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Listeria monocytogenes serotype 4b strains replicate in monocytes/macrophages more than the other serotypes

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ABSTRACT. We analyzed the pathogenicity of various serotypes of *Listeria monocytogenes* using a Balb/c mouse intravenous injection model. The survival rates of mice inoculated with strains NS1/2b (serotype 1/2b), NS3b (serotype 3b) and NS 4b (serotype 4b) were 60, 63.6 and 63.6%, respectively. Although the survival rates were similar, the bacterial growth in the liver of NS3b-infected mice was 144.5-fold higher than that in the liver of NS4b-infected mice. Histopathological analyses suggest that the NS4b strain replicated more in monocytes/macrophages, whereas the NS3b strain replicated more in hepatocytes. These results raise a possibility that the serotype 4b strains replicated more in monocytes/macrophages compared to the other serotype strains. To assess this, we isolated CD11b-positive cells from mouse livers infected with EGDe (serotype 1/2a), NS1/2b, NS3b, NS4b and the serotype 4b strains 51414 and F17 and counted the number of live bacteria in these cells. CD11b-positive cells from the NS4b-, 51414- and F17-infected mice possessed 24.4- to 42.7-fold higher numbers of live bacteria than those from mice infected with EGDe and NS3b strains. These results suggest that serotype 4b strains replicated more in monocytes/macrophages than the other serotypes, and this may be involved in the pathogenicity of serotype 4b strains, particularly in the dissemination of *L. monocytogenes* through the host body.

KEY WORDS: *Listeria monocytogenes*, monocyte/macrophage, pathogenicity, serotype

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Listeria monocytogenes (*L. monocytogenes*) is a gram positive, facultative intracellular bacterium that causes listeriosis in humans and animals. *L. monocytogenes* infection in healthy adult individuals induces influenza-like symptoms and mild gastroenteritis [3]. Food-borne outbreaks have been reported in many countries since an outbreak due to contaminated coleslaw was reported in Canada in 1981 [22, 24]. The elderly, immunocompromised hosts, pregnant women and neonates exhibit particularly severe symptoms, such as sepsis, meningitis, abortion and neonatal listeriosis [7, 13]. The fatal rate among high-risk patients is approximately 20–30%, which is relatively high among food-borne diseases [10].

L. monocytogenes is divided into 13 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 [18]. Most of the clinical isolates in sporadic and outbreak cases in humans belong to serotype 4b, followed by serotypes 1/2a and 1/2b. Nonetheless, several other serotypes, such as serotype 3b, also contribute to the incidence of sporadic listeriosis [14, 19]. Further, an outbreak from contaminated butter in Finland in 1998–1999 was caused by a serotype 3a strain [16]. To date, most of the studies regarding the pathogenicity of *L. monocytogenes* have been performed using the standard strains EGD, EGDe and 10403S that belong to serotype 1/2a, and some studies used serotypes 4b and 1/2b. A previous study by von Koenig *et al.* analyzed the pathogenicity of serotypes 1/2b, 3a, 4b and 4d in NMRI mice infected with *L. monocytogenes* serotypes via intraperitoneal and intravenous (i.v.) routes. Their results suggest that the pathogenicity of serotypes 3a and 4d was lower than that of serotypes 4b and 1/2b [27]. However, there are little information about the pathogenicity of serotypes other than 1/2a, 1/2b and 4b. Also, it still remains

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unclear if there are any differences among serotypes in the pathogenic mechanism.

In the current study, we compared the pathogenicity of 7 serotypes of *L. monocytogenes* as well as a standard strain EGDe in a Balb/c mouse i.v. injection model, which is an established model for analyzing the virulence of *L. monocytogenes* [21]. Our data suggest that serotype 3b might be as pathogenic as serotypes 4b and 1/2b. Our data also suggest that serotype 4b strains had a distinct feature in replication in monocytes/macrophages compared to the other serotypes.

MATERIALS AND METHODS

Bacterial strains

L. monocytogenes strains NS1/2a, NS1/2b, NS3a, NS3b, NS3c, NS4a and NS4b (serotypes 1/2a, 1/2b, 3a, 3b, 3c, 4a and 4b, respectively) were provided by the National Institute of Health, which was renamed as the National Institute of Infectious Diseases in 1997. The sources of these strains are unknown. Strains EGDe (serotype 1/2a, a mouse-passaged strain of EGD isolated from guinea pig) and 51414 (serotype 4b, isolated from raw milk associated with listeriosis outbreak in Massachusetts) were purchased from ATCC (Manassas, VA, U.S.A.). Strain F17 (serotype 4b complex) was isolated from the feces of a clinically healthy sheep. Bacteria were grown in Heart Infusion Broth or on Heart Infusion Agar (Nissui, Tokyo, Japan).

Mice

Total 209 of 6- to 7-week-old female Balb/c mice were purchased from CLEA Japan Inc. (Hamamatsu, Japan). All procedures were approved by the Animal Experiment Committee of the Graduate School of Veterinary Medicine, Hokkaido University. Mice were euthanized by overexposure to sevoflurane (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan).

Survival rate of mice in a Balb/c intravenous (i.v.) infection model

Seventy two mice were inoculated intravenously with 10^4 colony forming unit (CFU) of *L. monocytogenes* strains EGDe (n=8), NS1/2a (n=8), NS1/2b (n=10), NS3a (n=8), NS3b (n=11), NS3c (n=8), NS4a (n=8) or NS4b (n=11) in 50 μ l of Hank's balanced salt solution (HBSS) (Sigma-Aldrich, Tokyo, Japan). Fifty microliters of HBSS was inoculated in 3 mice as a negative control (mock). Mice were observed until 7 days post inoculation (dpi).

Bacterial growth in livers and spleens

Eighty eight mice were inoculated intravenously with *L. monocytogenes* strains EGDe (n=12), NS1/2a (n=12), NS1/2b (n=10), NS3a (n=11), NS3b (n=11), NS3c (n=11), NS4a (n=10) or NS4b (n=11). HBSS was inoculated in 9 mice for mock. At 1, 3 and 6 dpi, the livers and spleens were collected from 3 to 4 mice and homogenized in phosphate buffered saline (PBS) at 10% w/v. Ten-fold serial dilutions were made with PBS and spread onto the agar plates. The plates were incubated at 37°C for 24 hr, and the colonies were counted.

Histopathological analysis

Thirteen mice were inoculated intravenously with *L. monocytogenes* strains EGDe (n=4), NS1/2b (n=3), NS3b (n=3) or NS4b (n=3). HBSS was inoculated to 3 mice for mock. At 3 dpi, the livers, spleens, kidneys, hearts, lungs and brains were collected. For analysis of dead mice, we used the tissue from dead mice used for analysis of the survival rate. The number of dead mice available for the examination was 2 for NS1/2b-infected mice, 1 for NS3a-infected mice, 2 for NS3b-infected mice and 2 for NS4b-infected mice. The tissues were fixed with 10% phosphate-buffered formalin and embedded in paraffin. The sections were subjected to hematoxylin and eosin (HE) staining and immunostaining. For immunohistochemistry, anti-*L. monocytogenes* rabbit polyclonal antibodies (Abcam, Cambridge, U.K., No. 35132) were incubated at a 1:5,000 dilution as primary antibodies at 4°C overnight. EnVision+ System HRP Labeled Polymer Anti-Rabbit (Dako, Tokyo, Japan, no. K4002) were incubated for 30 min at room temperature. The ImmPACT DAB Substrate (Vector Laboratories, Burlingame, CA, U.S.A.) was reacted to visualize the immunoreactivity. Nuclei were counterstained with hematoxylin. For double immunofluorescence staining, the sections were incubated with anti-*L. monocytogenes* antibodies as described above, and Alexa Fluor 488-labeled anti-Rabbit IgG (H+L) F (ab')² goat polyclonal antibodies (Thermo Fisher Scientific, Yokohama, Japan) were used as secondary antibodies. After being fixed with 4% paraformaldehyde, the sections were incubated with Alexa Fluor 555-labeled anti-Iba1 rabbit IgG (order made by Sigma). Fluorescence labeling was performed using the Alexa Fluor 555 Antibody Labeling Kit (Thermo Fisher Scientific).

Isolation of CD11b-positive cells from livers

Twenty one mice were inoculated intravenously with *L. monocytogenes* strains EGDe (n=4), NS1/2b (n=4), NS3b (n=4), NS4b (n=3), 51414 (n=3) or F17 (n=3). At 3 dpi, livers were collected, minced and digested with 0.1 U/ml of Dispase II (Roche, Basel, Switzerland) at 37°C for 10 min. After the cells were dispersed by pipetting, the debris was removed by filtration using 100 μ m pore filter. The cell suspension was centrifuged at 300 g for 10 min, and the cells were resuspended in 0.5% bovine serum albumin (Sigma) in HBSS. CD11b-positive cells were isolated using CD11b microbeads, LS column and a MACS separator according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were serially diluted and spread onto the agar plates. After incubation at 37°C for 24 hr, the number of colonies was counted.

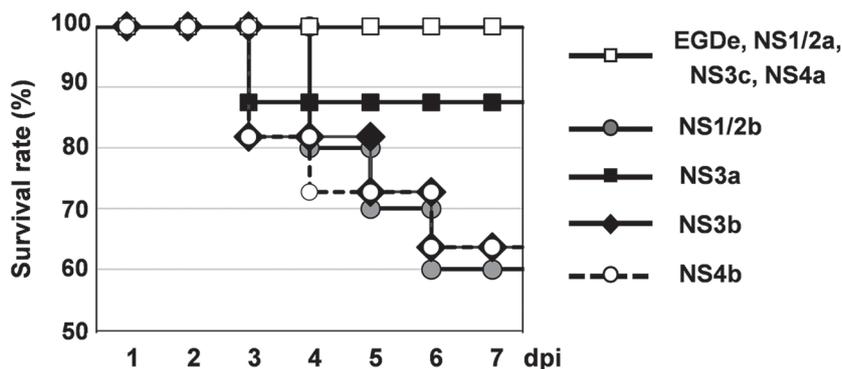


Fig. 1. Survival curve of Balb/c mice infected with 7 serotypes of *L. monocytogenes*. Eight to 11 mice were inoculated intravenously with each strain. Survival of mice was observed until 7 dpi.

RESULTS

Survival rate of Balb/c mice infected intravenously with 7 serotypes of L. monocytogenes

To compare the pathogenicity among serotypes of *L. monocytogenes*, we inoculated *L. monocytogenes* to Balb/c mice intravenously (Fig. 1). All mice showed ruffled fur at 2 dpi, but NS3c-, NS4a- and mock-infected mice did not. Mice inoculated with the NS1/2b, NS3b and NS4b strains died from 3 to 6 dpi, and the survival rates were 60% (6/10 mice), 63.6% (7/11 mice) and 63.6% (7/11 mice), respectively. Mice that survived until 7 dpi appeared to recover from the infection as they did not show any clinical signs. One of the eight mice inoculated with the NS3a strain died at 3 dpi, and the survival rate was 87.5%. The survival rates of mice inoculated with the EGDe, NS1/2a, NS3c and NS4a strains were 100% (8/8 mice for each).

Growth of 7 serotypes of L. monocytogenes in the livers and spleens of Balb/c mice

After an i.v. inoculation to mice, *L. monocytogenes* grew in the liver and spleen [26]. To examine if there were any differences in the bacterial growth among the serotypes, we counted the number of live bacteria in the liver and spleen at 1, 3 and 6 dpi (Fig. 2a and 2b). In the EGDe-, NS1/2b-, NS3a-, NS3b- and NS4b-infected mice, bacterial counts reached a peak at 3 dpi and decreased at 6 dpi both in the livers and spleens. Interestingly, the average bacterial count in the livers of NS3b-infected mice ($10^{6.72}$ CFU/g tissue) was 144.5-fold higher than that of NS4b-infected mice ($10^{4.57}$ CFU/g tissue; Fig. 2a, dashed circles), although their survival rates were the same. Further, the average bacterial count in the livers of EGDe-infected mice ($10^{6.25}$ CFU/g tissue) was 49-fold higher than those in the livers of the 4b-infected mice (Fig. 2a, dashed circles), although no mice died by EGDe infection. The bacterial growth of the NS1/2a, NS3c and NS4a strains was much lower than that of the EGDe, NS1/2b, NS3b and NS4b strains. No bacterial colony was observed in the livers and spleens of mock-infected mice.

Different cell tropism in the liver among the strains EGDe, NS1/2b, NS3b and NS4b

To explore the reason for low bacterial growth in the livers of NS4b-infected mice, we performed a histopathological analysis at 3 dpi (Figs. 3 and 4). In the liver, multiple abscesses were observed in EGDe-, NS1/2b-, NS3b- and NS4b-infected mice (Fig. 3a), although the sizes of the abscesses were smaller in EGDe-infected mice compared to the others. In addition, infiltration of macrophages around blood vessels and bile ducts was prominent in the livers of NS4b-infected mice (Fig. 3c and 3d). In EGDe- and NS3b-infected mice, bacterial antigens were mainly detected in the hepatocytes (Fig. 4a and 4b). On the other hand, in NS4b-infected mice, bacterial antigens were mainly detected in the foci of inflammatory cells (Fig. 4f–4h). The bacterial antigen-positive cells in the livers of NS4b-infected mice were positive for a monocyte/macrophage marker, Iba1 (Fig. 4i–4k). In NS1/2b-infected mice, bacterial antigens were detected in both foci of inflammatory cells (Fig. 4d) and hepatocytes (Fig. 4e). The histological findings were observed in all examined mice. These results suggest that the NS4b strain infected monocytes/macrophages more than the other strains. There was no significant histopathological change and bacterial antigen in the livers of mock-infected mice (Figs. 3b and 4c).

Histopathological findings in the tissues other than the livers

We further performed histopathological analysis on the spleens, kidneys, hearts, lungs and brains of EGDe-, NS1/2b-, NS3b- and NS4b-infected mice at 3 dpi (Fig. 3e–3h). In the spleens, migration of monocytes/macrophages was prominent in EGDe-, NS1/2b-, NS3b- and NS4b-infected mice (Fig. 3e), but not in mock-infected mice (Fig. 3f). There were no histopathological lesions in the kidneys, hearts, lungs and brains (data not shown). Bacterial antigens were mainly detected in the dendritic cells of the lymphoid follicles in the spleens of EGDe-, NS1/2b-, NS3b- and NS4b-infected mice (Fig. 4l and 4m). Bacterial antigens were occasionally in monocytes/macrophages of the splenic sinus of EGDe-, NS1/2b-, NS3b- and NS4b-infected mice. No bacterial antigen was detected in mock-infected mice (Fig. 4n). We also examined dead mice infected with NS1/2b, NS3a, NS3b and NS4b strains. Bacterial clumps were observed in the livers, spleens, and kidneys regardless of the *L. monocytogenes* strain (Fig. 3g and 3h). Multifocal necrosis were observed in the livers and spleens of all dead mice, although there were no histological lesions in

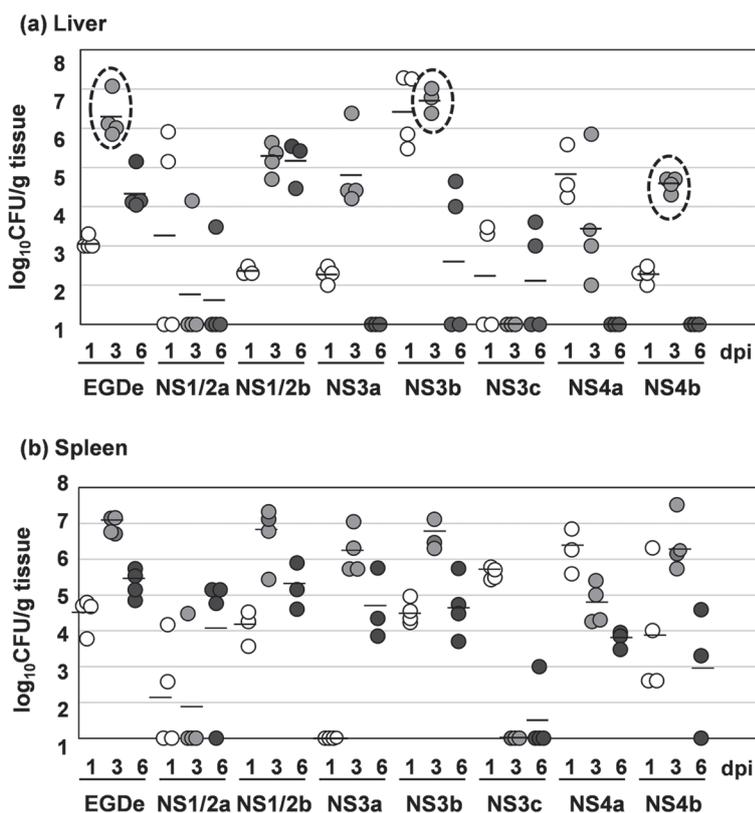


Fig. 2. Bacterial growth of 7 serotypes of *L. monocytogenes*. (a) Bacterial growth in the liver. (b) Bacterial growth in the spleen. Mice were inoculated intravenously with each strain. At 1, 3 and 6 dpi, the livers and spleens were harvested from 3 or 4 mice for each strain, and live bacteria were counted. The detection limit was 10^2 CFU/g tissue. The bacterial counts in the livers of EGDe-, NS3b- and NS4b-infected mice at 3 dpi were enclosed by dashed circles for emphasis. White dots, 1 dpi; light gray dots, 3 dpi; dark gray dots, 6 dpi. Bars indicate the means of three or four samples.

the other tissues. Bacterial antigens were also observed in the blood vessels of lungs and meninges of an NS4b-infected mouse, although any histopathological lesions were not observed in these tissues (data not shown).

Difference in the replication in monocytes/macrophages between serotype 4b strains and the other serotypes

The histopathological analysis of the livers raised a possibility that the NS4b strain replicated in monocytes/macrophages more than the other strains. We hypothesized that this feature might be common among the serotype 4b strains. To assess this, we counted the number of live bacteria in CD11b-positive cells from the livers of mice infected with strains EGDe, NS1/2b, NS3b, NS4b and the serotype 4b strains 51414 and F17 at 3 dpi (Fig. 5). In 10^5 of CD11b-positive cells from the livers of EGDe-, NS1/2b- and NS3b-infected mice, 2.5×10^2 , 2.2×10^2 and 2.2×10^2 CFU of live bacteria were detected, respectively. On the other hand, 6.1×10^3 CFU of bacteria were detected in those from NS4b-infected mouse livers, which was significantly higher than those from EGDe- and NS3b-infected mouse livers ($P < 0.05$, Dunnett's test). Further, as we expected, 9.4×10^3 and 7×10^3 CFU of bacteria were detected in the CD11b-positive cells from mouse livers infected with the serotype 4b strains 51414 and F17, and these bacterial counts were significantly higher than those from EGDe-, NS1/2b- and NS3b-infected mouse livers ($P < 0.05$, Dunnett's test). These results suggest that *L. monocytogenes* serotype 4b strains replicated in CD11b-positive monocytes/macrophages more than the other serotypes.

DISCUSSION

In the current study, our data suggest a possibility that the pathogenesis of listeriosis caused by serotype 4b strains may be different from the others, because *L. monocytogenes* serotype 4b strains replicated more than the other serotypes in CD11b-positive monocytes/macrophages in the liver. This feature may be involved in the pathogenicity of *L. monocytogenes* serotype 4b strains, particularly in the dissemination of *L. monocytogenes* through the host body. Drevets *et al.* [5] suggest that intracellular *L. monocytogenes* in phagocytes play an important role in the systemic spread of bacteria replicated in the liver and spleen. In the current study, we isolated cells using anti-CD11b antibody-coated microbeads. Although neutrophils as well as monocytes/macrophages are CD11b-positive, the results of Fig. 5 were more likely to be indicative of *L. monocytogenes* infection to monocytes/macrophages because the antigen-positive cells in the livers of NS4b-infected mice were positive for a monocyte/macrophage marker, Iba1 (Fig. 4i–4k). The importance of monocytes in the dissemination of bacteria during systemic

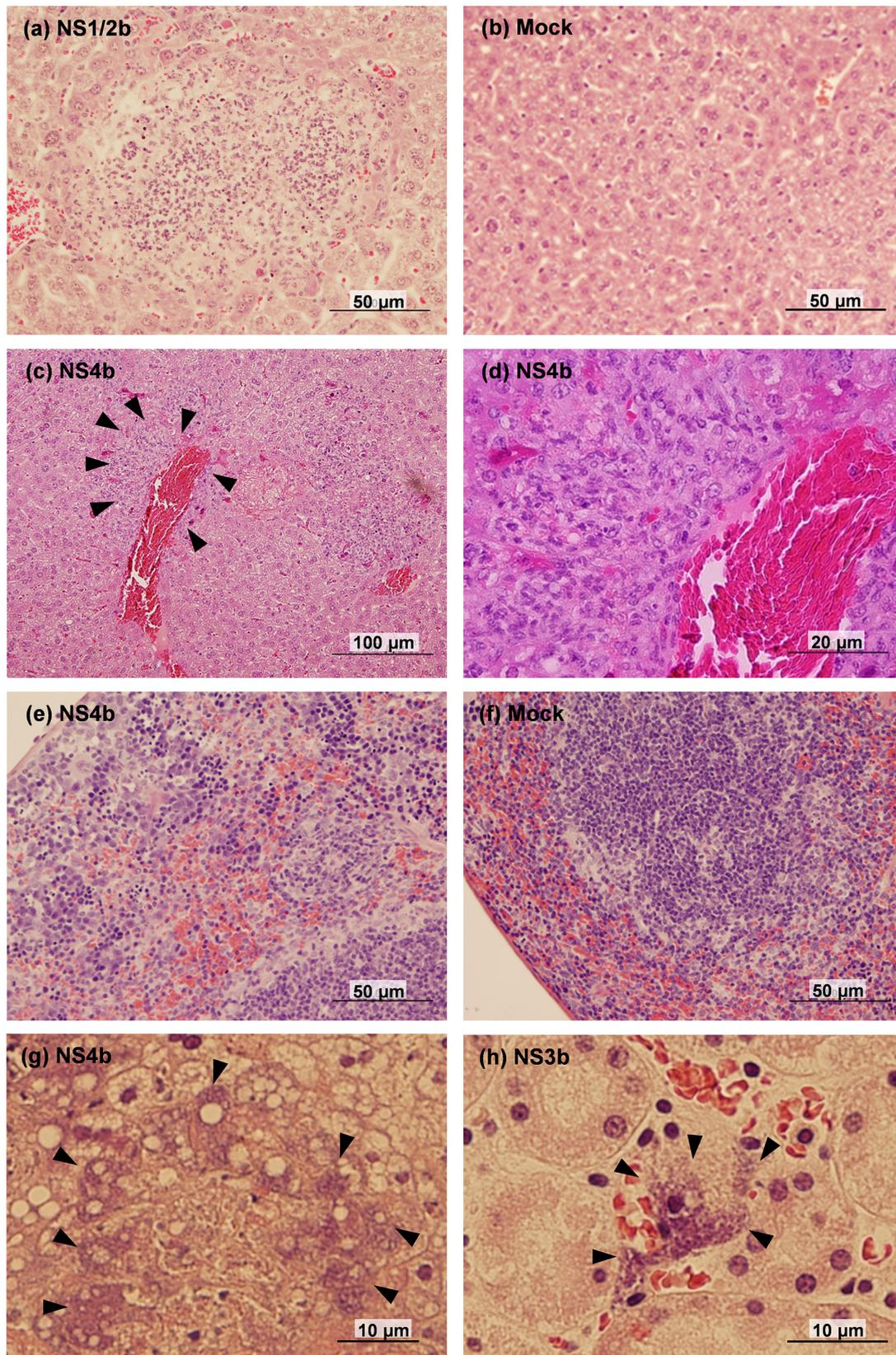


Fig. 3. Histopathology of *L. monocytogenes*-infected Balb/c mice. (a) Abscess in the liver of NS1/2b-infected mouse. (b) Liver of mock-infected mouse. (c) Perivascular infiltration of macrophages in the liver of NS4b-infected mouse. Arrowheads surround the area with macrophage infiltration. (d) Magnified image of the area surrounded by arrowheads in (c). (e) Migration of macrophages in the splenic sinus of NS4b-infected mouse. (f) Spleen of mock-infected mouse. (g, h) Bacterial clumps in the dead mice, observed in the liver of NS4b-infected mouse (g) and in the kidney of NS3b-infected mouse (h). Arrowheads show the bacterial clumps.

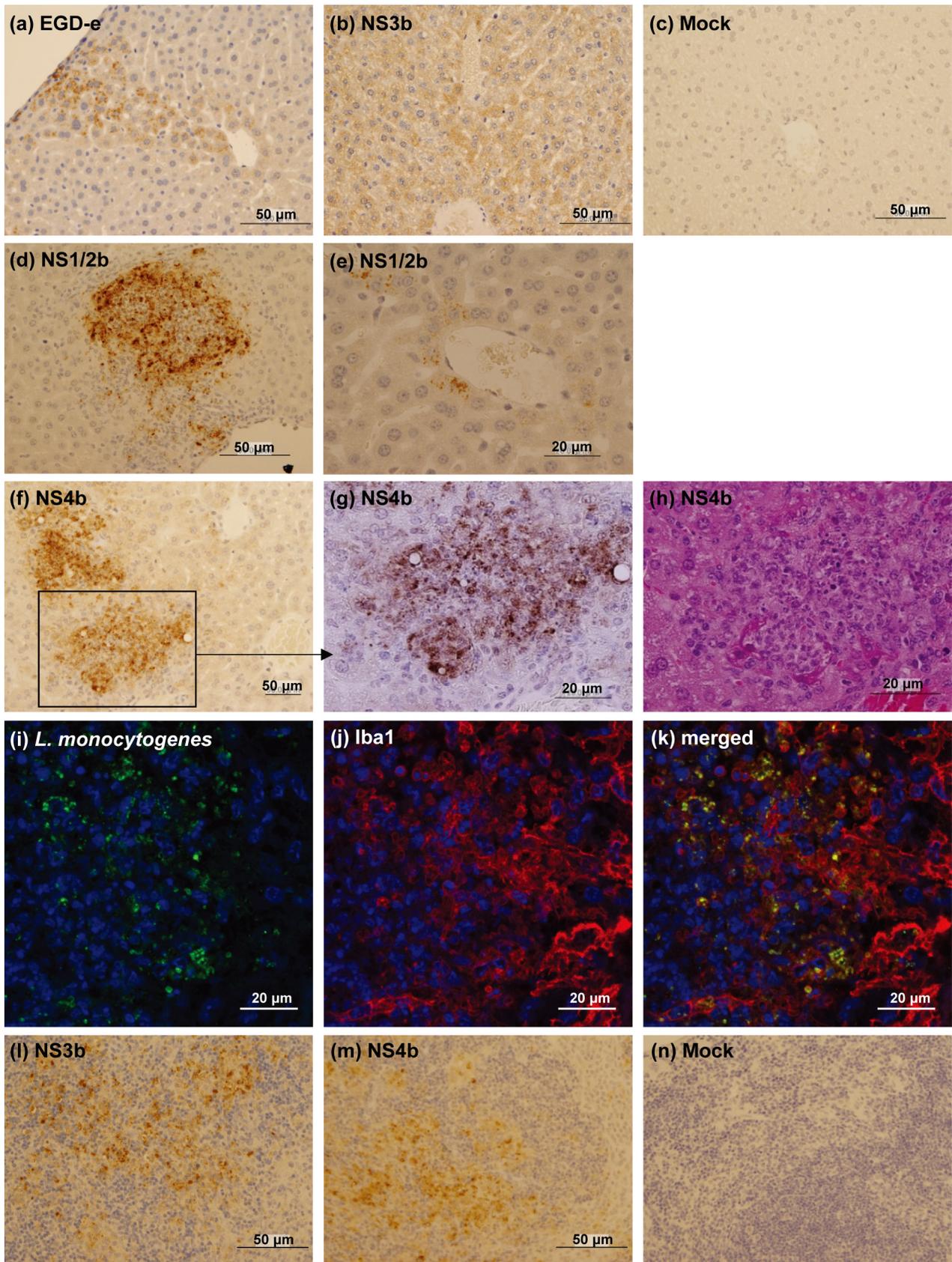


Fig. 4. Immunostaining for *L. monocytogenes* antigens. (a–g) Distribution of the *L. monocytogenes* antigens in the livers of EGDe-(a), NS3b-(b), mock-(c), NS-1/2b-(d, e) and NS4b-infected (f, g) mice. (g) Magnified image of the enclosed area of (f). (h) HE staining of the serial sections of (g). (i–k) Double immunofluorescence staining of *L. monocytogenes* antigens and Iba1 in the liver of NS4b-infected mouse. Green, *L. monocytogenes*; Red, Iba1; Blue, Nuclei. (l–n) Distribution of *L. monocytogenes* antigens in the spleens of NS3b-(l), NS4b-(m) and mock-infected (n) mice.

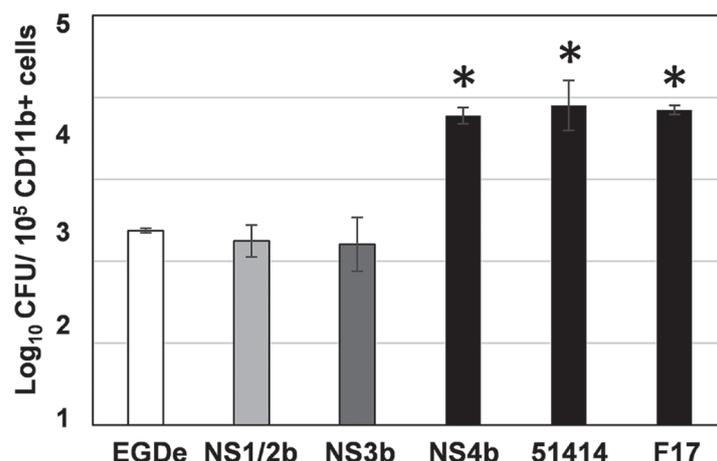


Fig. 5. Bacterial counts in CD11b-positive cells isolated from *L. monocytogenes*-infected mouse livers. Three or four mice were inoculated intravenously with each strain. At 3 dpi, CD11b-positive cells were isolated from the liver, and live bacteria were counted. * $P < 0.05$ vs EGDe (Dunnett's test).

L. monocytogenes infection has been suggested by previous studies. Most of the *L. monocytogenes*-infected leukocytes in the blood have been morphologically identified as mononuclear phagocytes [4]. Further, Drevets *et al.* [6] suggested that Ly6C^{high} monocytes contributed to the transport of *L. monocytogenes* into the brain. In contrast to the livers, there was no difference in the bacterial growth in the spleen between serotype 4b strains and the others. Most of the *L. monocytogenes* antigen-positive cells appeared to be dendritic cells in the splenic follicles, while some monocytes/macrophages in the sinus were also positive. The identical cell tropism may result in the similar bacterial growth in the spleens of serotype 4b strains and the others.

A serotype 3b strain, NS3b, was as pathogenic as the NS4b strain in Balb/c mice when injected intravenously. Human clinical cases caused by serotype 3b strains are not common, although serotype 3b strains are occasionally isolated from food and the environment [2, 23]. One possible reason for this is that the successful invasion of serotype 3b strain via the oral route may be quite limited compared with that of serotypes 4b, 1/2a and 1/2b strains. Indeed, the serotype 3b strain seems to be less invasive to intestinal epithelial cells than serotypes 4b and 1/2b strains, as Bueno *et al.* demonstrated using Caco-2 cells [1]. Further, it may be possible that serotype 3b strains are pathogenic only when invasion to the bloodstream has been established. The low infectivity to monocytes/macrophages may result in an insufficient invasion into the bloodstream.

Serotypes 1/2a and 1/2b strains are also often isolated from human clinical cases and foods. The majority of food isolates belong to serotype 1/2a, while food-borne outbreaks of human listeriosis are mainly caused by serotype 4b strains [8, 9, 11, 12, 17, 20, 25]. However, multistate outbreaks by serotypes 1/2a and 1/2b strains occurred in the United States in 2011 [17]. In Canada, serotype 1/2a isolates with very similar pulsed-field gel electrophoresis patterns predominantly caused human listeriosis from 1988 to 2010 [12]. Therefore, the pathogenicity of serotypes 1/2a and 1/2b strains may be variable depending on the strain or host immune condition. In the current study, the NS1/2a strain seemed to be low pathogenic to a Balb/c i.v. injection model, because the mortality late was low, while the NS1/2b strain caused the highest mortality. *L. monocytogenes* antigens in the livers of NS1/2b-infected mice were detected both in hepatocytes and monocytes/macrophages. However, the NS1/2b strain did not replicate in CD11b-positive macrophages as successfully as serotype 4b strains. Although the reason for unsuccessful replication of the NS1/2b strain in macrophages is unknown for now, it would be of interest to analyze the factors that determine the replication ability of *L. monocytogenes* in macrophages in the future.

The cause of death for the mice infected with NS1/2b, NS3b and NS4b strains was most likely due to sepsis, because bacterial clumps were often observed in the livers and spleens of dead animals regardless of the strains. Further, *L. monocytogenes* antigens were detected in the blood vessels in the lungs, kidneys and meninges. These results suggest that replication ability in macrophages did not affect the occurrence of sepsis, once adequate amount of *L. monocytogenes* invade the blood stream. As we injected *L. monocytogenes* into the tail vein here, significance of replication ability in macrophages on bacterial dissemination in the host body should be assessed in the future by using an oral exposure of a transgenic mouse model expressing human E-cadherin in the intestinal epithelium, in which *L. monocytogenes* invade from intestinal epithelium more efficiently than from those of wild type mice [15]. Meningoencephalitis is one of the clinical conditions caused by *L. monocytogenes* infection in humans and animals. However, meningoencephalitis hardly occurred in the mouse model used in the current study. Although we speculate that the sufficient replication of *L. monocytogenes* in macrophages might increase the incidence of meningoencephalitis, this should be also examined by using an appropriate model in the future, such as intraperitoneal inoculation of *L. monocytogenes*-infected macrophages as described by Drevets *et al.* [5].

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