Induction of Protective Cellular Immunity against *Mycobacterium tuberculosis* by Recombinant Attenuated Self-Destructing *Listeria monocytogenes* Strains Harboring Eukaryotic Expression Plasmids for Antigen 85 Complex and MPB/MPT51

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We report here the induction of specific protective cellular immunity against *Mycobacterium tuberculosis* by the employment of vaccination with recombinant attenuated *Listeria monocytogenes* strains. We constructed self-destructing attenuated *L. monocytogenes Δ2* strains carrying eukaryotic expression plasmids for the antigen 85 complex (Ag85A and Ag85B) and for MPB/MPT51 (mycobacterial protein secreted by *M. bovis* BCG/mycobacterial protein secreted by *M. tuberculosis*) molecules. Infection of these recombinant bacteria allowed expression of the genes in the J774A.1 murine macrophage cell line. Intraperitoneal vaccination of C57BL/6 mice with these recombinant bacteria was capable of inducing purified protein derivative-specific cellular immune responses, such as foot pad reactions, proliferative responses of splenocytes, and gamma interferon production from splenocytes, suggesting the efficacy of vaccination against mycobacterial infection by use of these recombinant *L. monocytogenes* strains. Furthermore, intravenous vaccination with recombinant bacteria carrying expression plasmids for Ag85A, Ag85B, or MPB/MPT51 in BALB/c mice elicited significant protective responses, comparable to those evoked by a live *Mycobacterium bovis* BCG vaccine. Notably, this is the first report to show that MPB/MPT51 is a major protective antigen in addition to Ag85A and Ag85B, which have been reported to be major mycobacterial protective antigens.

Tuberculosis (TB) remains an urgent public health problem worldwide, resulting in 8 million new cases and 2 million deaths each year (14). Outbreaks of TB, especially in immunocompromised people, such as aged groups and AIDS patients, have also been reported. In addition, the appearance of multidrug-resistant *Mycobacterium tuberculosis* strains is also a serious issue in the world.

The only TB vaccine currently available is the attenuated *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG), which has been reported to have a variable protective efficacy, ranging from 0 to 85% in different controlled studies (6). Therefore, there remains an urgent need for an improved vaccine. A DNA vaccine is one of the most promising candidates for future TB vaccines. Many reports on DNA vaccination against TB have been accumulating. Secreted molecules have been known to be recognized by the protective immune response against TB. In these reports, various target antigens (Ags) for TB DNA vaccination have been reported, including the Ag85 complex molecules, Hsp65, Hsp70, the 38-kDa Ag, and ESAT-6 (reviewed in reference 28).

Ag85 complex molecules have been reported to be the dominant secreted Ags expressed by nearly all mycobacterial species analyzed so far (reviewed in reference 39). The complex consists of three structurally related components, namely Ag85A (p32A; 32-kDa Ag), Ag85B (p30; 30-kDa Ag, also termed α Ag), and Ag85C. Ag85 complex molecules are cross-reactive Ags and are highly conserved among *Mycobacterium* spp. The genes encode proteins with fibronectin-binding capacities (1) and mycolyltransferase activities, which are involved in the final stage of mycobacterial cell wall assembly (5). Ag85A and Ag85B have been reported to stimulate B- and T-cell responses in TB patients (24, 25), and immunization with Ag85A and Ag85B proteins induced protection against an aerosol challenge with *M. tuberculosis* in mice and guinea pigs, respectively (19). In addition, reports of successful naked DNA vaccines against TB, employing the Ag85A (3, 4, 9, 13, 21, 29, 36, 37) and Ag85B (22, 29, 37) genes, have also accumulated. According to these reports, the Ag85A and Ag85B molecules seem to be two of the most promising candidates for future subunit TB vaccines. Another molecule, MPB/MPT51 (mycobacterial protein secreted by *M. bovis* BCG/mycobacterial protein secreted by *M. tuberculosis*), has also been reported to be related to this family (31). The amino acid sequence deduced for MPB51 (GenBank/EMBL/DDBJ accession number D26486) is identical to the sequence deduced for MPT51 of *M. tuberculosis* strains H37Rv (AJ022076) and CDC1551 (AE007185). So far, MPB/MPT51 has not been reported as a target Ag for vaccination against *M. tuberculosis*.

DNA vaccines offer many advantages over other methods.
of immunization: they have a relatively easy design and construction by recombinant DNA techniques, a strong induction of cellular immunity, chemical stability, a relatively low cost, and so on (reviewed in references 2 and 12). For successful results with DNA vaccination (for example, in the case of Ag85A), however, intramuscular immunization with large amounts (50 to 100 μg) of plasmid DNA was reported to be necessary (36), and the induction of immunity with intramuscular immunization of plasmid DNA is poor in terms of reproducibility (41). Recently, several investigators used attenuated intracellular bacteria as the carriers of DNA vaccines (reviewed in reference 11). These bacterial carrier systems have several special features, including direct delivery of the plasmid DNA to professional antigen-presenting cells and the possibility of oral administration. Bacteria utilized as this type of vaccine carrier include *Salmonella* (8) and *Shigella* (35) as well as *Listeria* (10). Gram-negative carriers such as *Salmonella* and *Shigella* have the disadvantage of containing abundant amounts of toxic lipopolysaccharide. Therefore, *Listeria monocytogenes*, a gram-positive bacterium, is a good candidate for a carrier. Furthermore, this bacterium is considered a possible effective recombinant vaccine vector based on its predilection for professional antigen-presenting cells such as macrophages and dendritic cells and its capacity to escape from phagolysosomes and to live in the cytoplasm of host cells (34, 38). In addition, this bacterium has been reported to have the ability to induce T-helper cell type 1 (Th1) immune responses (20). These features are favorable for eliciting effective cellular immunity against TB. Dietrich et al. (10) reported a DNA vaccination system using an attenuated self-destructing *L. monocytogenes* strain. They demonstrated the feasibility of the system in a cell culture system. They used a deletion mutant of *L. monocytogenes* Δ2 that lacks the entire lecithinase operon, including the virulence-associated genes actA, mpl, and plcB (17). This strain can infect macrophages and replicate in the cytoplasm but cannot spread to adjacent cells. This attenuated mutant was introduced with a plasmid containing the gene for lysis protein PLY118 of the listerial bacteriophage A118. PLY118 expression was controlled by the actA promoter, which is active when *L. monocytogenes* is in the host cell cytoplasm. Thus, this *L. monocytogenes* mutant escapes from the phagosome and then lyses when the PLY118 gene is expressed in the cytoplasm. Autolysis of the *L. monocytogenes* mutant apparently releases the plasmid DNA into the host cell cytoplasm, allowing expression of the transgene in the host cells. However, it was still unknown whether this DNA vaccine carrier system is capable of inducing specific immunity and protective immunity against infection in vivo. For this study, we examined the inducibility of protective cellular immunity against *M. tuberculosis* by immunization of mice with this attenuated *L. monocytogenes* strain carrying a cukaryotic expression plasmid for Ag85A, Ag85B, or MPBS1. The results showed that vaccination with the attenuated self-destructing *L. monocytogenes* strain could induce protective cellular immunity against *M. tuberculosis* infection. Furthermore, we show for the first time that MPB/MPT51, which is related to Ag85 family molecules, is a major protective Ag.
Mice. C57BL/6 and BALB/c mice (Japan SLC, Hamamatsu, Japan) were kept under specific-pathogen-free conditions and fed autoclaved food and water ad libitum at the Experimental Animal Institute of the Hamamatsu University School of Medicine. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

Immunization procedures. Mice were immunized intraperitoneally (i.p.) (C57BL/6: \( \sim 10^7 \) CFU) or intravenously (i.v.) (BALB/c: \( \sim 10^8 \) CFU) with a recombinant attenuated L. monocytogenes \( \Delta \)2 strain three times at 2-week intervals. As a control, mice were also immunized with BCG i.p. once (C57BL/6: \( \sim 10^7 \) CFU) or subcutaneously twice at a 2-week interval (BALB/c: \( \sim 10^9 \) CFU).

Genomic DNA PCR. \( \Delta p3L118R-MPB51 \) or \( \Delta p3L118R-Listeria \) (strain \( \sim 10^8 \) CFU) was injected i.v. into BALB/c mice. One day after the injection, tissue cell suspensions from injected mice were prepared and washed three times after the lysis of erythrocytes with Tris-buffered 0.83% ammonium chloride solution. After a brief centrifugation, the cells were added to 10 volumes of proteinase K solution (1 mg/ml; Boehringer Mannheim GmbH, Mannheim, Germany) in 10 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl, and 0.4% sodium dodecyl sulfate and were incubated for 15 min at 65°C. The cells were further incubated in the same solution overnight at 37°C. Genomic DNA was prepared from the cells after phenol extraction and ethanol precipitation. A nested PCR was performed with 1 \( \mu \)g of genomic DNA for MPB51 gene detection. The first-round PCR was performed with the same primer pairs that were used for RT-PCR analysis, and the second-round PCR was performed with 1 \( \mu \)l of the first-round PCR solution (20- \( \mu \)l total volume) and the following primer pairs located just inside of the first-round PCR primers: 5'-GCCGCGTAAAGCGCTGGAACAC-3' and 5'-CACACGCCGCGATTGCTGCAT-3'. For both PCRs, the conditions were 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The expected size of the MPB51 PCR product was 341 bp.

Delayed-type hypersensitivity (DTH) reaction. Purified protein derivative (PPD) was purchased from Japan BCG Inc. C57BL/6 mice were injected with 5 \( \mu \)g of PPD in 50 \( \mu \)l of phosphate-buffered saline (PBS) in the left hind foot pad. As controls, mice were injected with 50 \( \mu \)l of PBS alone in the right hind foot pad. The swelling of foot pads was measured with a caliper meter (Mitsutoyo Corp., Osaka, Japan) 48 h after injection. Naïve mice treated in the same way as the controls for nonspecific swelling.

Lymphocyte proliferation assay. Spleen cells (10^7) isolated from the immunized C57BL/6 mice were cultured for 48 h at 37°C in 96-well round-bottom tissue culture plates in the presence or absence of 5 \( \mu \)l of PPD/ml. After 48 h of incubation, de novo DNA synthesis was assessed by the addition of 0.5 \( \mu \)Ci of [methyl-\( ^3 \)H]thymidine (10 Ci/mmol; ICN Biochemicals, Irvine, Calif.) well for the last 12 h of culture. QuantiCult plates were harvested onto glass fiber filters and the radioactivity was counted by liquid scintillation. The [methyl-\( ^3 \)H]thymidine incorporation was calculated in counts per minute per 10^4 cells.

Cytokine ELISA. Spleen cells were harvested from the immunized C57BL/6 mice. Recovered cells were incubated for 4 days in 24-well plates at 2 \times 10^5 cells/well in RPMI-10% fetal bovine serum in the presence or absence of 5 \( \mu \)g of PPD solution/ml. Concentrations of gamma interferon (IFN-\( \gamma \)), interleukin-4 (IL-4), and IL-5 in the culture supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) as described elsewhere (40). For the sandwich ELISA, the following combinations of coating and biotinylated enzyme-linked immunosorbent assay (ELISA) were used (40). For the sandwich ELISA, the following combinations of coating and biotinylated enzyme-linked immunosorbent assay (ELISA) were used (40).

Semiquantitative RT-PCR for IFN-\( \gamma \) gene. Immune spleen cells (C57BL/6 mice) were cultured for 48 h at 10^7 cells/ml in the presence or absence of 5 \( \mu \)g of PPD solution/ml. Total RNAs were extracted from cells by use of Iogen RNA extraction solution (Nippon Gene). Single-stranded cDNAs were synthesized with Moloney murine leukemia virus reverse transcriptase (Life Technologies) and then used in PCRs for IFN-\( \gamma \) gene detection as described elsewhere (40).

In the protection assay, immunized BALB/c mice were infected with \( 5 \times 10^8 \) CFU of M. tuberculosis H37Rv iv. 2 months after the last immunization. Mice were sacrificed 10 weeks later, and the bacterial numbers in the spleens, livers, and lungs were counted in CFU on Middlebrook 7H11 medium (Becton Dickinson).

Statistics. Data from multiple experiments were expressed as means \( \pm \) standard deviation (SD). Statistical analyses were performed with the StatView-J 5.02 statistics program (Abacus Concepts, Berkeley, Calif.). Data were analyzed by Fisher’s protected least significant difference.

RESULTS

Infection of recombinant L. monocytogenes allowed expression of genes in J774A.1 murine macrophage cell line. J774A.1 murine macrophage-like cells were infected with an L. monocytogenes \( \Delta \)2 mutant carrying the plasmid p3L118R, p3L118R-
Ag85A, p3L118R-Ag85B, or p3L118R-MPB51. Thirty-six hours after infection, the infected cells were harvested for the isolation of total RNA. RT-PCR was then performed to confirm the expression of Ag85A, Ag85B, or MPB51 mRNA in the cells. As shown in Fig. 1A, clear bands for these mRNAs were detected after RT of total RNA solutions but not before reverse transcriptase treatment. In addition, to refute the possibility of contamination of plasmids p3L118R-Ag85A, p3L118R-Ag85B, and p3L118R-MPB51 in the cDNA pools used, we subjected the cDNA pools to PCRs with relevant primer sets, by which only the plasmid DNAs, but not the transcripts, were detected (see Materials and Methods for details). The results showed that no bands were detected with the cDNA pools by PCR, while p3L118R-Ag85A, p3L118R-Ag85B, and p3L118R-MPB51 controls gave specific bands, indicating no contamination of plasmids in the cDNA pools. These data indicate that the Ag85A, Ag85B, and MPB51 genes were expressed in J774A.1 cells by this attenuated L. monocytogenes system.

Detection of injected plasmid DNA in tissues of mice infected with recombinant attenuated L. monocytogenes. Δ2/p3L118R-MPB51 recombinant Listeria was injected i.p. (C57BL/6 mice) or i.v. (BALB/c mice). In order to check for p3L118R-MPB51 plasmid transfer to tissues of the injected mice, we prepared genomic DNAs from cells of the tissues and performed PCR analysis for MPB51 DNA detection. As shown in Fig. 2, we observed an MPB51-specific band only for DNAs derived from mice injected with Δ2/p3L118R-MPB51 Listeria. The PCR was performed with tissue cells washed with PBS, suggesting that the p3L118R-MPB51 plasmid was transferred into host cells after recombinant Listeria injection. It is noteworthy that we observed no colonies of carrier L. monocytogenes in the spleens of the i.p. immunized C57BL/6 mice by plating of the tissue homogenates on Trypticase soy agar (data not shown).

PPD-specific DTH reaction with recombinant attenuated L. monocytogenes vaccination. For effective protective immunity against M. tuberculosis, specific cellular immunity against the bacterium plays a critical role. We first examined DTH responses of C57BL/6 mice immunized with Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51. C57BL/6 mice were immunized with Δ2/p3L118R, Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51 three times at 2-week intervals. The data for mice immunized once with M. bovis BCG are also shown as a control. One month after the last immunization, foot pad swelling responses directed against PPD were examined. Black bars, footpad swelling after in vivo PPD stimulation; hatched bars, foot pad swelling with PBS alone. The means ± SD of four to five mice per group are shown. Asterisks indicate statistically significant (P ≤ 0.001) differences with the value for a control (Δ2/p3L118R) immunization.
PPD-specific cytokine production with recombinant attenuated L. monocytogenes vaccination. IFN-γ is known to be a key factor for the elicitation of effective protection against M. tuberculosis. Therefore, employing RT-PCR analysis, we semiquantitatively assessed IFN-γ mRNA expression from splenocytes of immunized C57BL/6 mice upon PPD stimulation. As shown in Fig. 5, IFN-γ mRNA-specific bands were clearly detected in PPD-stimulated splenocytes of C57BL/6 mice immunized with recombinant Listeria strain Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51. The strengths of the bands were comparable to that of mice immunized with M. bovis BCG. Again, splenocytes of mice immunized with the Δ2/p3L118R control gave only a faint IFN-γ mRNA-specific band.

In addition, we examined the cytokine production of splenocytes from immunized C57BL/6 mice by a sandwich ELISA for IFN-γ, IL-4, and IL-5 (Table 1). Correlating with the results of RT-PCRs, splenocytes from mice immunized with M. bovis BCG or a recombinant Listeria strain harboring the Ag85A, Ag85B, or MPB51 gene produced high amounts of IFN-γ after in vitro stimulation with PPD. We observed the production of moderate levels of IFN-γ from spleen cells of naïve mice and control Listeria (Δ2/p3L118R)-immunized mice upon PPD stimulation. We did not detect significantly enhanced production of IL-4 or IL-5 for any of the mice examined.

Recombinant attenuated L. monocytogenes vaccination conferred protective immunity against M. tuberculosis infection comparable to M. bovis BCG immunization in BALB/c mice.

### Table 1. Cytokine production by spleen cells from mice immunized with Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>Stimulation with PPD</th>
<th>Cytokine production (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-γ</td>
<td>IL-4</td>
</tr>
<tr>
<td>Naïve</td>
<td>–</td>
<td>131</td>
</tr>
<tr>
<td>BCG immunized</td>
<td>+</td>
<td>868</td>
</tr>
<tr>
<td>Δ2/p3L118R immunized</td>
<td>–</td>
<td>186</td>
</tr>
<tr>
<td>Δ2/p3L118R-Ag85A</td>
<td>+</td>
<td>4,103</td>
</tr>
<tr>
<td>Δ2/p3L118R-Ag85B</td>
<td>+</td>
<td>51</td>
</tr>
<tr>
<td>Δ2/p3L118R-Ag85A</td>
<td>+</td>
<td>740</td>
</tr>
<tr>
<td>Δ2/p3L118R-Ag85B</td>
<td>+</td>
<td>218</td>
</tr>
<tr>
<td>Δ2/p3L118R-MPB51</td>
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<td>1,728</td>
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<td>Δ2/p3L118R-MPB51</td>
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<td>210</td>
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<tr>
<td>Δ2/p3L118R-MPB51</td>
<td>+</td>
<td>3,432</td>
</tr>
<tr>
<td>Δ2/p3L118R-MPB51</td>
<td>+</td>
<td>191</td>
</tr>
<tr>
<td>Δ2/p3L118R-MPB51</td>
<td>+</td>
<td>4,093</td>
</tr>
</tbody>
</table>

* a: Splenocytes from immunized C57BL/6 mice (2 × 10⁶ cells per well) were cultured in the presence (+) or absence (−) of 5 µg of PPD/ml.

* b: After 4 days, cytokine concentrations in culture supernatants were quantified by IFN-γ, IL-4, and IL-5-specific ELISA, as described in Materials and Methods. The values for naïve and BCG-immunized mice are also shown as controls. Averages of duplicate representative data from several similar experiments are shown.
We evaluated the effects of recombinant attenuated *Listeria* vaccination on protective immunity against *M. tuberculosis* H37Rv infection and compared them with those of *M. bovis* BCG vaccination. At first, we used C57BL/6 mice for the experiments, but the relative resistance against *M. tuberculosis* infection of the strain hampered the evaluation of the vaccination effects. Therefore, we used BALB/c mice for the evaluation. Ten weeks after i.v. injection with *M. tuberculosis* H37Rv, spleens, livers, and lungs were prepared from the immunized mice and the numbers of CFU of *M. tuberculosis* H37Rv in these tissues were evaluated. Figure 6 shows viable H37Rv, spleens, livers, and lungs were prepared from the immunization. The data for mice immunized once with *M. bovis* BCG are also shown as a control. The mice were challenged i.v. with live *M. tuberculosis* H37Rv. Numbers of CFU in the spleens, livers, and lungs were determined 10 weeks later. The means ± SD of six mice are shown. Asterisks indicate statistically significant (P < 0.05) differences with the value for a control (Δ2/p3L118R) immunization.

DISCUSSION

From the findings described in this paper, we drew the following conclusions concerning the attenuated self-destructing *L. monocytogenes*-harboring DNA vaccine against *M. tuberculosis*. (i) Inoculation with recombinant *L. monocytogenes*-harboring plasmid DNA vaccines for Ag85 complex and MPB/MPT51 molecules is able to induce specific type 1 cellular immune responses in spleen cells of mice. (ii) Inoculation with these vaccines can confer protective immunity against TB. (iii) The MPB/MPT51 molecule, which is related to the Ag85 family, appears to be a major protective Ag, in addition to Ag85A and Ag85B.

We detected a significant level of PPD-specific IFN-γ secretion, which is a hallmark of type 1 immune responses and is considered an important factor in the protective immunity against *M. tuberculosis* (7, 15, 23), in splenocytes of mice immunized with attenuated recombinant *Listeria* harboring an Ag85A, Ag85B, or MPB/MPT51 DNA vaccine (Table 1). The production of moderate levels of IFN-γ from splenocytes of naive mice and control *Listeria* (Δ2/p3L118R)-immunized mice may be caused by nonspecific responses of these mice against PPD.

Cellular immunity, including CD8+ cytotoxic T lymphocytes and CD4+ Th1 cells, has been reported to play critical roles in effective protective immunity against *M. tuberculosis* (reviewed in references 16 and 32). In this context, the attenuated *Listeria* immunization system shown here should be a favorable immunization method, as it is able to elicit effective type 1 cellular immune responses against *M. tuberculosis*. Furthermore, an attenuated *Listeria* strain harboring the suicide gene *pHy118* was revealed to be almost nontoxic, since inoculation with ∼10⁶ CFU of the attenuated *Listeria*, but not virulent *L. monocytogenes*, failed to kill even IFN-γ receptor knockout mice as well as C57BL/6 wild-type mice (data not shown). Also, we could not detect carrier *L. monocytogenes*, although the plasmid DNA vaccines were detected in the spleens of i.p. immunized C57BL/6 mice (data not shown).

Several heterologous carrier systems for mycobacterial Ags have been reported. Zhu et al. (42) showed that the recombinant vaccinia virus system for *M. tuberculosis*-derived 19- and 38-kDa glycolipoproteins is effective for protection against murine *M. tuberculosis* infection. Hess et al. (18) reported that a recombinant *Salmonella enterica* serovar Typhimurium vaccine which secretes Ag85B is effective for the induction of pathogen-specific IFN-γ and tumor necrosis factor and also for protection against murine TB. It will be interesting to compare the system shown here with these systems in terms of the induction of protective immunity against *M. tuberculosis*.

As a general rule, the determination of a target Ag is very important for the development of effective DNA vaccines.
against bacterial infection. Many reports have already shown the effectiveness of Ag85A and Ag85B for eliciting protective immunity against M. tuberculosis. We also confirmed with our system that Ag85A and Ag85B are capable of inducing cellular and protective immunity. In addition, we evaluated the effectiveness of MBP/MPT51 as a target Ag for an anti-TB vaccine. Our results indicate that MBP/MPT51 is also a protective Ag and is comparable to Ag85A and Ag85B. In particular, immunization with Δ2/p3L118R-MBP51 induced enhanced PPD-specific IFN-γ production from splenocytes, the expression level of which was comparable to that by BCG immunization. So far, MBP/MPT51 has not been reported as a target Ag for vaccination. Therefore, it is interesting and important to examine the antigenicity of the molecule in detail to study, for example, the capacity to induce specific CD4+ and CD8+ T-cell effectors, and to identify the T-cell epitopes in the molecule. We identified T-cell epitopes in C57BL/6 and BALB/c mice (M. Suzuki, T. Aoshi, T. Nagata, and Y. Koide, submitted for publication). The spleen cells derived from Δ2/p3L118R-MBP51-immunized mice were able to induce IFN-γ in response to these epitope peptides, indicating that the responses are MBP51 specific (data not shown).

For the induction of effective immunity, the route of vaccination is a critical factor. The Listeria carrier system is suitable for the induction of mucosal immunity. Particularly, intranasal inoculation of our recombinant Listeria strains may be capable of inducing protective T-cell immune responses in the lung. That study is now in progress. For the present study, however, we immunized mice with Listeria i.p. (C57BL/6 mice) or i.v. (BALB/c mice). The main reason for not choosing the oral route for immunization is that the mouse was reported not to be a good model for the entry of L. monocytogenes into intestinal epithelium due to a Glu-to-Pro substitution in mouse E-cadherin, which serves as a receptor for internalin A of L. monocytogenes (26, 27). In humans, however, oral immunization with L. monocytogenes-harboring plasmid DNA vaccines seems to be a possible choice for DNA vaccine delivery. Although mice are devoid of the E-cadherin molecule, L. monocytogenes may have a capacity to enter into M cells located in the intestinal epithelium. Therefore, oral administration of attenuated L. monocytogenes strains is also worthwhile to try in mice.

Taken together, we show here that DNA vaccines with the attenuated self-destructing L. monocytogenes carrier system may be favorable DNA vaccination systems in vivo when accompanied with the adjuvanticity to induce Th1-type immune responses and the predilection of the bacterium to interact with macrophages.

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