-GalCer

Vehicle 122 32

L. monocytogenes cytokine is essential in defense against Nil 85 25

35), we measured IFN-

To determine whether IFN-γ is involved in enhanced bac-

tericial activity of Mφ by α-GalCer, IFN-γ was neutralized.

Bactericidal activity of peritoneal Mφ from α-GalCer-treated C57BL/6 mice was significantly, although incompletely, reduced in the presence of anti-IFN-γ neutralizing MAb (Fig. 5D). These results suggest that IFN-γ participates, at least in part, in enhanced bactericidal activity of Mφ after α-GalCer treatment.

NO does not play a major role in enhanced bactericidal activity of Mφ by α-GalCer treatment. NO is produced by Mφ after L. monocytogenes infection and plays a crucial role in intracellular killing of L. monocytogenes (48, 52, 69, 71). Moreover, NO production is induced by IFN-γ (4, 7, 38, 48). We thus examined NO concentrations in peritoneal fluids. NO was undetectable in peritoneal fluids not only from vehicle-treated and untreated C57BL/6 mice but also from α-GalCer-treated C57BL/6 mice (data not shown). However, considerable levels of NO were detected in culture supernatants of peritoneal Mφ from α-GalCer-treated, but not untreated, C57BL/6 mice (Fig. 5C). Some NO was detected in culture supernatants of peritoneal Mφ from vehicle-treated mice, but at lower levels than after α-GalCer treatment.

To examine the role of NO in bactericidal activity of Mφ by α-GalCer, an NO inhibitor was used. Bactericidal activity of peritoneal Mφ from α-GalCer-treated C57BL/6 mice was not prevented by NO inhibitor, although the effect of NO inhibitor was slightly enhanced by anti-IFN-γ neutralizing MAb (Fig. 5D). These results argue against a major role of

Since IFN-γ plays a central role in Mφ activation and this cytokine is essential in defense against L. monocytogenes (31, 35), we measured IFN-γ concentrations in peritoneal fluids. IFN-γ was undetectable in peritoneal fluids drawn from untreated C57BL/6 mice, but high levels of IFN-γ were detected in fluids from α-GalCer-treated C57BL/6 mice (Fig. 5B). Although IFN-γ was detectable in peritoneal fluids from vehicle-treated mice, it was less abundant than that for α-GalCer-treated mice.

To determine whether IFN-γ is involved in enhanced bac-

TABLE 1. Numbers of L. monocytogenes bacteria ingested by peritoneal Mφ from C57BL/6 mice and numbers of Mφ infected with L. monocytogenes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of bacteria/500 Mφ</th>
<th>Infected Mφ/500 Mφ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>85</td>
<td>25</td>
</tr>
<tr>
<td>Vehicle</td>
<td>122</td>
<td>32</td>
</tr>
<tr>
<td>α-GalCer</td>
<td>2,263*</td>
<td>363*</td>
</tr>
</tbody>
</table>

a Mice were left untreated or were treated with vehicle or α-GalCer 24 h before L. monocytogenes infection. Peritoneal Mφ were infected with L. monocytogenes (strain EGD) (MOI = 10) for 60 min, and the phagocytic activities were determined. After being washed with prewarmed RPMI 1640 containing 10% FCS and gentamicin (5 μg/ml), the cells were stained with rabbit anti-Listeria Ab, followed by Alexa 594-conjugated anti-rabbit IgG Ab. Experiments were performed twice with comparable results. * P < 0.01 (vehicle or nil versus α-GalCer).

b Numbers of L. monocytogenes bacteria in 500 Mφ were counted (100 Mφ in five different fields randomly taken).

c Numbers of Mφ infected with L. monocytogenes among 500 Mφ were counted (100 Mφ in five different fields randomly taken).

FIG. 3. Influence of in vivo administration of α-GalCer on the phagocytic activity of resident and TG-induced peritoneal Mφ. (A) C57BL/6 mice were left untreated or were treated with α-GalCer or vehicle, and peritoneal Mφ were prepared 24 h after treatment. Cells were infected with L. monocytogenes (strain EGD) (MOI = 10) for 60 min and stained with rabbit anti-Listeria Ab, followed by Alexa 594-conjugated anti-rabbit IgG Ab (red) after a washing step. Magnification, ×200. (B) Resident peritoneal Mφ from C57BL/6 mice were infected with L. monocytogenes (strain EGD or Δhly strain; MOI = 10) for 1, 2, and 4 h. The cells were stained with Alexa 488-conjugated phalloidin (green) and rabbit anti-Listeria Ab, followed by Alexa 594-conjugated anti-rabbit IgG Ab (red) after a washing step. Magnification, ×1,000. (C and D) C57BL/6 mice were injected i.p. with TG on day 0, and peritoneal Mφ were prepared 4 days after treatment. Cells were infected with L. monocytogenes (strain EGD [C and D] or the Δhly strain [C]; MOI = 10) for 1, 2, and 4 h. The cells were stained with Alexa 488-conjugated phalloidin (green) and rabbit anti-Listeria Ab, followed by Alexa 594-conjugated anti-rabbit IgG Ab (red) after a washing step. (C) Magnification ×400; (D) magnification, ×1,000. Experiments were performed at least twice with comparable results.

...
NO in enhanced bactericidal activity of Mφ by α-GalCer treatment.

**Respiratory burst within the phagosome is promoted by α-GalCer.** ROI are produced by Mφ in response to *L. monocytogenes* infection, and they play a central role in listerial killing within phagosomes (52, 53, 59). To determine the role of ROI experimentally, the respiratory bursts in Mφ from α-GalCer- and vehicle-treated C57BL/6 mice were compared. The maximal CL responses were significantly higher in peritoneal Mφ from α-GalCer-treated mice than in those from vehicle-treated mice (Table 2). The CL emission during phagocytosis by peritoneal Mφ from α-GalCer-treated mice reached a maximum within 160 s (see Table 2) and then gradually decayed (data not shown). In contrast, the emission by peritoneal Mφ from vehicle-treated mice was slower (see Table 2) and rapidly decayed after reaching its maximum (data not shown). Thus, α-GalCer promoted a profound respiratory burst in Mφ after ingestion of *L. monocytogenes*.

**α-GalCer promotes killing of *L. monocytogenes** within the phagosomes of peritoneal Mφ. To evade phagosomal destruction, *L. monocytogenes* escapes from the phagosome into the cytosol by means of LLO (10, 22, 26, 42, 62, 65). However, in the cytosol, NO is capable of killing *L. monocytogenes* (48, 52, 69, 71). We therefore determined whether α-GalCer promotes the killing of *L. monocytogenes* after escape into the cytosol of Mφ. Subsequent to listerial escape from the phagosome, actin tails are formed in the cytosol of Mφ (12, 30, 32, 63, 74). We used immunohistochemistry, specifically fluorescein-labeled phalloidin, for detection of actin tails in peritoneal Mφ. In contrast to previous findings (12, 30, 32, 63, 74), the actin tail was undetectable in Mφ even 4 h p.i. regardless of α-GalCer treatment (Fig. 3B, upper panel). This not only indicates that *L. monocytogenes* does not escape from the phagosome into the cytosol but also suggests that the killing of *L. monocytogenes* occurs in the phagosome, prior to entry into the cytosol.

To decide whether α-GalCer promotes killing of *L. monocytogenes* within the phagosome, the Δhly *L. monocytogenes* strain, which has lost the capacity to escape from the phagosome into the cytosol due to the absence of LLO, was used. Bactericidal and phagocytic activities of Mφ from C57BL/6 mice, but not from IL-15−/− and SJL mice, were enhanced by α-GalCer treatment (Fig. 2, lower panel, and Fig. 3B, lower panel). We wondered whether our failure to obtain evidence for phagosomal escape of *L. monocytogenes* was related to the source of Mφ used in our experiments. We thus used TG-induced peritoneal Mφ. In contrast to resident Mφ, the actin tail was detected in TG-induced Mφ infected with strain EGD but not with the Δhly strain (Fig. 3C and D). The proportion of Mφ with the actin tail was rare, although detectable, 1 h after infection. However, the numbers of Mφ with actin tails were increased thereafter, and actin tails became detectable in most of infected Mφ 4 h after infection (see Fig. 3D). Our results suggest that α-GalCer promotes the killing of *L. monocytogenes* within the phagosomes of Mφ.

**DISCUSSION**

We show here that Mφ activation by iNKT cells is involved in the amelioration of listeriosis after α-GalCer intervention. Listericidal activity of peritoneal Mφ was enhanced by α-GalCer, at least in part, via IFN-γ secreted by iNKT cells. We conclude that effects of α-GalCer in listeriosis are influenced by enhanced bactericidal Mφ activities and mediated by IFN-γ from iNKT cells.

In our experiments, IL-15−/− mice were used as iNKT cell-deficient mice. Since IL-15−/− mice are devoid not only of iNKT cells but also of NK cells, we cannot formally exclude the...
FIG. 5. IFN-γ levels in peritoneal fluids and NO concentrations in Mφ culture supernatants after α-GalCer stimulation, and the influence of IFN-γ neutralization or NO inhibition on bactericidal activity of Mφ. (A) C57BL/6 mice were left untreated or were treated with α-GalCer or vehicle, and the peritoneal fluids were collected 24 h after treatment. Resident peritoneal Mφ from C57BL/6 mice were incubated with the peritoneal fluids. Cells were then infected with L. monocytogenes (strain EGD; MOI = 10), and the bactericidal activities were determined. The data represent the killing index (nil = 1) and are expressed as means ± SD of three mice. Experiments were performed twice with comparable results. *, P < 0.05 (vehicle or nil versus α-GalCer). (B) C57BL/6 mice were left untreated or were treated with α-GalCer or vehicle, and the peritoneal fluids were collected 24 h after treatment. The IFN-γ levels in the peritoneal fluids were determined by enzyme-linked immunosorbent assay. The data are expressed as means ± SD of three mice. Experiments were performed twice with comparable results. *, P < 0.01 (vehicle or nil versus α-GalCer). (C) C57BL/6 mice were left untreated or were treated with α-GalCer or vehicle, and the peritoneal fluids were prepared 24 h after treatment. Cells were incubated for 2 h in a 96-well flat-bottom tissue culture plate, and the NO concentrations in the culture supernatants were determined. The data are expressed as means ± SD of three mice. Experiments were performed twice with comparable results. *, P < 0.01 (vehicle or nil versus α-GalCer). (D) Resident peritoneal Mφ from C57BL/6 mice were incubated with peritoneal fluids from C57BL/6 mice treated with α-GalCer or vehicle in the presence or absence of anti-IFN-γ MAb and/or Nω-monomethyl-L-arginine for 24 h. The cells were then infected with L. monocytogenes (strains EGD; MOI = 10), and the bactericidal activities were determined. The data represent the killing index (nil = 1) and are expressed as means ± SD of three mice. Experiments were performed twice with comparable results. *, P < 0.05 (α-GalCer versus α-GalCer + anti-IFN-γ MAb); **, P < 0.01 (α-GalCer versus α-GalCer + anti-IFN-γ MAb + NO inhibitor).

TABLE 2. CL response to L. monocytogenes by peritoneal Mφ from C57BL/6 mice treated with α-GalCer or vehicle

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean ± SD</th>
<th>Intensity of CL</th>
<th>Peak (RLU, 10^4)</th>
<th>Peak time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-GalCer</td>
<td>11.01 ± 0.85^a</td>
<td>1.80 ± 0.33</td>
<td>163 ± 12^a</td>
<td>375 ± 27</td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.57 ± 1.32</td>
<td>1.71 ± 0.17</td>
<td>375 ± 27</td>
<td></td>
</tr>
</tbody>
</table>

a Resistant peritoneal Mφ (1.2 × 10^6 to 1.5 × 10^6 cells) were mixed with luminol solution (10^-6 M) and opsonized L. monocytogenes (10^6 CFU) at 37°C. Light emission was measured at 37°C for 15 min. Experiments were performed four times with comparable results. In each assay, the CL measurement was performed in duplicate for the same sample (n = 8). *, P < 0.05 (vehicle versus α-GalCer).

b Intensities are expressed as peak RLU values.

The possible contribution of NK cells to amelioration of listeriosis and to enhanced bactericidal activity of Mφ by α-GalCer. However, amelioration of listeriosis and enhanced bactericidal activity of Mφ by α-GalCer were not found in SJL mice, with a genetic background different from that of C57BL/6 mice, and amelioration of listeriosis by α-GalCer was not found in Jα18^-/- mice, which harbor NK cells but not iNKT cells (19). Thus, we assume that iNKT cells are essential for amelioration of listeriosis and Mφ activation by α-GalCer.

Numbers of viable L. monocytogenes bacteria in Mφ from α-GalCer-treated C57BL/6 mice were significantly lower than those from vehicle-treated and untreated mice 60 min after infection (as determined by the colony count method). Nevertheless, by immunohistochemical analysis, comparable numbers of L. monocytogenes bacteria were detected in Mφ from α-GalCer-treated C57BL/6 mice, and these were markedly higher than those from vehicle-treated and untreated mice. The discrepancy is probably caused by the difference in killing activity of each Mφ, i.e., L. monocytogenes organisms are killed by Mφ from α-GalCer-treated mice immediately after infection. Indeed, the numbers of viable L. monocytogenes bacteria in Mφ from α-GalCer-treated C57BL/6 mice were markedly higher than those from vehicle-treated and untreated mice 20 min after infection (M. Emoto et al., unpublished observations). These results indicate that α-GalCer has a great potential to ingest and kill L. monocytogenes by Mφ through iNKT cell activation. However, because it is possible
that pinocytosis is accelerated by α-GalCer, we cannot formally exclude the possibility that this effect is influenced by gentamicin, although comparable numbers of viable L. monocytogenes bacteria were detected in Mφ cultured in the presence or absence of gentamicin.

Abundant numbers of iNKT cells have been detected in the peritoneal cavities of C57BL/6 mice by flow cytometry (36). In contrast, we did not detect iNKT cells in the peritoneal cavity even at mRNA levels. The discrepancy is probably caused by nonspecific staining in the study of Ito et al. (36), because cells are diagonally stained and a distinct population of NKT cells is invisible. Since (i) iNKT cells secrete IFN-γ in response to α-GalCer (6, 40), (ii) high levels of IFN-γ were detected in peritoneal fluids of α-GalCer-treated C57BL/6, but not IL-15−/− and SJL, mice, and (iii) iNKT cells were undetectable in the peritoneal cavity even at mRNA levels, we assume that IFN-γ was derived from iNKT cells residing in an organ(s) other than the peritoneal cavity. Indeed, high numbers of IFN-γ-secreting cells were detected among liver infiltrating iNKT cells 1 h after α-GalCer treatment (18). Consistent with this notion, IFN-γ was detected in the peripheral blood of C57BL/6, but not IL-15−/− and SJL, mice after in vivo administration of α-GalCer (21, 43, 54; Emoto et al., unpublished). It is possible that iNKT cells infiltrate the peritoneal cavity after α-GalCer treatment. Because iNKT cells were undetectable in the peritoneal cavity after α-GalCer treatment not only at the cellular level but also at the mRNA level (this study) and because most L. monocytogenes bacteria are trapped in the liver and spleen immediately after i.p. injection (see Fig. 1), we assume that IFN-γ detected in the peritoneal cavity is derived from other organs, such as the liver and spleen.

NO participates in the killing of L. monocytogenes in Mφ (48, 52, 69, 71). Consistent with this, considerable NO levels were detected in culture supernatants of peritoneal Mφ from α-GalCer-treated mice. However, the enhanced listericidal activity of peritoneal Mφ by α-GalCer was only marginally affected by NO inhibitor. At first sight, these findings seem to argue against a major role of NO in killing of L. monocytogenes. However, as shown here, the escape of L. monocytogenes from the phagosome into the cytosol was not detectable in peritoneal Mφ. Since (i) NO is produced in the cytosol (45, 60) and (ii) killing of L. monocytogenes in TG-induced peritoneal Mφ that permit escape of L. monocytogenes from the phagosome into the cytosol was inhibited by NO inhibitor (Emoto et al., unpublished), it is possible that NO plays only a minor role in prompt phagosomal killing as observed in our model.

α-GalCer promoted a profound respiratory burst in Mφ after ingestion of opsonized L. monocytogenes. ROI are produced by Mφ in response to L. monocytogenes infection, and they play a central role in listerial killing within phagosomes (52, 53, 59). We therefore assume that enhanced listerial killing in Mφ by α-GalCer is similarly caused by rapid and abundant production of ROI.

Further, in our hands, actin tails were undetectable in resident peritoneal Mφ even 4 h after L. monocytogenes infection in vitro. This finding could be taken as evidence against listerial escape from the phagosome into the cytosol (10, 22, 26, 42, 62, 65). However, actin tails were detected in TG-induced Mφ already 1 h p.i. in vitro. We thus consider the possibility that L. monocytogenes escapes from the phagosome in inflammatory, but not resident, peritoneal Mφ. Resident and inflammatory Mφ differ in maturation, differentiation, and activation status (14, 34, 57, 68, 72, 73, 75). Bone marrow-derived monocytes are immature compared to resident Mφ (8, 11, 47, 51, 77), and Mφ colony-stimulating factor provides an essential signal for Mφ maturation (8, 11, 47, 51, 77). After the i.p. administration of TG or protease peptone, monocytes from bone marrow infiltrate the peritoneal cavity (2, 9). We assume that L. monocytogenes escapes from the phagosome into the cytosol in freshly immigrating monocytes, but not in resident peritoneal Mφ. Consistent with this notion, escape of L. monocytogenes from the phagosome has been described to occur in bone marrow-derived Mφ (32, 63). LLO is essential for the escape of L. monocytogenes from the phagosome into the cytosol (10, 22, 26, 42, 62, 65), and its activity is highly dependent on low pH (3, 24, 25, 66) and on the abundance of ROI (49, 59). Bacterial numbers ingested by Mφ were comparable for the hly-expressing strain L. monocytogenes EGD and for the L. monocytogenes Δhly strain (see Fig. 3B and C), indicating that phagocytosis occurred independently of LLO. The numbers of engulfed L. monocytogenes bacteria were higher in TG-induced Mφ than in resident peritoneal Mφ (see Fig. 3B and C). We assume that inflammatory Mφ have a higher phagocytic potential than resident peritoneal Mφ. Since L. monocytogenes escaped from the phagosome into the cytosol in TG-induced Mφ but not resident peritoneal Mφ, we assume that killing capacities are influenced by the degree of escape from the phagosome into the cytosol. Because bacterial escape from the phagosome into the cytosol is determined by whether the actin tail can be detected or not, it would be better to verify this with a confocal microscope to obtain direct evidence.

In conclusion, we describe here profound bactericidal activities of Mφ after α-GalCer treatment. Mφ activation was mediated by IFN-γ from iNKT cells. Our findings therefore define α-GalCer as an anti-intracellular bacterial agent.

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