Regression of Established B16F10 Melanoma with a Recombinant Listeria monocytogenes Vaccine

Zhen-Kun Pan, Larry M. Weiskirch, and Yvonne Paterson

Department of Microbiology and the University of Pennsylvania Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6076

ABSTRACT

We have previously shown that Listeria monocytogenes, a Gram-positive, facultative intracellular bacterium, is a potent vector for targeting tumor-specific antigens to the immune system. In the present study, we extend these studies to the highly tumorigenic mouse melanoma B16F10, transduced with a model tumor antigen. We are able to induce the regression of primary tumors and established lung metastases by parenteral immunization with a L. monocytogenes recombinant that expresses the same antigen. Adjunctive therapy with granulocyte macrophage colony-stimulating factor or a vaccinia-based vaccine does not result in an improved cure rate over the L. monocytogenes vaccine alone. Tumor regression is accompanied by the expression of inflammatory cytokines in the tumor.

INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultative intracellular bacterium that has the unusual ability to escape from the phagolysosome and live in the cytoplasm of the cell. It has, thus, been scrutinized as a potential vaccine vector for both infectious and neoplastic disease (reviewed in Ref. 1). Using a model system consisting of colon (CT26) and renal (Renca) carcinomas that express the influenza virus NP and a recombinant L. monocytogenes strain that secretes this antigen (Lm-NP), we have shown that Listeria has an exceptional capacity to induce antitumor immunity to a tumor-associated antigen (2, 3). NP serves as a good model tumor antigen because it is well characterized immunologically and it does not alter the intrinsic immunogenicity and tumorogenicity of the parental tumor (2). We showed that Lm-NP delivered i.p. could protect mice against lethal challenge with tumor cells that express the antigen and cause regression of established macroscopic tumors (2). CD8+ cells were the critical effectors, although CD4+ cells were also important mediators of antitumor immunity (2). Because the normal route of L. monocytogenes infection is through the gut (1), we have also explored the potential of Lm-NP to cause regression of macroscopic tumors when delivered p.o. We found that we could cure 50–60% of mice of established CT26-NP and Renca-NP tumors by the oral administration of Lm-NP. However, although we did see a slowing of the growth of established B16F10-NP, we could not effect a cure of any animals by this route of administration (3).

B16F10 melanoma is among the most aggressive, poorly immunogenic murine tumors (4). In addition, B16F10-NP tumor cells lose expression of the NP antigen transgene very rapidly with the emergence of antigen loss tumor cells in vivo (3). Thus, among transplantable mouse tumors, this model tumor system provides a stringent test of the potency of any immunotherapeutic approach. In this study, therefore, we have attempted to optimize a parenteral vaccine protocol using Lm-NP to determine whether conditions exist under which we can cure mice of established s.c. tumors and of experimental lung “metastases” established by tail-vein injection with B16F10-NP cells.

MATERIALS AND METHODS

Animals. Specific pathogen-free, female C57BL/6 mice were obtained from The Jackson Laboratory. Mice were used at 6–8 weeks of age.

Cell Lines. B16F10 is a spontaneously arising melanoma cell line derived from the C57BL/6 mouse that shows very low expression of MHC class I and no expression of MHC class II (4). It was transduced with a NP gene (A/PR8/34) using a replication-defective Moloney murine leukemia retrovirus containing both the NP gene and a neomycin phosphotransferase gene, as described previously (5, 6). Transductants were selected in G418 and retain the NP gene in vivo in the presence of G418. The expression of NP in this tumor does not enhance its immunogenicity or decrease its intrinsic tumorogenicity in that the minimal tumoricidal dose in mice is identical to the parent line; for B16F10-NP, 2 × 103/mouse. Thus, when expressed in the tumor, NP behaves indistinguishably from an endogenous tumor antigen.

B16F10-GM-CSF is B16F10 transduced with murine GM-CSF, as described previously (7). It was kindly provided by Drew Pardoll (Johns Hopkins School of Medicine, Baltimore, MD).

Cells were maintained in RPMI 1640 containing 10% FCS, 2 mm L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

L. monocytogenes Strains and Propagation. The L. monocytogenes vaccine strain used in this study is DP-L2028, which was constructed from the hemolytic wild-type strain 10403S to stably express and secrete a fusion protein of listeriolysin, an essential virulence factor of the bacterium and influenza (A/PR8/34) nucleoprotein, LLO-NP. The construction of the L. monocytogenes recombinant DP-L2028 (Lm-NP) has been described previously (8). In some experiments, as a control for nonspecific effects mediated by the bacterial vector alone, we used a stable chromosomal recominant of L. monocytogenes that synthesizes and secretes the HIV Gag protein (9). In this study, we refer to these strains as Lm-Gag and Lm-NP to draw attention to their antigenic difference. Lm-Gag has a similar LLO content in C57BL/6 mice to that of Lm-NP (~4 × 107 CFU; Ref. 8). Both strains have attenuated virulence, of about three logs, compared with the parental strain 10403S from which they were constructed. The bacteria are propagated in brain-heart infusion medium broth and agar. Lm-NP is maintained in medium supplemented with 25 μg/ml of chloramphenicol to select for the LLO-NP-containing plasmid.

Vaccinations. Lm-NP and Lm-Gag were administered i.p. using the doses described. Vaccination by vaccinia (107 PFU) was via the tail vein. Vaccinia expressing the NP gene of PR8 was kindly provided by Dr. Jack Bennink (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Disease). Vaccinia expressing the E7 gene of human papilloma virus strain 16 was kindly provided by Dr. T-C. Wu (Johns Hopkins University, Baltimore, MD). B16F10-GM-CSF immunizations were performed with a total of 1.5 × 107 irradiated (6000 rads) cells. Mice received 3 × 106 B16F10-GM-CSF tumor cells in each limb, plus the back.

Tumor Regression Studies. B16F10-NP (2 or 3 × 105) was introduced s.c. into C57BL/6 mice. After measurable (4–5-mm) tumors had grown between 7 and 11 days after tumor challenge, treatment was initiated. Groups of 8 or 10 mice were used for each treatment, and all animals were randomized before receiving treatment. Tumor growth was then measured using calipers every 2 days and was recorded as the narrowest and longest surface length. Values shown for tumor size (mm) are the mean of these two lengths for each animal in the group. Mice were sacrificed when tumor sizes reached ~20–25 mm in average diameter. Statistical significance was determined by t test.
(Statworks, version 1.2). In all experiments, a P ≤ 0.05 was considered significant. Each experiment was performed at least twice with essentially similar results, unless otherwise described.

**Treatment of B16F10-NP Pulmonary Metastases.** Fifty mice were injected with $4 \times 10^4$ B16F10-NP tumor cells in the tail vein to establish pulmonary metastases. All animals were randomized before receiving treatment and divided into five groups of 10 mice. As a control, one group of 10 mice was left untreated. Two groups were immunized with $4 \times 10^4$ CFU, $10^5$ CFU, and $4 \times 10^4$ CFU of Lm-NP on either day 6, 12, and 18 or day 9, 15, and 21 after i.v. challenge with tumor cells. The other two groups received identical doses of Lm-Gag on a similar schedule. The animals were sacrificed on day 50, and lungs from the mice were inflated with PBS and then fixed in 10% buffered formalin phosphate. Metastases were counted under a dissecting microscope.

**Analysis of Cytokine mRNA Expression in B16F10-NP Tumors by RT-PCR.** Mice were injected s.c. with $2 \times 10^5$ B16F10-NP. At a time when the tumors reached 5 mm in size, the mice were immunized i.p. with $2 \times 10^6$ CFU Lm-NP or Lm-Gag; seven days later, a second dose of Lm-NP was administered. One, 3, or 7 days after the second immunization, tumors were harvested and frozen immediately in liquid nitrogen for RT-PCR analysis. The tissue was then homogenized and lysed in a guanidinium-based solution for purification of total RNA using the RNeasy total RNA kit (QIAGEN), according to the manufacturer’s directions. For first-strand cDNA synthesis the SuperScript kit (Life Technologies, Inc.) was used. For amplification of the target cDNA, PCR primers were designed to encompass a length of ~300 bp of cDNA. Primer sequences are described in Table 1 and have been shown competent to amplify the appropriate gene product. After PCR amplification, the cDNA products were run on a 0.9% agarose gel at 100 V for 1–2 h with ethidium bromide staining and compared with a 123-bp ladder marker to detect the cDNA samples were run on a 0.9% agarose gel at 100 V for 1–2 h with ethidium bromide staining and compared with a 123-bp ladder marker to detect cDNA products of appropriate size. β-Arcin levels were used to standardize cDNA template concentrations used in PCR reactions. Representative results are presented from a single set of experiments; the isolation of RNA from the various groups of tumor-bearing mice was performed three times, and each set of RNA isolates was analyzed by RT-PCR at least three times.

**RESULTS**

**Established Primary B16F10-NP Tumors Can Be Eliminated by Lm-NP Immunization.** We have previously shown that two i.p. immunizations with $2 \times 10^6$ CFU of Lm-NP are sufficient to cure 90% of BALB/c mice of established syngeneic RENCA-NP tumors (2). In the case of B16F10-NP, however, it causes the regression of only about 40% of the tumors (Fig. 1A). The animals that were cured of established B16F10-NP tumor resisted further challenge with the tumor given on day 30. In an attempt to improve this cure rate, we investigated the effects of repeated immunization at weekly intervals with the Lm-NP vaccine, which may provide long-term activation of anti-NP CTL (10). In the first experiment, B16F10-NP tumors were established in eight mice and immunization began at day 7; twenty-five percent of the mice were cured by a single immunization, and an additional 25% were cured after a second immunization on day 14. After the third immunization on day 21, the tumor was eliminated in one mouse and tumor growth was arrested in an additional mouse. No additional cures were observed after the fourth immunization on day 28 or the fifth immunization on day 35, although tumor growth was halted in all but one mouse so that at 6 weeks after tumor challenge seven of eight mice in the Lm-NP-immunized mice survived, whereas all of the mice in the other groups had been euthanized (Fig. 1B). In a second experiment, five mice in each group were immunized weekly for 7 weeks. After five immunizations, a total of 60% of mice were cured by this protocol and remained tumor free until we terminated the experiment at around 3 months after tumor challenge (data not shown). No further cures were effected by the last two immunizations in this experiment. Thus, repeated immunization with $2 \times 10^6$ CFU of Lm-NP improves the cure rate of established B16F10-NP tumors slightly and also inhibits tumor growth in nonregressing tumor-bearing mice.

Because antilisterial immunity induced by the first immunization with Lm-NP causes rapid clearance of subsequent bacterial challenges and protects against lethal challenge, we sought to improve the cure rate by using a 2-fold higher dose for the initial immunization and escalating doses for booster immunizations. Fig. 1C shows the effect on regression of B16F10-NP by delivering Lm-NP or Lm-Gag on day 7 (4 $\times 10^6$ CFU), day 14 (10$^5$ CFU), and day 21 (2 $\times 10^7$ CFU). This did not increase the cure rate of B16F10-NP to greater than that achieved using lower doses (Fig. 1, A, B, and C). We conclude that repeated immunizations of small doses are more effective than increased doses of the *L. monocytogenes* vaccine.

In the experiments shown in Fig. 1, B and C, we included a control for nonspecific immunity. We had not observed any slowing of tumor growth due to the i.p. delivery of 2 $\times 10^6$ CFU of vector alone with RENCA-NP and CT-26-NP (2) or $10^8$ to $10^9$ PFU p.o. with RENCA, CT-26-NP, and tumor growth was similar to that seen in the Lm-Gag group. We had previously shown that increased doses of the vector alone did not increase the cure rate (10).

**Table 1. Oligonucleotide primers used for RT-PCR**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primer sequence (5′ → 3′)</th>
<th>Reaction product</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>GCAACTGTCTCTGTTGAACTCA</td>
<td>382</td>
</tr>
<tr>
<td>IL-2</td>
<td>CTGCAAGCTTCTTACCTGCA</td>
<td>442</td>
</tr>
<tr>
<td>IL-6</td>
<td>AGGAGAGAGGAGGAGGAGGAGGAG</td>
<td>432</td>
</tr>
<tr>
<td>IL-12β</td>
<td>ATCGCCTGTGGGAGCTTGAG</td>
<td>325</td>
</tr>
<tr>
<td>TNF-α</td>
<td>TGGCTGACGGGCGCTGAGTACG</td>
<td>406</td>
</tr>
<tr>
<td>TNF-β</td>
<td>TGCTGACACCGGCGCTGACG</td>
<td>320</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>AACTGGGTCTTTGAGACCTGTG</td>
<td>320</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>CGCTGTCTGACCTGAGCAGAGCT</td>
<td>509</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>GCCTGACACCGGCGCTGAGTACG</td>
<td>294</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>TGGTTGACTCTGAGCTGACG</td>
<td>573</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GTGGGGGCGCTTACGACGGACT</td>
<td>540</td>
</tr>
</tbody>
</table>

*These primers amplify the p40 chain.

*These primers amplify the following IFN-α genes: α-2 (Genbank accession numbers K01238 and X01969); α-1-9 (Genbank accession number M13360); α-7 (M13710); and α-B (Genbank accession number L38608).
CT-26, or B16F10-NP (3). However, because the dose was repeated (Fig. 1B) or escalated (Fig. 1C) for the subsequent immunizations we included Lm-Gag, a strain of L. monocytogenes that synthesizes and secretes the HIV Gag protein, which has similar virulence to Lm-NP. In Fig. 1, B and C, we see a slowing of tumor growth in animals who received Lm-Gag compared with naive mice that by day 14 and later time points in the experiments shown in Fig. 1, B and C, was statistically significant (on day 14: Fig. 1B, P > 0.015 for Lm-Gag relative to naive mice compared with P > 0.0001 for Lm-NP relative to naive mice; Fig. 1C, P > 0.005 for Lm-Gag relative to naive mice compared with P > 0.0001 for Lm-NP relative to naive mice). Although Lm-Gag clearly exercises a potent adjuvant effect on tumor growth, we have never observed long-term survivors in this treatment group of mice, in contrast to mice cured of established tumors by Lm-NP that typically remain tumor free for several months and resist subsequent tumor challenges.

**Combined Therapy with L. monocytogenes-NP and Vaccinia-NP Does Not Improve the Cure Rate of B16F10-NP Tumors.** Another approach to eliminating the limits imposed by pre-exposure to the L. monocytogenes vector is to use a combination therapy that delivers the tumor antigen with a different vector. Vaccinia virus that expresses the same NP gene as Lm-NP does not impact greatly on the growth of established B16F10-NP. In Fig. 2A, only one of eight mice is cured by multiple immunizations of vaccinia-NP. Nevertheless, a booster immunization using a different NP-expressing vector might preferentially activate NP-specific, rather than L. monocytogenes-specific, T cells. We, thus, used Lm-NP for primary immunization and vaccinia-NP for subsequent boosters (Fig. 2B). This adjunctive therapy did not improve the best cure rate that we obtained using Lm-NP alone (Fig. 2B compared with Fig. 1).

**Combined Therapy with L. monocytogenes-NP and GM-CSF-secreting Irradiated B16F10 Cells Does Not Improve the Cure Rate of B16F10-NP Tumors.** We have shown that regression of NP-containing tumors by immunization with Lm-NP is associated with infiltration of the tumors with CD8+ and CD4+ T cells and that both T-cell subsets are important in antitumor immunity (2). Immunization with Lm-NP induces strong anti-NP T cell responses that are most likely responsible for tumor elimination; indeed, outgrowth of tumors in LM-NP immunized animals is associated with loss of NP expression (2). Induction of immunity against endogenous tumor antigens expressed by B16F10 is induced by immunizing with B16F10-GM-CSF (7). We, thus, examined whether complementing Lm-NP-induced immunity with B16F10-GM-CSF-induced immunity would improve the cure rate of B16F10-NP tumors in this model system. As Fig. 2C shows, five of eight animals that received both Lm-NP plus irradiated B16F10-GM-CSF became tumor free, four of eight mice that received Lm-NP became tumor free, and three of eight mice that received irradiated B16F10-GM-CSF alone became tumor free. On day 35, the average tumor size for naive animals was 23.6 ± 2.16 compared with 10.8 ± 11.74 for B16-GM-CSF-immunized mice, 4.3 ± 4.65 for Lm-NP-immunized mice, and 1.8 ± 2.43 for mice that received the combined therapy. The difference between the groups of animals that received Lm-NP or Lm-NP and B16F10-GM-CSF combined was not statistically significant (P = 0.16), nor was the difference between animals that received Lm-NP or B16F10-GM-CSF (P = 0.15). However, the difference between the animals that received combined therapy or B16F10-GM-CSF was marginally significant (P = 0.04). Thus, the tumors progressed more rapidly in mice treated with irradiated B16F10-GM-CSF alone, and the tumors in the mice that received the combined therapy grew the slowest (Fig. 2B).

We repeated this experiment including a group of mice that received Lm-NP plus irradiated B16F10 tumor cells that did not express GM-CSF. We found no statistical difference between the numbers of mice that became tumor free in the groups of mice that received Lm-NP alone, Lm-NP plus B16F10-GM-CSF, or Lm-NP plus B16F10. In all groups, 35–50% of the mice showed regression of B16F10 tumors of about 2 mm (data not shown). Taken together, these data suggest that a combined therapy of Lm-NP with irradiated B16F10-GM-CSF did not greatly improve the Lm-NP single therapy.

**GM-CSF Is Found in B16F10-NP Tumors from Mice Immunized with Lm-NP.** The lack of synergy between Lm-NP and B16F10-GM-CSF suggests that GM-CSF is not a limiting factor in Lm-NP therapy. These data also suggest a lack of synergy between the anti-NP response and the antimelanoma antigen response facilitated by GM-CSF. Conceivably, cellular infiltrates into necrotizing B16F10-NP tumors could be secreting GM-CSF after treatment with
To test this hypothesis, we examined whether GM-CSF was, indeed, found in tumors for mice immunized with Lm-NP using RT-PCR. Fig. 3 shows that GM-CSF is detected in B16F10-NP at day 7 after immunization with Lm-NP, whereas GM-CSF is not detected after treatment with Lm-Gag.

B16F10-NP Experimental Pulmonary Metastases Can Be Eliminated by Lm-NP Immunization. A primary objective of immunological approaches to cancer therapy is the elimination of metastatic disease. Although mouse transplanted tumors do metastasize to the lung (4), the growth rate of the primary tumor is usually so rapid that at this stage it is refractory to therapy. This problem can be overcome by establishing experimental metastases by tail vein immunization and starting treatment some days later (11).

Mice received two immunizations 6 days apart on days 12 and 18 or days 15 and 21 after i.v. challenge. Metastases were counted on day 50 after tumor challenge. Table 2 shows that 5 of 10 mice who received Lm-NP on days 6, 12, and 18 were metastases free and 3 of 10 mice who received Lm-NP on days 9, 15, and 21 were metastases free. Although there is a strong nonspecific slowing of tumor growth in Lm-Gag-immunized mice, only 1 of 10 mice who received Lm-Gag...
on days 6, 12, and 18 were free of metastases and none of the mice who received Lm-Gag on days 9, 15, and 21 were tumor free. Clearly, Lm-NP is as effective at treating micrometastatic disease as it is at treating established primary tumors.

**Antigen Nonspecific Effects of *L. monocytogenes* on B16F10-NP: Induction of Cytokine mRNA Expression.** The ability of the recombinant control strain Lm-Gag to weakly inhibit B16F10-NP tumor growth (Fig. 1B and Table 2) indicates a nonantigen-specific component of therapy with live *L. monocytogenes* vaccines. Although Lm-Gag only slows tumor progression and does not cure mice with established tumors, infection with *L. monocytogenes* clearly results in the production of soluble mediators that either directly kill B16F10-NP or slows their growth rate. *L. monocytogenes* infections are associated with strong Th1 CD4+ T cell responses, including the production of IL-12, IFN-γ, and TNF-α (1). We, thus, examined the profile of mRNA expression for IFN-γ, IL-2, IL-12, IL-12 p40; IFN-α, -β, and -γ; TNF-α and -β in B16F10-NP tumors from mice immunized with Lm-NP versus Lm-Gag using RT-PCR. Fig. 3 shows the up-regulation of mRNA for all of these cytokines, except IFN-γ and TNF-α in tumor-bearing mice treated with Lm-NP at days 1, 3, and/or 7 after immunization. However, tumors from mice immunized with Lm-Gag also showed elevated levels of mRNA for most of these cytokines compared with tumors grown in unimmunized animals, suggesting that they could play a role in slowing tumor growth. Curiously, IFN-γ and TNF-α, which are the hallmark of a listerial infection, were undetectable in the tumors at these time points (data not shown). It is possible, however, that they are expressed in the tumor nodule at later time points than day 7, the last time point evaluated.

**DISCUSSION**

In the last few years, there has been a burgeoning interest in T cell-based immunotherapies for cancer that has emerged from the discovery that tumor-specific antigens can be naturally processed and expressed in the context of MHC on the surface of tumor cells, thus rendering them targets for T cell recognition (12, 13). With the identification of an increasing number of tumor-specific antigens for a wide variety of cancers, various strategies for targeting tumor-associated antigens to the cellular arm of the immune response have been attempted. These have included strategies designed to allow the passage of an exogenous antigen from the endosomal compartment to the cytosol (14), or that use recombinant viral vectors (15) or peptide-associated heat shock proteins (16). We have been developing an antigen vector approach that takes advantage of the unusual life cycle of the facultative intracellular bacterium *L. monocytogenes*. This bacterium enters the host cell and is taken up in a phagosome but, unlike most other intracellular bacteria such as Salmonella or Bacillus Calmette-Guérin, it escapes into the cytoplasm of the cell by disrupting the phagosomal membrane, primarily through the action of a secreted virulence factor, listeriolysin O (1). Thus, any protein that the bacterium constitutively secretes, or is engineered to secrete, is targeted to both the MHC class I and class II pathways of the infected cell for antigen presentation. This property, which is rare for most intracellular bacteria, results in the induction of a strong cellular immune response (1). Indeed, in a number of studies, we have verified that stable recombinants of *L. monocytogenes* that express foreign viral and bacterial antigens can elicit strong CD8+ T cell responses to these antigens (1, 17). In addition, we have demonstrated that this cell-mediated immunity can be harnessed effectively in the therapy of transplanted mouse tumors (Ref. 2 and 3 and the present study).

In the present study, we have extended our findings that an *L. monocytogenes* vector can cure mice of established tumors to a more tumorogenic model, that of B16F10. We show that we can effectively and permanently cure 50–60% of syngeneic C57BL/6 mice of palpable primary s.c. tumors and established experimental lung metastases using Lm-NP alone as the therapy and that this is just as effective as combination therapies with recombinant vaccinia vectors or GM-CSF-complemented autologous irradiated tumor cells. The lack of synergy between B16F10-GM-CSF-induced immunity and Lm-NP was unexpected. GM-CSF-based tumor immunotherapy is directed at inducing cell-mediated immunity to endogenous tumor antigens (7). It has recently been shown that the induction of this immunity is crucially dependent on CD4+ T cells of both the Th1 and Th2 phenotype in that B16F10-GM-CSF-primed IFN-γ−/− and IL-4−/− mice are impaired in their ability to reject B16F10 tumors (18). Th2 cells are thought to induce eosinophils that are abundant at the site of tumor challenge (18). It is possible that the strong Th1 immunity induced by Lm-NP impairs the ability of B16F10-GM-CSF to induce appropriate CD4+ effector cells. On the other hand, the presence of mRNA for GM-CSF in regressing tumors from Lm-NP immunized mice indicates that this cytokine is present in tumors after Lm-NP infection. The potent cytokine response induced by treatment of tumor-bearing mice with Lm-NP could conceivably function to activate T cell-immunity against endogenous melanoma antigens, in addition to NP, in the absence of the irradiated B16F10-GM-CSF vaccine.

These studies provide the first demonstration that, in addition to inducing potent antigen-specific therapy, *L. monocytogenes* has an adjuvant effect that results in significant nonspecific slowing of tumor growth. This correlates with expression of mRNA for IL-1, IL-2, IL-6, IL-12 p40; IFN-α, -β, and -γ; TNF-α and -β in B16F10-NP tumors from mice immunized with Lm-NP versus Lm-Gag using RT-PCR. Fig. 3 shows the up-regulation of mRNA for all of these cytokines, except IFN-γ and TNF-α in tumor-bearing mice treated with Lm-NP at days 1, 3, and/or 7 after immunization. However, tumors from mice immunized with Lm-Gag also showed elevated levels of mRNA for most of these cytokines compared with tumors grown in unimmunized animals, suggesting that they could play a role in slowing tumor growth. Curiously, IFN-γ and TNF-α, which are the hallmark of a listerial infection, were undetectable in the tumors at these time points (data not shown). It is possible, however, that they are expressed in the tumor nodule at later time points than day 7, the last time point evaluated.

**Table 2. Effect of immunization of *L. monocytogenes* vaccines on the development of experimental metastases in the lung by day 50 after tumor challenge with 4 × 10⁶ B16F10-NP tumor cells in the tail vein**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average no. metastases/mouse (±SD)</th>
<th>Average no. metastases/tumor-bearing mouse (±SD)</th>
<th>No. mice metastases free (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>70 ± 16.8</td>
<td>70 ± 16.8</td>
<td>0</td>
</tr>
<tr>
<td>Lm-Gag (days 9, 15, and 21)</td>
<td>21 ± 15.1</td>
<td>21 ± 15.1</td>
<td>0</td>
</tr>
<tr>
<td>Lm-Gag (days 6, 12, and 18)</td>
<td>18 ± 12.5</td>
<td>20 ± 11.6</td>
<td>1</td>
</tr>
<tr>
<td>Lm-NP (days 9, 15, and 21)</td>
<td>11 ± 14.6</td>
<td>18 ± 15.1</td>
<td>4</td>
</tr>
<tr>
<td>Lm-NP (days 6, 12, and 18)</td>
<td>6 ± 9.9</td>
<td>12 ± 11.9</td>
<td>5</td>
</tr>
</tbody>
</table>

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tory cytokines by intracellular bacteria such as *Bacillus Calmette-Guérin* has been used for decades in cancer therapy (23, 24). Recently *Salmonella typhimurium* has also been introduced into the armory of antitumor agents (25). The potency of *L. monocytogenes* as an anti-cancer immunotherapeutic may be the result of the combination of the adjuvant property of this bacterium and its ability to induce potent antigen-specific cell-mediated immunity. The contribution of the adjuvant effects of the vector vehicle to antitumor immunity induced by the vectored antigen clearly warrants further investigation.

**ACKNOWLEDGMENTS**

We thank Dr. Drew Pardoll (Johns Hopkins University, Baltimore, MD) for generous donation of reagents. We also thank Dr. Pardoll, Dr. Claudine Bruck, and Gregory Beatty for helpful discussions.

**REFERENCES**


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