Listeria-based cancer vaccines that segregate immunogenicity from toxicity

Dirk G. Brockstedt*†, Martin A. Giedlin*, Meredith L. Leong*, Keith S. Bahjat*, Yi Gao*, William Luckett*, Weiqun Liu*, David N. Cook*, Daniel A. Portnoy‡§, and Thomas W. Dubensky, Jr.*

*Cerus, Concord, CA 94520; and ‡Department of Molecular and Cell Biology and §School of Public Health, University of California, Berkeley, CA 94720

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The facultative intracellular bacterium *Listeria monocytogenes* is being developed as a cancer vaccine platform because of its ability to induce potent innate and adaptive immunity. For successful clinical application, it is essential to develop a *Listeria* platform strain that is safe yet retains the potency of vaccines based on wild-type bacteria. Here, we report the development of a recombinant live-attenuated vaccine platform strain that retains the potency of the fully virulent pathogen, combined with a >1,000-fold reduction in toxicity, as compared with wild-type *Listeria*. By selectively deleting two virulence factors, ActA (Δ*actA*) and Internalin B (Δ*inlB*), the immunopotency of *Listeria* was maintained and its toxicity was diminished *in vivo*, largely by blocking the direct internalin B-mediated infection of nonphagocytic cells, such as hepatocytes, and the indirect ActA-mediated infection by cell-to-cell spread from adjacent phagocytic cells. In contrast, infection of phagocytic cells was not affected, leaving intact the ability of *Listeria* to stimulate innate immunity and to induce antigen-specific cellular responses. *Listeria* Δ*actA*/Δ*inlB*-based vaccines were rapidly cleared from mice after immunization and induced potent and durable effector and memory T-cell responses with no measurable liver toxicity. Therapeutic vaccination of BALB/c mice bearing murine CT26 colon tumor lung metastases or palpable s.c. tumors (>100 mm³) with recombinant *Listeria* Δ*actA*/Δ*inlB* expressing an endogenous tumor antigen resulted in breaking of self-tolerance and long-term survival. We propose that recombinant *Listeria* Δ*actA*/Δ*inlB* expressing human tumor-associated antigens represents an attractive therapeutic strategy for further development and testing in human clinical trials.

Cancer immunotherapy represents a promising treatment strategy that has produced some tantalizing clinical responses for a variety of malignant diseases. Although promising, there continues to be a strong need for a practical immunization strategy that can be routinely adopted to specific malignancies and that consistently yields durable and robust therapeutic antitumor responses.

Progress in molecular and cellular immunology, combined with increasing understanding of pathogen physiology and host–pathogen interaction has facilitated the design and use of attenuated bacteria as conventional vaccine vectors. However, the practical utility of live attenuated vaccines relies on achieving a proper balance between the virulence/toxicity and immunogenicity of the vaccine. The potency of a pathogen to elicit adaptive immunity is related in part to its ability to stimulate significant innate immunity through recognition of microbial pathogen-associated molecular patterns by *Toll*-like receptors. Microbial encounter with professional antigen-presenting cells (APC), such as dendritic cells, results in activation and maturation (1) as well as secretion of high levels of T helper-1-type cytokines (2). This interaction initiates adaptive immune responses and therefore links innate and adaptive immunity.

Recombinant microbial-based vectors expressing model tumor antigens, including *Listeria monocytogenes*, have been shown to elicit robust innate and antigen-specific cellular responses in a number of models, including tumor-bearing animals (3, 4). Despite the potential safety concerns for using wild-type *Listeria* as a vaccine vector, there are a number of desirable features of the natural biology of this bacterium that make it an attractive platform for continued development toward clinical evaluation. The central rationale is that the intracellular lifecycle of *Listeria* enables effective stimulation of $CD4^+/CD8^+$ T cell immunity. There are also numerous practical features of *Listeria*-based vaccines, including its anticipated ease of production in defined media, combined with relatively simple vaccine construction by using well developed techniques for bacterial engineering (5). *Listeria* is not hindered by constraints on the size of the heterologous sequence, a limitation common to viral-based vector systems. However, *Listeria* is a food-borne pathogen, and unique approaches to retain the immunogenicity of this platform while attenuating its pathogenicity are essential for eventual clinical efficacy trials in humans.

Here, we describe our work constructing a live-attenuated vaccine strain, *Listeria* Δ*actA*/Δ*inlB* (InlB, Internalin B). We hypothesized that by combining specific attenuating genetic mutations affecting *Listeria* cell-to-cell spread and tropism for nonphagocytic cells, it should be possible to segregate vaccine immunogenicity because of uptake of bacteria by phagocytic cells, including professional APC, from overt *Listeria* toxicity due in part to infection of nonphagocytic cells, such as hepatocytes. By deletion of two distinct virulence determinants, we believe that the Δ*actA*/Δ*inlB*-based vaccine strain appropriately addresses toxicity concerns related to *Listeria* while preserving its immunogenicity and is thus an attractive vaccine platform strain for continued development.

Materials and Methods

Bacterial Host Strains and Recombinant Vectors. All *Listeria* strains used in this study were derived from the *L. monocytogenes* wild-type strain 10403S (6) (*Supporting Methods* and Table 1, which are published as supporting information on the PNAS web site). *Listeria* strains with in-frame deletions of the indicated genes were generated by splicing by overlapping extension PCR and allelic exchange with established methods (7). The pPL2 genetic integration vector (5) was used to derive ovalbumin (OVA) and AH1-A5 recombinant *Listeria* strains containing a single copy of the antigen expression cassette within the *Listeria* genome adjacent to the gene encoding tRNA arginine. Expression of secreted heterologous antigens was driven from the *hly* promoter as a fusion protein with the N-terminal region of listeriolysin O (LLO) as described in ref. 8. The AH1-A5 epitope was inserted in-frame within OVA by using a unique *Ava*II site.

***In Vitro* Infectivity.** Infectivity of human cell lines and primary cells, such as monocytes and hepatocytes, was assessed *in vitro* as described in ref. 9. Human primary hepatocytes were obtained

Abbreviations: OVA, ovalbumin; LLO, listeriolysin O; APC, antigen-presenting cells; CFSE, carboxyfluorescein diacetate-succinimidyl ester; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TNF-α, tumor necrosis factor type α.

†To whom correspondence should be addressed at: Cerus Corporation, 2411 Stanwell Drive, Concord, CA 94520. E-mail: dirk_brockstedt@cerus.com.

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from In Vitro Technologies (Baltimore). Human primary monocytes were purified from a buffy-coat from healthy volunteers (Sacramento Blood Bank Center, Sacramento, CA) by using a Ficoll–Hypaque gradient and CD14 microbeads (Miltenyi Biotec, Auburn, CA). HepG2 cells (human hepatocellular carcinoma cell line, American Type Culture Collection) and primary human hepatocytes were infected at a multiplicity of infection of 10. THP-1 cells (human monocyte cell line, American Type Culture Collection) and primary human monoctyes were infected at an multiplicity of infection of 1 and 100, respectively. After the incubation with gentamycin (50 μg/ml), cells were lysed with sterile water, and serial dilutions were plated onto brain heart infusion agar to determine the level of infectivity.

Listeria Pathogenicity Studies. To determine liver enzyme levels, mice were infected i.v. with *Listeria* strains at a dose equal to 0.1 LD_{50}. Serum liver enzyme levels for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined at the indicated time after vaccination. The *in vivo* growth of attenuated *Listeria* mutants was determined as described in ref. 10. Briefly, C57BL/6 mice were infected with the indicated *Listeria* strains at a dose equal to 0.1 LD_{50}. The level of infection in each mouse was determined at the indicated time after *Listeria* challenge by enumerating bacteria in liver and spleen organ homogenates. All animals were treated according to National Institutes of Health guidelines, and protocols requiring animal experimentation received prior approval from the Cerus Animal Care and Use Committee.

Antigen-Specific T Cell Response. The frequency of IFN-γ and tumor necrosis factor type α (TNF-α) secreting $CD8^+$ T lymphocytes specific for OVA, glycoprotein gp70 (AH1 or AH1-A5), and LLO ($LLO_{190\text{-}201}$ and $LLO_{296\text{-}304}$) were determined by intracellular cytokine staining as described in refs. 11 and 12. For the detection of OVA-specific $CD8^+$ T cells the H-$2K^b$-restricted epitope SL8 (SIINFEKL) was used (13). gp70-specific immunity was assessed by using the endogenous H-$2L^d$-restricted epitope AH1 (SPSYVYHQF) and the altered peptide ligand AH1-A5 (SPSYAYHQF) (14). Cells were stained for cell-surface markers with anti-CD8α-peridinin chlorophyll protein (clone 53-6.7, Pharmingen). Cells were fixed in 2% paraformaldehyde, permeabilized with Perm/Wash buffer (Pharmingen), and incubated with anti-IFN-γ-allophycocyanin (clone XMG1.2, eBioscience, San Diego) and anti-TNF-α-phycoerythrin (clone MP6-XT22, eBioscience). Samples were acquired on a FACSCalibur flow cytometer, and the data were analyzed with CELLQUEST software (Becton Dickinson Immunocytometry Systems).

***In Vivo* Cytotoxicity Assay.** *In vivo* cytotoxic activity of antigen-specific T cells was determined in female BALB/c mice vaccinated with *Listeria* Δ*actA*/Δ*inlB*-AH1-A5 by using the *in vivo* cytotoxicity assay as described in ref. 15. The carboxyfluorescein diacetate-succinimidyl ester $(CFSE)^{hi}$-labeled cells were pulsed with the AH1 or AH1-A5 peptide, whereas the $CFSE^{low}$-labeled cells were pulsed with the β-galactosidase control peptide. A 1:1 ratio of $CFSE^{low}$- to $CFSE^{hi}$-labeled cells was injected i.v. into BALB/c mice that were vaccinated 7 days prior. At 18 h after injection of the targets cells, spleens were removed and the ratio of $CFSE^{low}$ to $CFSE^{hi}$ cells was determined by flow cytometry.

***In Vivo* Tumor Studies.** Female BALB/c mice were implanted i.v. with 2×10^5 CT26 cells on day 0. Four days later, mice were randomized and vaccinated i.v. with the indicated *Listeria* strains or Hanks' balanced salt solution (HBSS) as negative control. Lungs were harvested 19 days after tumor challenge, fixed in Bouin's solution and the number of metastases was assessed. To assess survival, mice were killed when any signs of stress or labored breathing were observed.

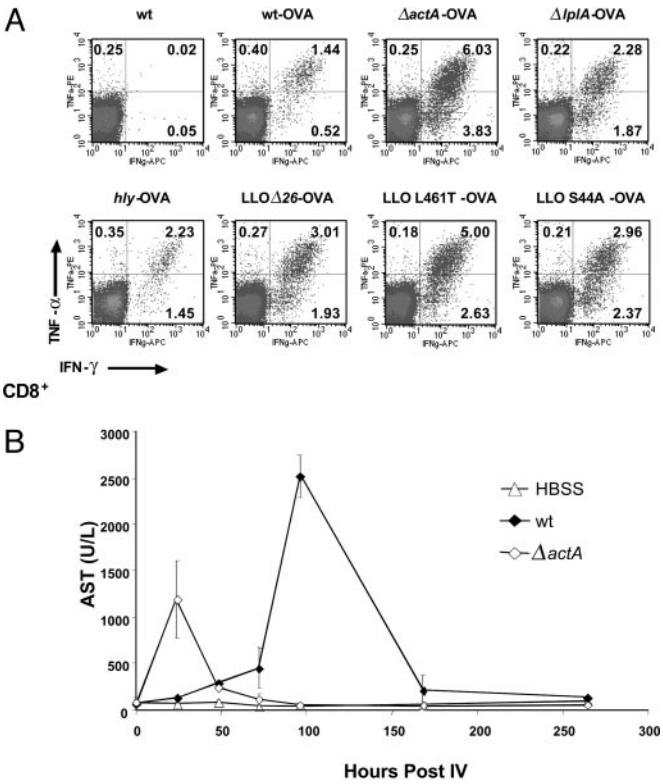

Fig. 1. Recombinant *Listeria* strains induce a potent OVA-specific effector T cell response that is associated with transient liver toxicity. (*A*) Female C57BL/6 mice were vaccinated i.v. (IV) with 0.1 LD_{50} of indicated attenuated *Listeria*-OVA strain. OVA-specific immunity was assessed by intracellular cytokine staining on day 7 after a single vaccination. Representative dot blots of SL8-stimulated spleen cells gated on $CD8^+$ T cells are shown. The percentage of IFN-γ^+CD8^+ T cells in the absence of SL8 stimulation was <0.03% (data not shown). (*B*) Vaccination of mice with wild type (wt) or the attenuated *Listeria* Δ*actA* mutant induces transient liver toxicity. Three C57BL/6 mice per group were vaccinated i.v. with 5×10^4 colony-forming units (CFU) of wild type or 1×10^7 colony-forming units of Δ*actA*. Serum levels of the liver enzyme AST (in units per liter) were determined at different times after infection.

Statistical Analyses. All experiments were repeated at least twice to confirm reproducibility. Tumor survival results were analyzed by one-way ANOVA and two-tailed Student's *t* test for statistical significance.

Results

Attenuated *Listeria* Strains Deleted in actA Induce OVA-Specific $CD8^+$ T Cell Responses with the Highest Magnitude. For clinical application as a tumor vaccine, two independent deletions in virulence genes are preferred to minimize the possibility for reversion to wild type. Identification of the vaccine platform strain was performed in a two-step process. First, we screened *Listeria* mutants deleted for a single virulence factor based on the relative potency to induce primary (Fig. 1) and memory T cell responses (Table 1). Second, we introduced deletions into the leading single mutant strains with the goal of maintaining potency but improving safety. Initially, we selected a wide range of attenuated mutants deficient or altered in a single virulence gene (e.g., *hly*, *actA*, or *lplA*) as well as gene products that might contribute to infection of nonphagocytic cells, such as InlB (16, 17). The loss of certain virulence determinants was demonstrated by an up to 10,000-fold attenuation in pathogenicity in

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mice, as determined by median lethality (LD_{50}). To determine the relative potency of these *Listeria* vaccine strains to induce antigen-specific effector and memory T cell-mediated immunity, OVA fused with a truncated form of LLO to facilitate antigen secretion was used as a model antigen. Because of the defined integration of a single copy of the LLO–OVA fusion gene into the genome, all recombinant strains had similar growth characteristics in liquid broth, expressed and secreted OVA at similar levels (within a 2-fold variation) based on Western Blot analysis, and the LD_{50} of the resulting strains was within a factor of five of the unmodified parental strains (data not shown). A single vaccination of mice induced potent OVA-specific effector $CD8^+$ T cell (Fig. 1*A*) as well as $LLO_{190\text{-}201}$-specific $CD4^+$ T cell responses (data not shown). Depending on the strain used for vaccination, differences in magnitude (percentage) of the response to OVA were observed, with the Δ*actA* and LLO L461T mutants routinely eliciting the strongest responses. However, no significant difference in spleen size or number of splenocytes in mice vaccinated with various *Listeria* mutants was observed. The potency to induce OVA-specific primary responses did not correlate with the number of bacteria administered or with the strain's ability to induce protective memory T cell responses. Certain defined genetic mutations, such as Δ*LLO*, ΔPEST, and Δ*lplA*, resulted in a significant reduction or complete loss of the ability to induce protective immunity. Vaccination of mice with the Δ*actA* or LLO L461T strain resulted in almost complete protection, comparable with mice vaccinated with wild-type *Listeria*. Based on the ability to induce potent effector and memory T cell responses as well as a 1,000-fold attenuation compared with wild type, we selected the *Listeria* Δ*actA* for further strain development.

Attenuated *Listeria* Strain Deleted in *actA* and *inlB* Maintains Its Immunogenicity. Next, we constructed double-deletion mutants by in-frame deletion of *actA* by allelic exchange in Δ*inlB,* LLO L461T, and Δ*lplA* strains. The deletion of *inlB* was chosen to test whether the potency of Δ*actA* to induce cellular immunity could be segregated from its toxicity (Fig. 1*B*) by altering its tropism for nonphagocytic cells, such as hepatocytes. The deletion of *inlB* on the background of Δ*actA* did not result in a significant reduction in lethality (LD_{50}) as compared with *Listeria* Δ*actA* (Table 1). Vaccination of mice with double mutants expressing OVA clearly demonstrated that Δ*actA*/Δ*inlB* maintained its ability to induce potent OVA-specific primary responses (Fig. 2) and memory responses (Table 1) that were comparable with the Δ*actA* single mutant. *Listeria* Δ*actA*/Δ*inlB* was significantly more potent compared with other double mutants tested. *Listeria* Δ*actA*/Δ*inlB* provided an increased therapeutic window, as shown by induction of OVA-specific $CD8^+$ T cell responses that were equivalent to that observed with wild-type *Listeria*, but at a 3-log lower dose (Fig. 5*A*, which is published as supporting information on the PNAS web site). We also determined whether *Listeria* Δ*actA*/Δ*inlB* induced potent OVA-specific T cell responses when administered by means of routes that are preferable for clinical applications. To date, most studies in mice have investigated the interaction of immune cells with *Listeria* when either given i.v., i.p., or by means of oral delivery. We tested these immunization routes and additional routes of administration, including s.c., intradermal, and i.m. Oral delivery was not assessed in this study based on previous findings by Cossart and coworkers (18), demonstrating that the mouse model is inappropriate to study oral delivery because of poor interaction between invasins, such as internalin A, and host cell receptors that are required for mucosal invasion by *Listeria*. i.m. but not s.c. or intradermal administration of attenuated Δ*actA*/Δ*inlB* resulted in potent OVA-specific $CD8^+$ T cell immunity, comparable with i.v. vaccinations (Fig. 5*B*).

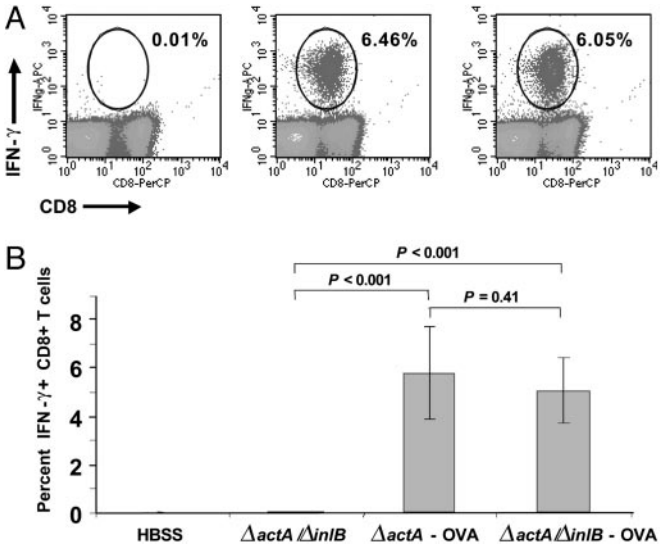

Fig. 2. The introduction of the *inlB* deletion on the Δ*actA* mutant does not abrogate the ability of strains to induce potent antigen-specific T cell responses. C57BL/6 mice were vaccinated i.v. with 1×10^7 colony-forming units (CFU) of the indicated strain. OVA-specific $CD8^+$ T cell immunity was assessed on day 7 after vaccination by using intracellular cytokine staining. (*A*) Representative dot blots of SL8-stimulated spleen cells are shown. (*Left*) Δ*actA*/Δ*inlB*. (*Center*) Δ*actA*-OVA. (*Right*) Δ*actA*/Δ*inlB*-OVA. PerCP, peridinin chlorophyll protein. (*B*) The average percentage of OVA-specific $CD8^+$ T cells of eight mice is shown.

Deletion of ActA and InlB Renders Bacteria Unable to Infect Nonphagocytic Parenchymal Cells *in Vitro*. Internalin proteins, such as InlB, have been shown to confer the unique ability of *Listeria* to infect nonphagocytic cells, such as hepatocytes. To assess the ability of the double mutant Δ*actA*/Δ*inlB* to infect phagocytic as well as nonphagocytic cells *in vitro*, we compared the infection by wild type, Δ*actA*, Δ*inlB*, and Δ*actA*/Δ*inlB* strains of *Listeria* in human nonphagocytic and phagocytic cell lines and primary cells. As shown in Fig. 3*A*, all strains were able to infect THP-1 cells and human monocytes to a similar extent, indicating that the absence of ActA or InlB does not affect infection of phagocytic cells. However, infection of hepatocytes was significantly decreased for *Listeria* strains lacking InlB. Infectivity was reduced by ≈60% in primary human hepatocytes and by ≈80% in HepG2 cells when infecting with either of the InlB-null mutant strains (Fig. 3*A*).

Accelerated Clearance of Δ*actA*/Δ*inlB* Infection Without the Induction of Liver Damage. Although attenuated strains of *Listeria* can be administered at higher doses compared with wild type, for the development of a safe vaccine it is important that the infection can be cleared rapidly, without damaging the primary organs of infection, i.e., liver or spleen. In that regard, we compared the *in vivo* growth kinetics of wild type, Δ*inlB*, Δ*actA*, and Δ*actA*/Δ*inlB* in female C57BL/6 mice (Fig. 3*B* and Fig. 6*A*, which is published as supporting information on the PNAS web site). Infection of mice with wild-type *Listeria* resolved within 7–11 days after administration as described in ref. 19. The number of wild-type *Listeria* steadily increased over the time period of 4 days by 10,000-fold and decreased to undetectable levels in spleen and liver by day 11. Interestingly, the Δ*inlB* mutant demonstrated similar kinetics and magnitude of expansion in spleen as well as liver. This result stands in contrast with previous studies by Lingnau *et al.* (17) in which retarded growth of the Δ*inlB* mutant in liver and spleen had been observed. The number of Δ*actA* *Listeria* increased 10-fold over the first 48 h in the liver but not

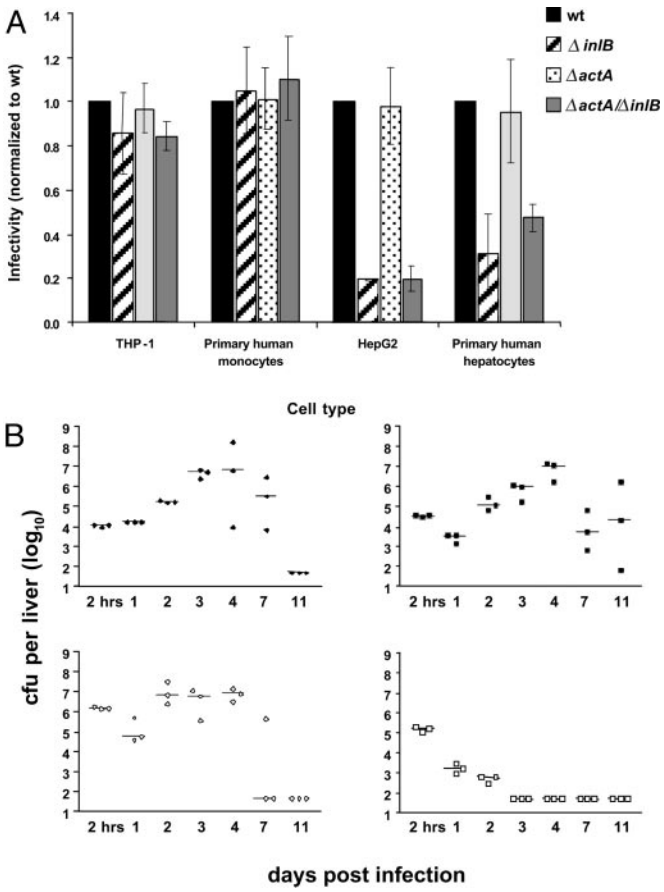

Fig. 3. The inability of *Listeria* Δ*actA*/Δ*inlB* to infect nonphagocytic cells *in vitro* results in accelerated clearance *in vivo*. (*A*) *In vitro* infectivity of the human hepatocyte cell line (HepG2), fresh human hepatocytes (two donors), a human monocyte cell line (THP-1), and primary human monocytes (three donors) is shown. For all strains, the rate of infection is normalized to the rate of infection by wild-type (wt) *Listeria*. The averages of three (or two for primary human hepatocytes) independent experiments are shown. (*B*) The *in vivo* growth kinetic in liver was assessed for wild type (*Upper Left*), Δ*actA* (*Lower Left*), Δ*inlB* (*Upper Right*), and Δ*actA*/Δ*inlB* (*Lower Right*). C57BL/6 mice were injected i.v. with 0.1 LD_{50} of the indicated strain and bacteria per organ were determined from three mice at each time point. A representative of two experiments is shown.

in the spleen, and the bacteria count was maintained for up to 4 days before it eventually decreased; the infection was cleared by day 7 after infection. In contrast, the Δ*actA*/Δ*inlB* double mutant was cleared significantly more rapidly from the liver and spleen by days 3–4. To assess the level of tissue damage and liver pathology, serum samples were collected at different time points during the course of an infection and liver enzyme levels and the proinflammatory cytokine profile (data not shown) were assessed. Δ*actA* but not Δ*actA*/Δ*inlB* induced liver enzymes, such as AST or ALT, 24 h after infection (Fig. 6*B*). Moreover, histopathological changes in the liver throughout the course of infection were significantly milder in mice receiving the Δ*actA*/Δ*inlB* double mutant when compared with wild type-, Δ*actA*-, or Δ*inlB*-infected mice (data not shown). Interestingly, wild type and Δ*actA* induced higher serum levels of IFN-γ, MCP-1, and IL-6 at 24 h after infection compared with the Δ*inlB* and Δ*actA*/Δ*inlB* mutants (data not shown). However, the induction of proinflammatory cytokines did not seem to correlate with the ability of a strain to induce potent antigen-specific T cell responses.

Vaccination with Recombinant Δ*actA*/Δ*inlB* Expressing AH1-A5 Breaks Self-Tolerance and Results in Tumor Regression and Prolongation of Life. The previous experiments demonstrated that a single vaccination with attenuated *Listeria* strains expressing a foreign antigen, such as OVA, elicits antigen-specific $CD8^+$ T cell immunity of high magnitude. However, the induction of immunity to a truly native tumor antigen requires breaking of self-tolerance. To test whether *Listeria*-based vaccinations result in breaking of self-tolerance, we constructed the Δ*actA*/Δ*inlB* that expresses the altered T cell epitope, AH1-A5, of the endogenous tumor antigen gp70 of the murine colon tumor CT26 (14, 20). A single vaccination with Δ*actA*/Δ*inlB*-AH1-A5 administered either i.v. or i.m. (data not shown) resulted in the induction of 2.2% AH1-A5-specific $CD8^+$ T cells in the spleen (Fig. 7*A*, which is published as supporting information on the PNAS web site). Half of the response (1.1%) was specific to the endogenous T cell epitope AH1. The AH1-specific $CD8^+$ T cell response was significantly increased by administering a boost vaccination 4 weeks after the primary vaccination (Fig. 7*B*). The boost vaccination could be administered as early as 2 weeks after the primary immunization with similar results (data not shown). Antigen-specific cytolytic activity in vaccinated mice was determined by using the *in vivo* cytotoxicity assay. Vaccination of mice with Δ*actA*/Δ*inlB* expressing AH1-A5 induced a potent cytotoxic T cell response demonstrated by the disappearance of AH1-A5 as well as AH1 peptide-loaded splenocytes in vaccinated mice but not HBSS- or Δ*actA*/Δ*inlB* parental control-immunized mice (Fig. 4*A*). We next tested whether a vaccination with Δ*actA*/Δ*inlB*-AH1-A5 induces therapeutic immunity in an experimental lung metastases tumor model by using the colon tumor-derived CT26 cell line (Fig. 4*B*). The therapeutic vaccination of mice with the Δ*actA*/Δ*inlB* double mutant expressing AH1-A5 resulted in a significant reduction of lung metastases compared with vehicle control group ($P < 0.05$). More importantly, therapeutic vaccination of CT26 tumor-bearing mice with Δ*actA*/Δ*inlB*-AH1-A5, but not with the Δ*actA* control strain, resulted in a significant prolongation of life with 40% long-term survivors ($P < 0.0001$) (Fig. 4*C*). Even at vaccination doses 10,000-fold below the experimental LD_{50}, a potent antitumor response was induced that resulted in a median survival of >35 days compared with 21 days for mice treated with buffer or the *Listeria* vector only ($P < 0.001$). The robustness of the therapeutic anti-CT26 tumor response after *Listeria*-based vaccination was furthermore demonstrated in that vaccination could be delayed as much as 10 days after tumor implantation when tumors reached a size of >100 mm^3 resulting in comparable tumor growth delay (Fig. 7*C*). The therapeutic anti-CT26 response was completely mediated by $CD8^+$ T (depletion data not shown).

Discussion

There exists a relatively abundant literature demonstrating the potency of *Listeria*-based vaccines targeting selected infectious disease or model tumor antigens, in both prophylactic and therapeutic settings in animal models of disease. However, to our knowledge, there has not been an effort to develop a genetically defined mutant vaccine platform that combines potency similar to wild-type *Listeria* with reduced pathogenicity. The results of this study demonstrate that by selectively deleting two determinants of *Listeria* pathogenesis, we can generate a vaccine strain that is more immunogenic yet considerably less toxic than wild-type bacteria. Immunization of tumor-bearing mice with this strain encoding a tumor antigen resulted in long-term survival as a result of breaking tolerance against an endogenous antigen.

The rationale for the combined deletions of *actA* and *inlB* was to separate the immunogenicity of *Listeria*-based vaccines from toxicity because of infection of nonphagocytic cells, such as

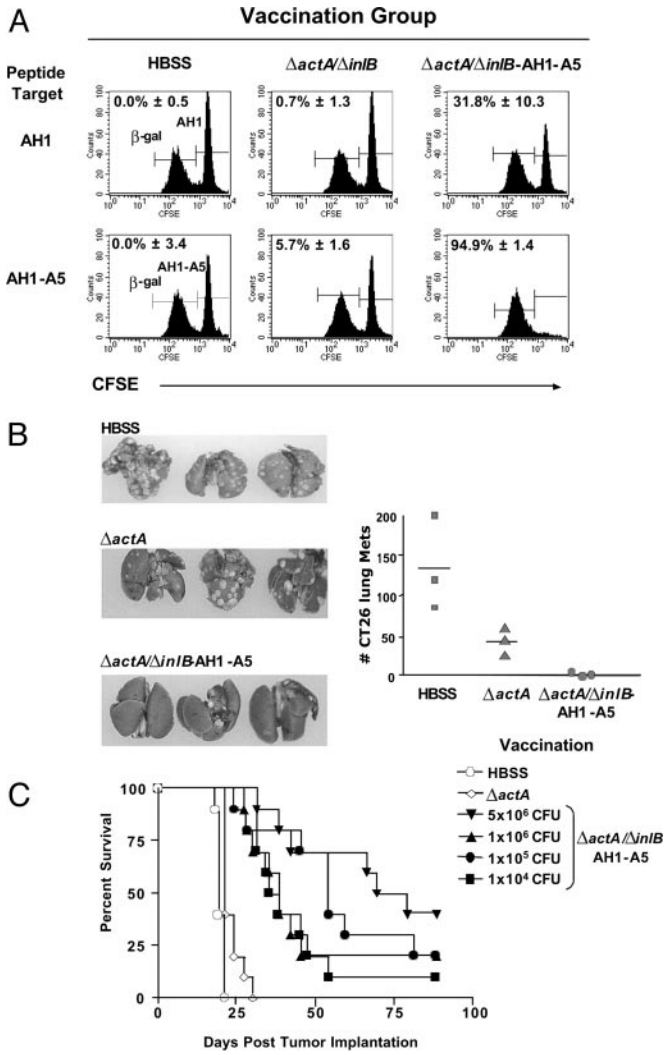

Fig. 4. Recombinant attenuated *Listeria* Δ*actA*/Δ*inlB AH1-A5* breaks tolerance to self that results in potent therapeutic antitumor activity and prolongation of life. (*A*) Female BALB/c mice were vaccinated once with 0.1 LD_{50} of the indicated strain. Cytotoxic activity was determined by *in vivo* cytotoxicity assay. $CFSE^{hi}$ AH1 (*Upper*) or AH1-A5 (*Lower*) peptide-pulsed BALB/c splenocytes (3×10^6) and $CFSE^{low}$ nonpulsed splenocytes (3×10^6) were coinjected i.v. into BALB/c mice that were naïve or 7 days prior to vaccination with the indicated *Listeria* strain. *In vivo* killing of CFSE-labeled target cells was assessed 18 h later. Histograms are gated on $CFSE^+$ cells in host mice. The number represents the average percentage of specific killing of three individual mice normalized to HBSS-injected mice. (*B*) Female BALB/c mice were implanted i.v. with 2×10^5 CT26 cells on day 0. Four days later, mice received a single vaccination with a dose equal to 0.1 LD_{50} of the indicated strain. Lungs were harvested on day 19, fixed in Bouin's solution, and the number of surface lung metastases were counted. Representative lungs are shown. (*C*) Female BALB/c mice were implanted i.v. with 2×10^5 CT26 cells on day 0. Four days later, mice were vaccinated with 0.1 LD_{50} of the indicated strain or vehicle control ($n = 10$). Survival was monitored over the course of the experiment. Therapeutic vaccination of mice with Δ*actA*/Δ*inlB AH1-A5* both resulted in a significant survival benefit (one-way ANOVA, $P < 0.0001$) compared with HBSS or Δ*actA* controls.

hepatocytes. Because Δ*inlB* single mutants can spread from cell to cell, infection of hepatocytes *in vivo* can occur indirectly by means of Kupffer cells. We hypothesized that the Δ*inlB in vivo* phenotype would be revealed only on the background of *Listeria* Δ*actA*, having defective cell-to-cell spread. This notion is supported by Gregory *et al*. (21), who demonstrated that *Listeria* Δ*inlB* infected and propagated within mouse hepatocytes *in vivo* despite its dramatically reduced capacity to infect nonphagocytic cells *in vitro*. As demonstrated here, the deletion of both *actA* and *inlB* resulted in an attenuated strain that is both unable to spread effectively from cell to cell and has a reduced capacity to infect hepatocytes directly or indirectly. Consequently, *Listeria* Δ*actA*/Δ*inlB* was cleared rapidly from the liver and spleen as compared to either single mutant alone. In contrast to the Δ*actA* or Δ*inlB* single mutants, vaccination of mice with *Listeria* Δ*actA*/Δ*inlB* did not result in any measurable induction of liver toxicity, as determined by release of liver enzymes. Furthermore, it has been suggested that the infection of the CNS by *Listeria* is mediated through routes that require cell-to-cell spread as well as InlB-dependent infection (22, 23). Preliminary results in an acute *Listeria* infection model in guinea pigs supports the hypothesis that the Δ*actA*/Δ*inlB* double mutant has lost its ability to infect the CNS.

The accelerated clearance of *Listeria* Δ*actA*/Δ*inlB* infection in mice after i.v. administration suggests strongly that *in vivo* bacterial proliferation and expansion in liver and spleen after 48 h is not required for the induction of a productive T cell response. This finding is supported by Mercado *et al*. (24), who demonstrated that the kinetics or magnitude of a T cell response after priming with *Listeria* is independent of the duration of infection *in* vivo. The idea that the innate immune response to *Listeria* during the first 24 h sets in motion an immunological program that determines the extent and duration of the T cell response has been also supported by Badovinac *et al*. (25), who demonstrated that the kinetics of the T cell response is independent of duration of infection or amount of antigen displayed. In contrast to the conclusions of Mercado *et al*., our dose–response data from mice given different doses of *Listeria* Δ*actA*/Δ*inlB*-OVA vaccines support the idea proposed by Badovinac *et al*. that the magnitude of the response depends on the amount of antigen displayed at the initiation of the immune response. More importantly, the attenuation of the double mutant (>1,000-fold) means that more bacteria can be administered for each vaccination, resulting in a much larger amount of antigen presented during the crucial time of T cell priming. This result means that because of the attenuated phenotype and the associated accelerated clearance, antigen-specific T cell responses are initiated without the potentially harmful *in vivo* expansion of *Listeria* that is associated with wild-type *Listeria* infection.

Listeria has evolved mechanisms for intracellular growth and spread while minimizing cytotoxicity, largely through confining the activity of the pore-forming cytolysin LLO to the low-pH environment of the phagosome. Particular *Listeria hly* mutants have a cytotoxic phenotype because of the retention of activity of LLO in the cytoplasm, which results in premature host cell lysis. Somewhat paradoxically, cytotoxic mutants have diminished virulence. We evaluated a number of *Listeria* mutants with various degrees of cytotoxicity (related to the cytoplasmic enzymatic activity of LLO) for their capacity to induce an effector immune response. Interestingly, although the LLO L461T mutant strain was highly immunogenic, the combination of that mutation with Δ*actA* resulted in a strain that was poorly immunogenic. Other single mutants with phenotypes of increased cytotoxicity, including LLO Δ26, LLO S44A, and LLO S44A/LLO L461T were considerably impaired in their capacity to induce a primary immune response. We speculate that the appropriate level of *Listeria* infection-induced cell death may be a critical factor for direct priming or crosspriming of $CD8^+$ T cells, to induce an effector and memory T cell immunity (26). Early events during infection may cause qualitatively and quantitatively differences in activation of APC at the site of infection and may result in the induction of distinct T cell responses, i.e., effector versus memory T cell immunity (27–30). By using a range of cytotoxic mutants, we are currently in the process of characterizing these early events during infection and the role of

$CD4^+$ T helper cells for the induction of effector and memory $CD8^+$ T cell responses.

The Δ*actA*/Δ*inlB* double mutant vaccine platform was systematically selected from a large panel of genetically defined attenuated *Listeria* strains. A single immunization of mice with *Listeria* Δ*actA*/Δ*inlB* induced protective immunity against lethal challenge with wild-type *Listeria*, which requires secondary $CD8^+$ T cell expansion and formation of memory of $CD8^+$ T cells (31). A single immunization with the vaccine strain given through a variety of routes also induced potent $CD8^+$ T cell immunity to a strong foreign antigen (OVA) and to the native tumor-associated antigen gp70. Furthermore, *Listeria* Δ*actA*/Δ*inlB*-based vaccination broke self-tolerance after a single immunization that translated into a therapeutic antitumor response and long-term survival. In stark contrast, delays in tumor growth but not complete tumor regressions have been observed in a therapeutic setting with other broadly used vaccine strategies targeting gp70, including antigen-pulsed dendritic cells, recombinant vaccinia virus, or adenovirus (14, 32–34). Furthermore, immunization of B16 melanoma-bearing mice with a *Listeria* vaccine containing a single attenuating mutation expressing tyrosinase-related protein 2 resulted in a significant decrease in pulmonary tumor nodules, demonstrating further that recombinant *Listeria* can overcome tolerance to an endogenous tumor-associated antigen (19). *Listeria* Δ*actA*/Δ*inlB* AH1-A5 vaccination resulted in complete tumor regression, even though CT26 tumors have been shown to evade anti-tumor immunity by down-regulating gp70 expression upon exposure to IFN-γ (35). *Listeria* Δ*actA*/Δ*inlB* maintained its immunogenicity, even when administered at doses that were several logs below its LD_{50}. Thus, deleting *actA* and *inlB* increased the therapeutic window for the safe use of *Listeria*-based vaccines.

In summary, we have selected a genetically defined live-attenuated *Listeria* platform strain with potent immunogenicity. Because its capacity to directly or indirectly infect particular nonphagocytic cells is largely abrogated and as a consequence of an accelerated clearance *in vivo*, we believe that this strain is an ideal candidate for further clinical development of potent *Listeria*-based vectors for active cancer immunotherapy. An evaluation of *Listeria* Δ*actA*/Δ*inlB* vaccines encoding antigens related to human cancer in preclinical animal studies would prove useful for the clinical development of this candidate.

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