American Journal of Pathology, Vol. 158, No. 1, January 2001

Role of Macrophage Scavenger Receptors in Response to *Listeria monocytogenes* Infection in Mice

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Type I and type II macrophage scavenger receptors (SR-A I/II) recognize a variety of polyanions including bacterial cell-wall products such as lipopolysaccharide, suggesting a role for SR-A I/II in immunity against bacterial infection. SR-A I/II-deficient (MSR-A−/−) mice were more susceptible to infection with listeriolysin-O (LLO)-producing *Listeria monocytogenes*. After infection, Kupffer cells in wild-type (MSR-A+/+) mice phagocytized larger numbers of *Listeria* than those in MSR-A−/− mice. The number and the diameter of hepatic granulomas were larger in MSR-A−/− mice than MSR-A+/+ mice. *L. monocytogenes* replicated at higher levels in the liver of MSR-A−/− mice compared with MSR-A+/+ mice, and macrophages from MSR-A−/− mice showed impaired ability to kill *Listeria in vitro*. However, macrophages from MSR-A+/+ and MSR-A−/− mice showed similar levels of listericidal activity against isogenic mutant *L. monocytogenes* with an inactivated LLO gene. The listerial phagocytic activities of MSR-A+/+ macrophages treated with an anti-SR-A I/II antibody (2F8) and MSR-A−/− macrophages were significantly impaired compared with untreated MSR-A+/+ macrophages, indicating that SR-A I/II function as a receptor for *L. monocytogenes*. Electron microscopy revealed that most *L. monocytogenes* had been eliminated from the lysosomes of MSR-A+/+ macrophages *in vivo* and *in vitro*. In contrast, *L. monocytogenes* rapidly lysed the phagosomal membrane and escaped to the cytosol in MSR-A−/− macrophages and in MSR-A+/+ macrophages treated with 2F8 before phagosome-lysosome fusion. These findings imply that SR-A I/II plays a crucial role in host defense against listerial infection not only by functioning as a receptor but also by mediating listericidal mechanisms through the regulation of LLO-dependent listerial escape from the macrophages. *(Am J Pathol 2001, 158:179–188)*

Macrophage scavenger receptors are implicated in the deposition of cholesterol in arterial walls during atherogenesis through receptor-mediated endocytosis of chemically modified low density lipoproteins (LDL).[1–6] Because of the broad ligand-binding capacity of these receptors, they have a wide spectrum of biological roles in not only atherogenesis but also host defense against pathogens as well as the removal and clearance of various arrays of negatively charged macromolecules. The scavenger receptors are classified into class A [type I and type II macrophage scavenger receptors (SR-AI/II),[1–6] and macrophage receptor with collagenous structure (MARCO)[7,8]]; class B (CD36[9] and SR-BI[10,11]); class C (dSR-CI[12,13]); class D (CD68/macrosialin[14,15]); class E [(lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1)[16]]; class F [scavenger receptor expressed by endothelial cells (SREC)[17]]; and Fc receptors (Fc γ RII-B2).[18] SR-AI/II is a trimeric glycoprotein expressed in macrophages in various tissues and binds to a precursor of gram-negative bacterial lipid A[19,20] and lipoteichoic acid of gram-positive bacteria.[21] Recently it was shown that SR-AI/II-knockout (MSR-A−/−) mice were more susceptible than wild-type mice to *Listeria monocytogenes*, herpes simplex virus, and malaria infections,[22–24] indicating important roles for SR-AI/II in host defense. Recent reports have demonstrated that MSR-A-deficient macrophages are defective in the uptake ability of dead and live bacteria.[25,26] However, the precise role of SR-AI/II in bactericidal mechanism has been little investigated.

L. monocytogenes is a facultative, intracellular gram-positive bacterium responsible for severe infections in newborn, aged, and immunocompromised individuals,[27,28] that has been used as a model to study cell-mediated immunity.[29–31] In murine infections, the bacte-

Supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, and Japan Foundation Grant for Aging and Health.

Accepted for publication September 28, 2000.

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ria accumulate predominantly in the liver and replicate until the host develops an acquired cellular immune response.[27,28,32] A key aspect of the pathogenicity of *L. monocytogenes* is its ability to invade and multiply in macrophages.[27,28] Although most *Listeria* are killed during the first 6 hours after primary infection, some of them lyse the host vacuole membrane, and escape from phagosomes into the host cytoplasm where the bacteria multiply and spread from cell to cell.[32,33] A critical factor implicated in the escape of Listeria from phagosomes is listeriolysin O (LLO), a sulfhydryl-activated pore-forming hemolysin secreted by *Listeria*.[34–36] It has been reported that mutants lacking LLO remained in vacuoles without proliferating.[35,36] However, the mechanism controlling this process has not been fully elucidated. In this study, we compared susceptibility of MSR-A−/− and MSR-A+/+ mice to *L. monocytogenes*. Phagocytic and listericidal activity of macrophages obtained from MSR-A+/+ and MSR-A−/− mice was also investigated using LLO-producing and -nonproducing strains.

Materials and Methods

Animals

MSR-A−/− mice were generated by disrupting exon 4 of the MSR-A gene[22] and maintained under standard conditions at the Laboratory Animal Center, Niigata University School of Medicine. Eight-week-old male mice were used in the experiments and were killed by ether anesthesia. The liver was removed at various times after intravenous injection with *L. monocytogenes* for the preparation of tissue sections and enumeration of bacteria.

Microorganisms and Infection of Mice

Virulent *L. monocytogenes*, strain EGD, was used in all experiments. Bacterial virulence was maintained by serial passage in BALB/c mice. Fresh isolates were obtained from infected spleens, grown in tryptic soy broth (Difco Laboratories, Detroit, MI), washed repeatedly, resuspended in sterile phosphate-buffered saline (PBS), and then stored at −80°C in small aliquots. Mice were inoculated intravenously with *L. monocytogenes* at various doses. For the observation of granuloma formation, we injected intravenously 1×10^4 colony-forming units (CFUs) of *Listeria*, a dose not lethal to either MSR-A−/− or MSR-A+/+ mice. An isogenic LLO-defective mutant, *L. monocytogenes* EGD *hlyA*::pLSV2, was obtained from Eva Ng (University of Wurzburg, Wurzburg, Germany). This mutant has been constructed by insertional inactivation of gene encoding LLO (*hlyA*) by using a plasmid integration into *L. monocytogenes* EGD.[37] This avirulent strain was also used in the *in vitro* experiments.

Evaluation of Listerial Growth by Determination of CFU Counts

The number of viable bacteria in the inoculum, homogenates of the liver and spleen, and infected cells was determined by plating 10-fold serial dilutions on brain-heart infusion agar (Difco Laboratories) plates. The numbers of CFUs were counted after incubation for 24 hours at 37°C.

Antibodies

The rat monoclonal antibodies F4/80 and Mac-1 (BMA Biomedicals, Augst, Switzerland) were used at a dilution of 1:100. F4/80 recognizes mature tissue macrophages and monocytes.[38] Mac-1 recognizes exudate macrophages and neutrophils.[39,40] The rat monoclonal antibody for SR-AI/II, 2F8,[41] was kindly provided by Prof. S. Gordon (Oxford University).

Histology and Histochemistry

Tissues were fixed in 10% phosphate-buffered formalin and processed routinely for paraffin sections. Five-μm-thick sections were prepared, deparaffinized, and hydrated before incubation with the staining solution. Sections were stained with hematoxylin and eosin. For detecting bacilli, Gram staining was performed. Neutrophils were stained by the 3-hydroxy-2-naphtholic acid o-toluidide-chloroacetate esterase method.[42]

Immunohistochemistry

The livers were fixed for 4 hours at 4°C in periodate-lysine-paraformaldehyde, washed for 4 hours with PBS containing 10%, 15%, and 20% sucrose, and embedded in OCT compound (Miles, Elkhart, IN). These tissue specimens were frozen in dry-ice-acetone and cut by a cryostat (Bright, Huntington, UK) into 6-μm-thick sections. After inhibition of endogenous peroxidase activity by the method of Isobe and colleagues,[43] we performed immunohistochemistry using the monoclonal antibodies described above. As a secondary antibody, we used anti-rat Ig-horseradish peroxidase-linked F(ab)2 fragment (Amersham, Poole, UK). After visualization with 3,3′-diaminobenzidine (Dojin Chemical Co., Kumamoto, Japan), and nuclear staining with methylene green, the sections were mounted with resin.

Electron Microscopy, Ultrastructural Acid Phosphatase Cytochemistry, and Immunoelectron Microscopy

The liver tissues and cultured macrophages were fixed with 1.5% glutaraldehyde, postfixed with 1% osmium tetroxide (OsO4), dehydrated in a graded ethanol series, and embedded in Epok. Ultrathin sections were observed under an electron microscope (H-800; Hitachi, Tokyo, Japan) after staining with lead citrate.

For visualizing lysosomes in macrophages, ultrastructural acid phosphatase staining was performed. Infected cells were washed in Hanks' balanced salt solution (Sigma Chemical, Co., St. Louis, MO), fixed for 30 minutes at 4°C with 1.0% glutaraldehyde in cold sodium-cacodylate

buffer (SCB) (0.1 mol/L sodium cacodylate, 0.25 mol/L sucrose, pH 7.4), and then washed again with SCB. Washing was followed by two 30-minute incubations in acid-phosphatase reaction buffer (0.1 mol/L sodium acetate, 1 mmol/L glyceroacetate, 2 mmol/L CeCl3), pH 5.2, at 37°C with gentle shaking. Thereafter, the cells were rinsed three times with the acid-phosphatase reaction buffer, and refixed in 3% glutaraldehyde in SCB for 1 hour at 4°C. After three more washes in SCB, monolayers were postfixed with 1% OsO4 in cold SCB. The cells then were washed, dehydrated, and processed for electron microscopy.

For identifying the location of SR-AI/II, macrophages were fixed with periodate-lysine-paraformaldehyde, washed in 0.05 mol/L cacodylate buffer, and incubated with 2F8 as reported.[5]

Listericidal Assay

Resident peritoneal cells were washed from the peritoneal cavity of mice by lavage with 10 ml of cold sterile PBS. Peritoneal exudate cells were elicited by intraperitoneal injection of 2 ml of Brewer's Thioglycollate Medium (Difco Laboratories, Sparks, MD). Cells were harvested by peritoneal lavage 48 hours after stimulation and centrifuged (1,000 rpm, 10 minutes). After a wash, peritoneal macrophages were resuspended in RPMI 1640 (Flow Laboratories, Inc., MacLean, VA) containing heat-inactivated 10% (v/v) fetal calf serum and the cells were counted. Cells (1×10^6/ml) were plated on 35-mm tissue-culture Petri dishes (Corning Costar Corp., Cambridge, MA) and then incubated for 2 hours at 37°C in 5% CO_2 in air. For characterization of the peritoneal exudate cells, ultrastructural peroxidase cytochemistry[44,45] and immunoelectron microscopy were performed on harvested cells as described above before infection. *L. monocytogenes* were added to a dish and incubated for 30 minutes at 37°C in 5% CO_2 in air. *Listeria* macrophage ratios were set at 10:1[46] for evaluation of listericidal activity, and at 100:1 and 200:1 for listerial uptake ability. For determination of listerial counts, dishes were washed five times with 4°C PBS and deposited into sterile distilled water. After mixing vigorously for 15 seconds to lyse the infected cells, 10-fold serial dilutions were plated on brain-heart infusion agar to enumerate the number of the bacteria from 0 to 4 hours after infection. At 30 minutes after infection, gentamicin sulfate was added to kill extracellular Listeria without affecting the viability of intracellular bacteria.[47,48] To test the involvement of SR-AI/II in the listerial uptake, peritoneal exudate cells of MSR-A+/+ mice were pretreated by 2F8 in some experiments. Electron microscopy and listerial multiplication as described above were performed at given time points from 30 minutes to 4 hours after infection. The monolayers of cells were also processed for electron microscopy as described above.

Statistics

For the analysis of survival rate, the Wilcoxon method was used. The significance of the other data were evaluated by Student's *t*-test.

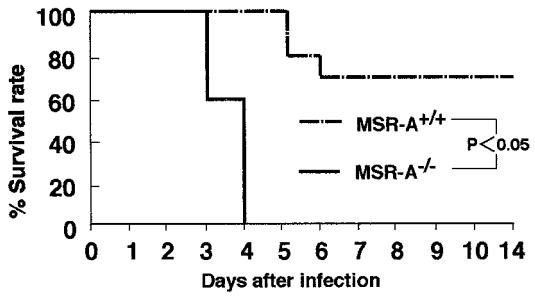

Figure 1. The survival rate of MSR-A+/+ and MSR-A−/− mice injected intravenously with 1×10^5 CFU of *L. monocytogenes* per mouse. Each genotype consisted of 10 mice. Their survival was assessed daily for 14 days.

Results

Decreased Survival of MSR-A−/− Mice after L. monocytogenes *Infection*

The survival rates for MSR-A−/− mice after injection of 1×10^5 CFU of Listeria were significantly lower than those for wild-type mice (Figure 1).

Increased Hepatic Granuloma Formation in MSR-A−/− Mice

In both MSR-A−/− and MSR-A+/+ mice injected intravenously with 1×10^4 CFU of *L. monocytogenes*, abscesses started to form in the liver from 2 days, and these changed into granulomas at 3 days. In MSR-A+/+ mice, SR-AI/II was expressed in Kupffer cells, sinusoidal endothelial cells, and macrophages in the granulomas (Figure 2A). In contrast, SR-AI/II was not expressed in any cell types in MSR-A−/− mice (Figure 2B). Significantly larger numbers of Mac-1-positive cells were accumulated in the granulomas of MSR-A−/− than MSR-A+/+ mice (Figure

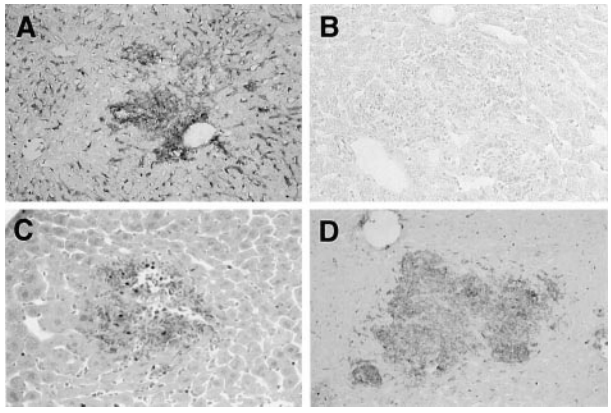

Figure 2. Immunohistochemistry of *Listeria*-infected livers of MSR-A+/+ and MSR-A−/− mice after intravenous injection of 1×10^4 CFU of *L. monocytogenes*. **A:** SR-AI/II is expressed in Kupffer cells, sinusoidal endothelial cells, and macrophages in the granulomas of MSR-A+/+ mice at 3 days after infection. Immunohistochemical staining using 2F8; original magnification, ×200. **B:** SR-AI/II is not expressed in any cell types in MSR-A−/− mice at 3 days after infection. Immunohistochemical staining using 2F8; original magnification, ×200. **C:** Mac-1-positive cells appear in granulomas of MSR-A+/+ mice at 3 days after infection. Immunohistochemical staining using Mac-1; original magnification, ×400. **D:** Abundant Mac-1-positive cells accumulate in large granulomas of MSR-A−/− mice at 3 days after infection. Immunohistochemical staining using Mac-1; original magnification, ×400.

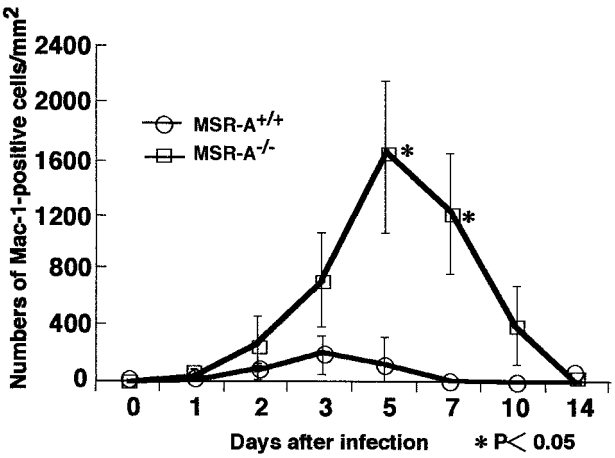

Figure 3. Changes in the numbers of Mac-1-positive cells in the granulomas of MSR-A+/+ and MSR-A−/− mice after intravenous injection of 1×10^4 CFU of *L. monocytogenes*. Data are expressed as the mean ± SD of five mice. *, $P < 0.05$.

2, C and D, and Figure 3). The numbers of granulomas in MSR-A+/+ mice peaked at 3 days and decreased thereafter. In MSR-A−/− mice, however, the numbers of granulomas increased remarkably up to day 5. At 7 and 10 days, significantly more granulomas remained in the liver of MSR-A−/− than MSR-A+/+ mice (Figure 4A). Granulomas in MSR-A+/+ mice had disappeared by day 10. In MSR-A−/− mice, granulomas were still found at day 10 (Figure 4A). The mean diameters of granulomas of MSR-A−/− mice were significantly larger than those of MSR-A+/+ mice (Figure 4B).

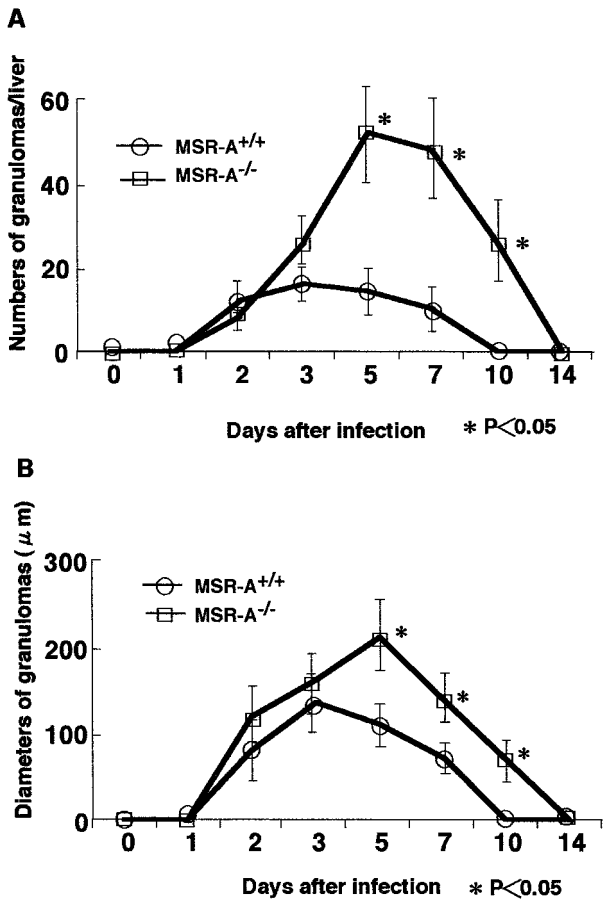

Figure 4. Changes in the numbers (**A**) and mean diameters (**B**) of granulomas in livers of MSR-A+/+ and MSR-A−/− mice after intravenous injection of 1×10^4 CFU of *L. monocytogenes*. Data are expressed as the mean ± SD for five mice. *, $P < 0.05$.

Decreased Listerial Phagocytosis by Kupffer Cells in MSR-A−/− Mice

At 30 minutes after infection, several Gram-positive rods were frequently present in Kupffer cells of MSR-A+/+ mice (Figure 5A and 6). In MSR-A−/− mice, however, only a few bacilli were detected in Kupffer cells (Figures 5B and 6). At 3 and 5 days, a few bacteria were detected in granulomas of MSR-A+/+ mice (Figure 5E). In contrast, a large number of bacilli were observed in the granulomas of MSR-A−/− mice (Figure 5F). Electron microscopy revealed that there was a remarkable difference in the fate of *Listeria* in Kupffer cells between MSR-A+/+ and MSR-A−/− mice. In MSR-A+/+ mice, the majority of bacteria had been incorporated into lysosomes of Kupffer cells and macrophages in the granulomas (Figure 5, C and G). In contrast, most of the bacteria were observed in single forms in MSR-A−/− mice, and endosomal membranes were often unclear (Figure 5, D and H).

Enhanced Multiplication of L. monocytogenes *in the Liver of MSR-A−/− Mice*

The multiplication of *L. monocytogenes* in liver is shown in Figure 7. The *Listeria* proliferated more in the liver of MSR-A−/− than MSR-A+/+ mice and the numbers of CFU in the liver of MSR-A−/− mice significantly increased from 3 to 5 days after infection. These results were consistent with the results of Gram staining, and indicated that listericidal ability was impaired in MSR-A−/− mice compared with that in MSR-A+/+ mice. The numbers of *Listeria* in the spleen did not differ significantly between the genotypes of mice (data not shown).

Decreased Phagocytosis of L. monocytogenes *by MSR-A−/− Macrophages* in Vitro

The numbers of phagocytosed *Listeria* of MSR-A−/− exudate peritoneal macrophages at 20 minutes after infection were significantly smaller than those of MSR-A+/+ macrophages (Figure 8). In addition, 2F8,[41] a monoclonal antibody for murine SR-AI/II, markedly inhibited the listerial uptake of MSR-A+/+ macrophages (Figure 8). These findings indicate that SR-AI/II functions as a crucial receptor for the uptake of *L. monocytogenes*.

Resident macrophages of both types of mice phagocytosed smaller numbers of *Listeria* than exudate macrophages, indicating that the listerial uptake of exudate macrophages is more active than resident macrophages (data not shown).

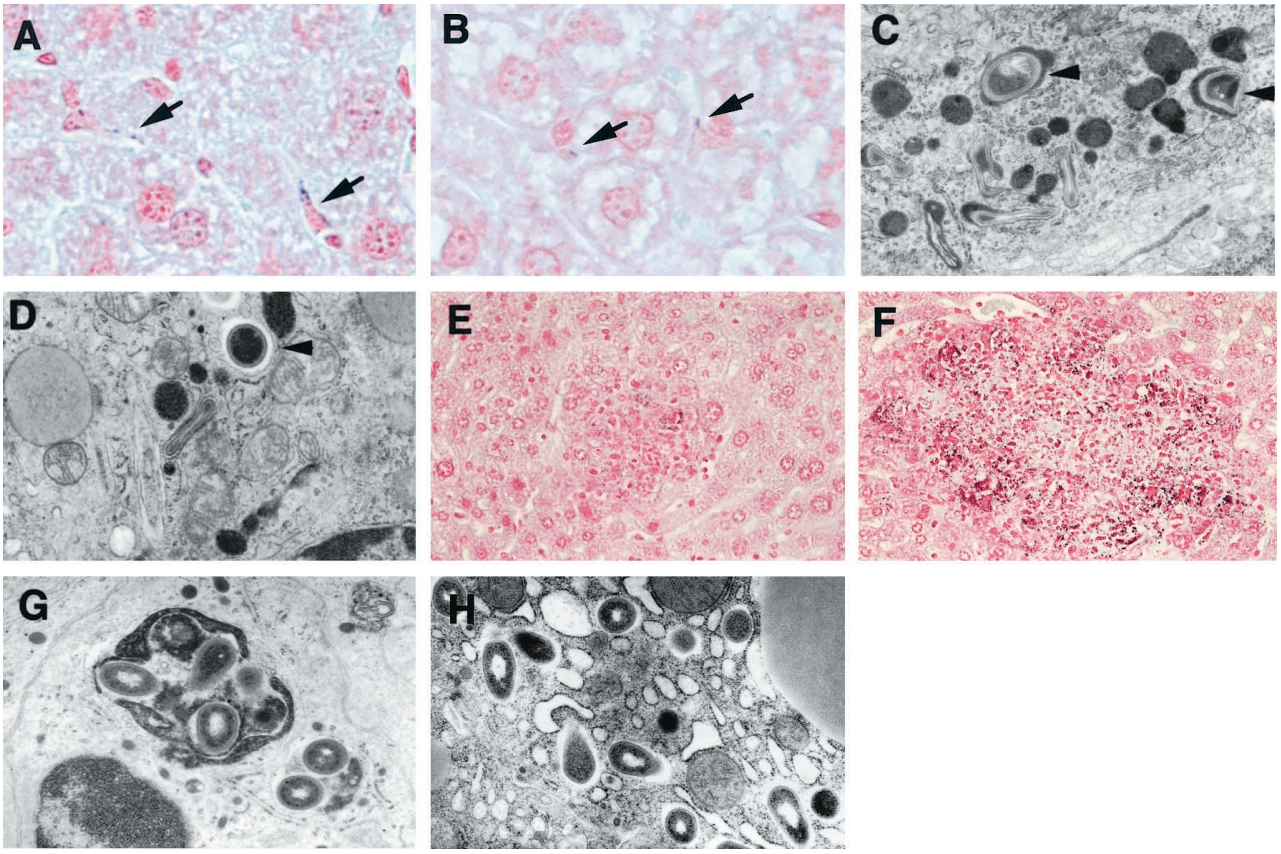

Figure 5. Gram staining and electron micrographs of *Listeria*-infected livers of MSR-A+/+ and MSR-A−/− mice after intravenous injection of 1×10^4 CFU of *L. monocytogenes*. **A:** Several *Listeria* are phagocytosed by Kupffer cells in MSR-A+/+ mice at 30 minutes after infection. Gram staining; original magnification, ×1,000. **B:** In MSR-A−/− mice, only a few bacilli are detected in Kupffer cells at 30 minutes after infection. Gram staining; original magnification, ×1,000. **C:** Most *Listeria* are incorporated into lysosomes (**arrowheads**) at 30 minutes after infection in Kupffer cells of MSR-A+/+ mice. Electron microscopy; original magnification, ×10,000. **D:** A few bacteria are present in Kupffer cells of MSR-A−/− mice at 30 minutes after infection. Some bacteria are free in the cytoplasm (**arrowhead**). Electron microscopy; original magnification, ×10,000. **E:** The bacteria are observed sparsely in the granulomas of MSR-A+/+ mice at 5 days after infection. Gram staining; original magnification, ×400. **F:** Numerous bacteria are observed in the granulomas of MSR-A−/− mice at 5 days after infection. Gram staining; original magnification, ×400. **G:** Most *Listeria* are confined within large phagolysosomes in macrophages of MSR-A+/+ mice at 5 days after infection. Electron microscopy; original magnification, ×10,000. **H:** In the macrophages of MSR-A−/− mice, many bacilli are located free in the cytoplasm at 5 days after infection. Electron microscopy; original magnification, ×10,000.

Decreased Killing of LLO-Producing L. monocytogenes *by Macrophages from MSR-A−/− Mice*

The multiplication of the EGD strain of *L. monocytogenes* in peritoneal macrophages was evaluated by determining CFUs (Figure 9A). The decreased number of bacterial colonies with time indicates bactericidal activity of macrophages. The listericidal capacity of MSR-A−/− macrophages was more impaired than that of MSR-A+/+ macrophages in both resident and exudate peritoneal macrophages. The listericidal activity of resident macrophages was impaired more in MSR-A−/− mice. MSR-A+/+ exudate macrophages efficiently suppressed the growth and continued to exhibit listericidal activity during the 4-hour experiment.

The replication of LLO-nonproducing isogenic mutant in exudate peritoneal macrophages was also examined. There was no significant difference in listericidal activity between MSR-A−/− and MSR-A+/+ exudate peritoneal macrophages (Figure 9B), indicating that LLO is one of the molecules responsible for the difference in listericidal capacity between MSR-A−/− and MSR-A+/+ mice.

Rapid Escape of Listeria *from the Phagosomes of MSR-A−/− Macrophages*

Electron microscopically, *Listeria* were rapidly phagocytosed by MSR-A+/+ and MSR-A−/− exudate macrophages *in vitro* (Figure 10, A and B). At 30 minutes after infection, most of the *Listeria* had been incorporated into large endosomes of MSR-A+/+ exudate macrophages (Figure 10, C and E). In contrast, most of the bacteria were observed in single forms at 30 minutes after infection in MSR-A−/− exudate macrophages, and a few bacteria were surrounded by an endosomal membrane (Figure 10, D and F). Some of the *Listeria* in MSR-A−/− macrophages were in the process of dividing (Figure 10F). These features of listerial behavior in macrophages *in vitro* were quite similar to those *in vivo*.

A striking difference was that approximately 90% of the phagosomes were perforated in MSR-A−/− exudate

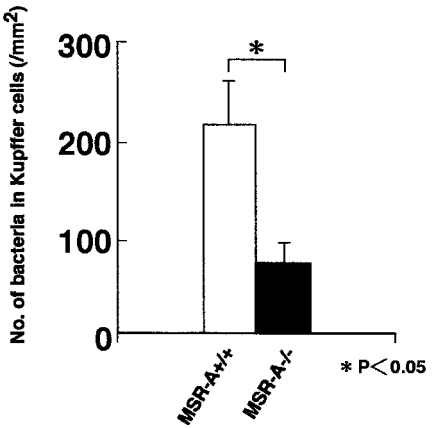

Figure 6. The numbers of *L. monocytogenes* in Kupffer cells of MSR+/+ and MSR−/− mice at 20 minutes after intravenous injection of 1×10^4 CFU of *L. monocytogenes*. *, $P < 0.05$.

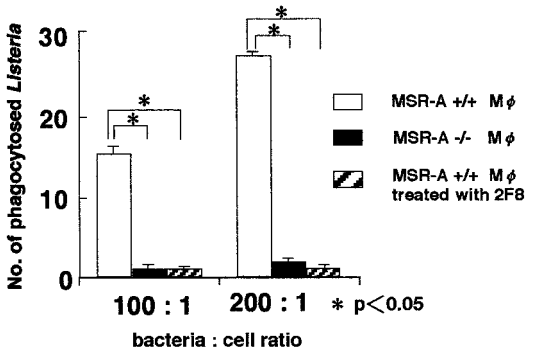

Figure 8. The numbers of phagocytosed *L. monocytogenes* per cell of MSR-A+/+, MSR-A−/−, and 2F8-treated MSR-A+/+ peritoneal macrophages at 20 minutes after infection *in vitro*. The *Listeria* cell ratios were set at 100:1 (1×10^7 *Listeria* for 1×10^5 cells), and 200:1 (2×10^7 *Listeria* for 1×10^5 cells). Data are the mean for 100 cells in each group. *, $P < 0.05$.

macrophages at 1 hour after infection, compared to <10% in MSR-A+/+ exudate macrophages (Figure 11). Anti-SR-AI/II antibody also enhanced the numbers of perforated phagosomes in MSR-A+/+ exudate macrophages (Figure 11).

Ultrastructural acid phosphatase staining for lysosomes revealed that most of the *Listeria*-bearing endosomes fused with lysosomes in MSR-A+/+ macrophages and many bacteria were undergoing degradation at 1 hour (Figure 10G). In contrast, few lysosomes fused with phagosomes in MSR-A−/− exudate macrophages (Figure 10H). In MSR-A+/+ resident macrophages, approximately half of the phagocytosed *L. monocytogenes* were eliminated in the same manner as in MSR-A+/+ exudate macrophages, but the residual *Listeria* were viable in nonperforated endosomes, supporting the fact that the listericidal activity of MSR-A+/+ resident macrophages was lower than that of MSR-A+/+ exudate macrophages (Figure 9A). These results imply that, in MSR-A−/− macrophages, especially MSR-A−/− exudate macrophages, *Listeria* rapidly escape from their phagosomes into cytoplasm before phagosome-lysosome fusion. The efficient replication of *Listeria* in macrophages reflects an impaired listericidal ability in MSR-A−/− mice.

Immunoelectron microscopy revealed that SR-AI/II was expressed continuously on the cell membranes of MSR-A+/+ macrophages as reported previously.[5] Immediately after bacterial phagocytosis, SR-AI/II was expressed on the endosomal membrane (Figure 10A, inset). However, no SR-AI/II expression was observed on the phagolysosomal membrane at 30 minutes after infection (Figure 10C, inset), suggesting the dissociation of SR-AI/II and bacteria before phagosome-lysosome fusion as reported previously.[49] Expression of SR-AI/II was not detected in any organelles of MSR-A−/− macrophages.

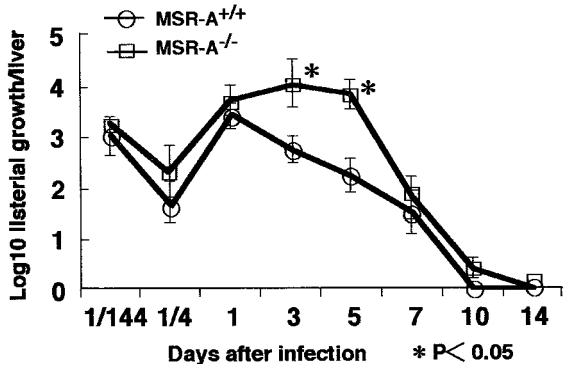

Figure 7. Proliferation of *L. monocytogenes* in livers of MSR-A+/+ and MSR-A−/− mice after intravenous injection of 1×10^4 CFU of *L. monocytogenes*. Data are expressed as the mean ± SD of five mice. *, $P < 0.05$.

Discussion

The impaired capacity of macrophages from MSR-A−/− mice to phagocytose *Listeria*, the increased susceptibility of MSR-A−/− *versus* MSR-A+/+ mice to lethal challenge

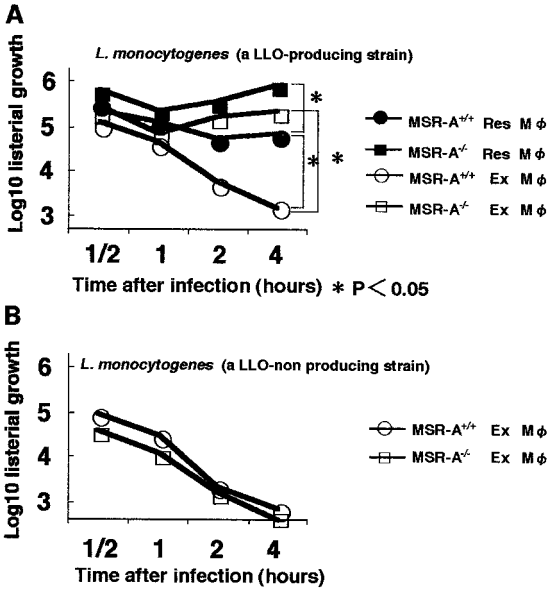

Figure 9. Growth of the bacteria *in vitro*. *Listeria* cell ratio was set at 10:1 (1×10^6 Listeria for 1×10^5 cells). **A:** Growth of a LLO-producing strain of *L. monocytogenes* in resident and exudate peritoneal macrophages obtained from MSR-A+/+ and MSR-A−/− mice. Data are the mean of three experiments. *, $P < 0.05$. **B:** Growth of a LLO-nonproducing mutant of *L. monocytogenes* in MSR-A+/+ and MSR-A−/− exudate peritoneal macrophages. Data are the mean for three experiments. Res, resident; Ex, exudate.

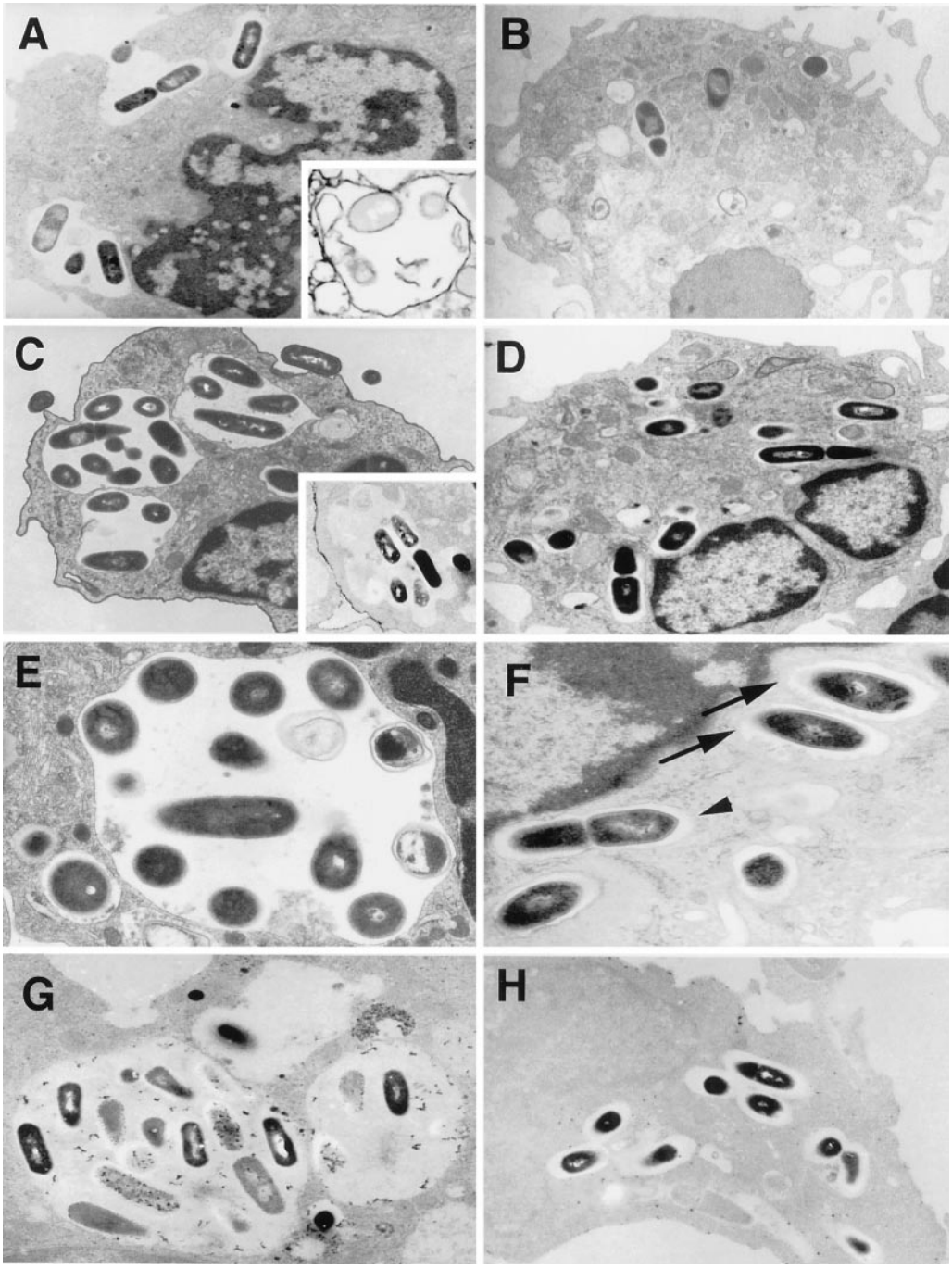

Figure 10. Electron micrographs of *Listeria*-infected macrophages. *Listeria* cell ratio was set at 10:1 (1×10^6 *Listeria* for 1×10^5 cells). **A:** *Listeria* are phagocytosed and incorporated in phagosomes of MSR-A+/+ macrophages at 20 minutes after infection. Original magnification, ×5,000. **Inset:** 2F8 is expressed on the phagosomal membrane. Immunoelectron microscopy using 2F8; original magnification, ×4,000. **B:** In MSR-A−/− macrophages, the majority of *Listeria* are present in single form at 20 minutes after infection. Original magnification, ×4,000. **C:** Most *Listeria* are confined within large vacuoles (phagolysosomes) at 30 minutes after infection in MSR-A+/+ macrophages. Original magnification, ×5,000. **Inset:** 2F8 is not expressed on the phagosomal membrane. Immunoelectron microscopy using 2F8; original magnification, ×4,000. **D:** Most *Listeria* are remaining in single form, and no large phagosomes are found at 30 minutes after infection in MSR-A−/− macrophages; original magnification, ×4,000. **E:** Vacuolar membranes encircle *Listeria* in MSR-A+/+ macrophages, and several *Listeria* are degraded at 30 minutes after infection. Original magnification, ×10,000. **F:** In MSR-A−/− macrophages, the vacuolar membranes are partially disrupted (**arrows**). A division of *Listeria* in the cytoplasm (**arrowhead**). Original magnification, ×20,000. **G:** In MSR-A+/+ macrophages, phagosomes are fused with lysosomes, and most *Listeria* are undergoing degradation at 1 hour after infection. Acid phosphatase staining; original magnification, ×4,000. **H:** Few phagosomes of MSR-A−/− macrophages have fused with lysosomes at 1 hour after infection. Acid phosphatase staining; original magnification, ×4,000.

with *Listeria*, prolonged and remarkable granulomatous inflammation in the liver of MSR-A−/− mice, and the impaired bactericidal activity in MSR-A−/− macrophages provide the direct evidence that SR-AI/II plays an essential role in host defense against *Listeria* by promoting phagocytosis and killing of the microorganisms. The impaired listericidal activity of macrophages from MSR-A−/− mice was attributed to the rapid escape of *Listeria*

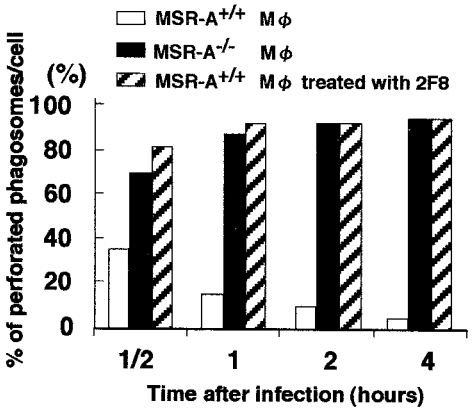

Figure 11. Changes in the proportion of perforated phagosomes in MSR-A+/+, MSR-A−/−, and 2F8-treated MSR-A+/+ peritoneal macrophages *in vitro*. The *Listeria* cell ratio was set at 10:1 (1×10^6 *Listeria* for 1×10^5 cells). Data are the mean for 100 cells in each group.

from the phagosomes into the host macrophage cytoplasm. LLO is in part responsible for the evasion.

Granulomas are part of host defense against pathogens including *L. monocytogenes.* The numbers of Mac-1-positive exudate macrophages[39,40] increased remarkably during the granuloma formation in MSR-A−/− mice. Therefore, we used thioglycollate-elicited exudate peritoneal macrophages for *in vitro* analysis and demonstrated the strong activity of elimination of *Listeria* by exudate macrophages, compared with that of resident macrophages. Hence, the impaired listericidal activity of MSR-A−/− exudate macrophages may chiefly account for the prominent granuloma formation in MSR-A−/− mice.

It has been reported that Kupffer cells are mainly responsible for the initial elimination of *Listeria* at the early stage of infection.[32] Several molecules, such as complement receptor type 3 (CR3),[50,51] and the receptor of internalin A (InlA)[52] mediate the uptake of *Listeria*. Previous reports have demonstrated a crucial role of SR-AI/II for the uptake of not only dead bacteria,[25] but live bacteria.[26] Thomas and colleagues[26] demonstrated that both opsonin-dependent and SR-AI/II mediated opsonin-independent phagocytosis of Gram-positive bacteria play an important role in host defense against bacterial infections. The SR-AI has been reported to recognize lipoteichoic acid and bind to Gram-positive bacteria including *L. monocytogenes in vitro*.[21] In the present *in vitro* study, listerial uptake activities of MSR-A−/− peritoneal macrophages and 2F8-treated MSR-A+/+ macrophages were more impaired than those of MSR-A+/+ macrophages. Furthermore, in the present *in vivo* study, initial uptake of *Listeria* by Kupffer cells were defective in MSR-A−/− mice. These findings clearly imply that SR-AI/II mediate the phagocytosis of *L. monocytogenes*.

The mechanism underlying the role of SR-AI/II in host defense against infections with intracellular pathogens is unclear. It is speculated that the impaired uptake of *L. monocytogenes* in MSR-A−/− macrophages results in decreased clearance of bacteria from the site of infection and extensive bacterial dissemination in various tissues. This is supported by the present study that demonstrated defective listericidal activity of MSR-A−/− macrophages. Decreased listericidal activity and increased listerial proliferation in MSR-A−/− macrophages may have resulted in recruitment of larger numbers of phagocytes and other immune cells, more remarkable formation of granulomas, and higher susceptibility to listerial infection in MSR-A−/− mice. This is the first evidence that SR-AI/II plays a critical role in the listericidal processes.

Which mechanism is responsible for the decreased listericidal capacity in MSR-A−/− macrophages? The present electron microscopy *in vivo* and *in vitro* demonstrated a significantly higher proportion of perforated *Listeria*-bearing phagosomes in MSR-A−/− macrophages than in MSR-A+/+ macrophages. Ultrastructural acid-phosphatase staining revealed that few perforated phagosomes of MSR-A−/− macrophages fused with lysosomes, whereas phagosome-lysosome fusion occurred frequently in MSR-A+/+ macrophages after infection. The importance of LLO for listerial escape was confirmed in the experiment using a LLO-nonproducing mutant strain. These findings suggest that *Listeria* use LLO efficiently to escape from the phagosomes before phagosome-lysosome fusion in MSR-A−/− macrophages. It has been shown that a key step to regulate the LLO activation is phagosomal acidification in macrophages. Vacuolar membrane perforation by LLO occurs frequently at acidic vacuolar pH, with a mean near 6.0.[53] On the other hand, phagosome-lysosome fusions require a more acidic pH, near 5.0 or less.[54,55] Our preliminary study demonstrated that bafilomycin A1, a proton pump inhibitor,[56,57] suppressed both the listerial multiplication and the perforation of the *Listeria*-containing phagosomes of MSR-A−/− macrophages (data not shown), suggesting that SR-AI/II may be involved in the mechanism of phagosomal acidification. Further studies are necessary to clarify the SR-AI/II-mediated listericidal mechanism in macrophages.

In conclusion, SR-AI/II functions positively in host defense against listerial infection not only by functioning as a receptor but also by mediating listericidal mechanisms through the regulation of LLO-dependent listerial escape.

Acknowledgments

We thank Mr. K. Sato, S. Momozaki, H. Sano, K. Ohyachi, Ms. M. Saito, and Ms. K. Moriki for their excellent technical assistance; and Dr. I. Fraser, Dr. D. A. Hughes, and Prof. S. Gordon, Oxford University, for the 2F8 monoclonal antibody.

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