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Impact of Preexisting Vector-Specific Immunity on Vaccine Potency: Characterization of *Listeria monocytogenes*-Specific Humoral and Cellular Immunity in Humans and Modeling Studies Using Recombinant Vaccines in Mice▽†

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Recombinant live-attenuated *Listeria monocytogenes* is currently being developed as a vaccine platform for treatment or prevention of malignant and infectious diseases. The effectiveness of complex biologic vaccines, such as recombinant viral and bacterial vectors, can be limited by either preexisting or vaccine-induced vector-specific immunity. We characterized the level of *L. monocytogenes*-specific cellular and humoral immunity present in more than 70 healthy adult subjects as a first step to understanding its possible impact on the efficacy of *L. monocytogenes*-based vaccines being evaluated in early-phase clinical trials. Significant *L. monocytogenes*-specific humoral immunity was not measured in humans, consistent with a lack of antibodies in mice immunized with wild-type *L. monocytogenes*. Cellular immune responses specific for listeriolysin O, a secreted bacterial protein required for potency of *L. monocytogenes*-derived vaccines, were detected in approximately 60% of human donors tested. In mice, while wild-type *L. monocytogenes* did not induce significant humoral immunity, attenuated *L. monocytogenes* vaccine strains induced high-titer *L. monocytogenes*-specific antibodies when given at high doses used for immunization. Passive transfer of *L. monocytogenes*-specific antiserum to naïve mice had no impact on priming antigen-specific immunity in mice immunized with a recombinant *L. monocytogenes* vaccine. In mice with preexisting *L. monocytogenes*-specific immunity, priming of naïve T cells was not prevented, and antigen-specific responses could be boosted by additional vaccinations. For the first time, our findings establish the level of *L. monocytogenes*-specific cellular immunity in healthy adults, and, together with modeling studies performed with mice, they support the scientific rationale for repeated *L. monocytogenes* vaccine immunization regimens to elicit a desired therapeutic effect.

There continues to be a strong need for vaccine platforms used in prophylactic or therapeutic vaccination settings that consistently elicit potent and broad-based cellular immunity against encoded antigens related to chronic infections or cancer, diseases that collectively represent a significant unmet medical need at a global scale. Multiple platforms based on recombinant strains of viruses and bacteria have been developed and are in various stages of evaluation in human clinical trials (1, 6–9, 15, 19, 36, 42). Attenuated and/or replication-defective vector platforms retain much of the natural biology of the parent organism and are sensed by the vaccinated host as an invading pathogen, and the host in turn responds with an appropriate innate and adaptive immune response. Yet, despite numerous publications demonstrating encouraging vaccine-induced cellular responses in humans, the parallel induction of neutralizing vector-specific antibodies and vector-specific cellular immune responses upon immunization ultimately limits the application and utility of these vectors (2, 11, 12, 38).

Recombinant vaccine platforms based on intracellular bacteria have a conceptual advantage compared to virus-based vectors, because unlike with viruses, the initial steps of interaction with a host cell surface receptor for cell entry and uncoating are not a prerequisite for intracellular bacteria to express encoded antigens. For example, it is known that uptake of the facultative intracellular bacterium *Listeria monocytogenes* by phagocytic cells, such as dendritic cells (DC) or macrophages, is not inhibited by antibodies, and Fc receptor-mediated uptake of opsonized bacteria is as efficient as direct bacterial internalization (25). It has been shown previously that infection of human monocyte-derived DC in vitro with *L. monocytogenes* was significantly increased by incubation with human plasma containing antibody specific for p60, a secreted bacterial protein (25). This property may contribute significantly to vaccine efficacy in settings of preexisting *L. monocytogenes*-specific immunity or with immunization regimens utilizing repeated vaccinations.

L. monocytogenes taken up by phagocytic cells is contained within a phagolysosome, and a genetic program induced by the *L. monocytogenes* transcription factor PrfA mediates escape from the vacuole in a process facilitated largely by the pore-forming cytolysin listeriolysin O (LLO). *L. monocytogenes* multiplies in the cytosol and expresses another PrfA-dependent

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virulence determinant, ActA, which facilitates polymerization of filamentous host cell actin to propel the bacterium through the cytosol and into neighboring cells to repeat the process, during which *L. monocytogenes* remains largely sequestered from the extracellular milieu (13, 31). In the mouse listeriosis model, acquired immunity is cell mediated and bacterium-specific antibodies play no role in protection (13, 31). Thus, the central rationale for utilizing *L. monocytogenes* as a vaccine platform is that its intracellular life cycle promotes effective stimulation of $CD4^+$ and $CD8^+$ T-cell immunity (to itself and to encoded heterologous antigens), which correlates with resolution of infection against intracellular pathogens and with protection for cancer antigen-targeted immunotherapies. Furthermore, although wild-type (WT) *L. monocytogenes* infects nonphagocytic cells such as enterocytes and hepatocytes through expression of a series of invasins known as internalins on the bacterial cell surface that interact with defined host cell receptors to facilitate uptake (14), the potency of *L. monocytogenes*-based vaccines is not dependent upon this property. Thus, attenuated vaccine strains with a reduced capacity for infection of nonphagocytic cells retain the immunogenicity of WT *L. monocytogenes*, since uptake by phagocytic cells, including DC, is unaffected with these strains (9).

Although much has been learned about the biology of *L. monocytogenes* in the murine model of listeriosis, little is known about the status of *L. monocytogenes*-specific immunity in humans. *L. monocytogenes* is a ubiquitous, food-borne bacterium, and it is estimated that people in the United States ingest several hundred thousand bacteria several times per year (17a), typically resulting in a subclinical infection among healthy individuals. Such routine infection may indicate an underlying level of *L. monocytogenes*-specific humoral and cellular immunity that is common among healthy individuals. While measurable antibodies to extracellular *L. monocytogenes* protein p60 have been demonstrated in a majority of human subjects tested (21) and LLO-reactive T cells have also been identified in human samples (22, 30a), the frequency of humoral and cell-mediated immunity in humans remains poorly defined. Thus, it is not possible to make predictions on whether preexisting immunity would limit the effectiveness of the *L. monocytogenes*-based vaccines that are currently undergoing evaluation in human clinical trials.

The impact of preexisting cellular immunity to *L. monocytogenes* on its effectiveness as a vaccine vector in mice has been investigated by several groups (5, 36, 37, 39, 40), but these studies have resulted in conflicting results. The impact of existing *L. monocytogenes*-specific immunity on the ability to prime naïve T cells to a newly introduced antigen using the same *L. monocytogenes* vaccine strain ranged from no (5, 36), to moderate (37, 39), to severe (40) attenuation. The previous studies were limited by their choice of vaccine strains that were based on WT *L. monocytogenes* (5, 36, 40) as well as the use of immunodominant antigens such as ovalbumin (OVA). The impact of preexisting immunity on the potency of live-attenuated *L. monocytogenes*-based vaccines will most likely be influenced by several factors, including the extent of preexisting immunity, the attenuation of the vaccine strain, and the choice of antigen. Additionally, the quality of the *L. monocytogenes*-specific response might depend on whether the individual was exposed to the WT or to an attenuated vaccine strain. Earlier studies focused exclusively on *L. monocytogenes*-specific cellular immunity, and the impact of existing *L. monocytogenes*-specific humoral immunity on vaccine efficacy is unknown. Here we show that when given at high immunization doses, attenuated strains of *L. monocytogenes* induce significant levels of *L. monocytogenes*-specific humoral immunity in mice, potentially interfering with subsequent administrations. Furthermore, emerging data suggest that antibodies to LLO can attenuate the infection with WT *L. monocytogenes* (16, 17).

Here, we characterized the level of *L. monocytogenes*-specific cellular and humoral immunity in more than 70 healthy adult volunteers. We did not detect significant levels of *L. monocytogenes*-specific antibodies, but we did detect LLO-specific cellular immunity in 60% of this volunteer cohort. We found that mice immunized with live-attenuated *L. monocytogenes* vaccines developed high-titer *L. monocytogenes*-specific antibodies; however, unlike viral vectors that are highly susceptible to neutralizing antibodies, the presence of *L. monocytogenes*-specific antibodies did not diminish the capacity of *L. monocytogenes* to infect cells in vitro or to induce cellular immunity in vivo. In mice with preexisting *L. monocytogenes*-specific cellular immunity comparable to levels we measured in humans, priming of naïve T cells specific for encoded heterologous antigens (foreign or self) was attenuated, and the impact was dependent on the dose and time of WT *L. monocytogenes* exposure. Importantly, we found that the responses could be boosted by additional vaccinations. These data support a rationale for ongoing human clinical trials utilizing repeated doses of live-attenuated *L. monocytogenes*-based vaccines.

MATERIALS AND METHODS

Mice. Six- to 8-week-old female BALB/c or C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) or Taconic (Germantown, NY). Mice were treated according to National Institutes of Health guidelines. All protocols requiring animal experimentation received prior approval from the Anza Animal Care and Use Committee.

In vitro cell culture. J774 cells (a murine macrophage cell line), DC2.4 cells (a human DC-like cell line), and B16 cells (a murine melanoma cell line) were cultured in RPMI medium (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 5×10^4 IU/5×10^4 μg penicillin-streptomycin (Cellgro, Herndon, VA), 1× nonessential amino acids (Cellgro), 2 mM L-glutamine (Cellgro), 1×10^{-2} M HEPES buffer (Gibco), 1 mM sodium pyruvate (Sigma), and 50 μM β-mercaptoethanol (Sigma). Caco2 cells (a human colon carcinoma cell line; American Type Culture Collection, Manassas, VA) were cultured in minimal essential medium (Gibco) supplemented as described above.

Complement fixation. Human serum samples from healthy donors were purchased from U.S. Biologicals (Swampscott, MA). Complement fixation assays were performed with an *L. monocytogenes*-specific complement fixation kit following the vendor's instructions (Virion, Rüschlikon, Switzerland). Naïve and immune mouse sera and serum isolated from pooled human cord blood (U.S. Biologicals) were used as controls.

***L. monocytogenes*-specific ELISAs.** Whole blood was collected from mice by retro-orbital bleeds at the indicated time points, and serum was isolated. Immulon 1B 96-well enzyme-linked immunosorbent assay (ELISA) plates (Nunc, Rochester, NY) were coated with ground WT *L. monocytogenes* and incubated overnight at 4°C. Plates were blocked with Superblock (Pierce Biotechnology, Rockford, IL) and incubated with serial dilutions of serum, followed by goat anti-mouse horseradish peroxidase-conjugated antibody. Plates were developed with one-step Slow TMB-ELISA substrate (Pierce Biotechnology). The reaction was stopped with 2 M H_2SO_4, and the optical density at 450 nm was measured with a spectrophotometer.

In vitro infectivity. Infectivities in J774, DC2.4, B16, and Caco2 cells were assessed in vitro as previously described (30). Briefly, WT *L. monocytogenes* was grown in brain heart infusion medium to stationary phase at 30°C overnight. The next day, *L. monocytogenes* was washed and incubated for 1 hour with either

saline or serum from mice vaccinated with *L. monocytogenes* Δ*actA*. J774, DC2.4, B16, and Caco2 cells were infected with *L. monocytogenes* for 1 hour at multiplicities of infection (MOI) of 0.1, 0.1, 1, and 10, respectively. Cells were incubated with gentamicin (50 μg/ml) and then were lysed with sterile water. Serial dilutions were plated on brain heart infusion agar. Infectivity was determined by the ratio of intracellular titer to the initial infection titer. This number was compared to the infectivity in a group injected with Hanks balanced salt solution (HBSS).

Peptides. Peptides for $OVA_{257–264}$ (SIINFEKL), AH1 (SPSYVYHQF) of gp70, $LLO_{91–99}$ (GYKDGNEYI), and $LLO_{190–201}$ (NEKYAQAYPNVS) were synthesized by Invitrogen (Carlsbad, CA). An LLO peptide library consisting of peptides that span the sequence of LLO (15-mers overlapping by 11 amino acids) was synthesized by SynPep (Dublin, CA).

***Listeria monocytogenes* and VV vaccines.** *L. monocytogenes* strains were derived from 10403S (4). *L. monocytogenes* strains with in-frame deletions of the indicated genes were generated by splicing by overlapping extension PCR and allelic exchange with established methods (10). The *L. monocytogenes* strain backgrounds used included WT (DP-L4056), Δ*hly* (DP-L4027), Δ*actA* (DP-L4029), and Δ*actA* Δ*inlB* (ANZ-100). The pPL2 integrational vector (26) was used to derive OVA and AH1/A5 recombinant *L. monocytogenes* strains containing a single copy of the antigen expression cassette within the *L. monocytogenes* chromosome adjacent to the gene encoding tRNA arginine. To make heat-killed *L. monocytogenes*, WT *L. monocytogenes* (DP-L4056) was incubated at 72°C for 2 h. For vaccination, *L. monocytogenes* was grown at 37°C in brain heart infusion broth (Sigma, St. Louis, MO) to log phase, washed with phosphate-buffered saline, and frozen in 8% dimethyl sulfoxide–phosphate-buffered saline at −80°C. For vaccination, aliquots were diluted in HBSS and administered by intravenous (i.v.) injections. Empty vaccinia virus (VV) and VV expressing OVA (VV-OVA) were kindly provided by Nicholas P. Restifo, NCI, Bethesda, MD. VV was administered intraperitoneally at the indicated doses.

ELISPOT assay. Healthy adult donors provided blood samples after providing consent in accordance with the company's Institutional Review Board guidelines (IRC, San Anselmo, CA). Lymphocytes were isolated using Ficoll-Hypaque (GE Amersham, Piscataway, NJ). Human enzyme-linked immunospot (ELISPOT) assays were performed with 4×10^5 cells/well using human gamma interferon (IFN-γ) and interleukin-2 (IL-2) kits according to their protocols (BD Biosciences, San Diego, CA). CEF peptide pool (a pool of HLA-A2-restricted T-cell epitopes from human cytomegalovirus, Epstein-Barr virus, and influenza virus) (Axxora, San Diego, CA) and *Staphylococcus aureus* enterotoxin B (Toxin Technologies, Sarasota, FL) served as positive controls. Murine ELISPOT assays were performed with lymphocytes isolated from whole mouse blood using Lympholyte-Mammal (Cedarlane Labs, Burlington, NC) and a murine IFN-γ ELISPOT pair (BD Biosciences). A total of 2×10^5 cells/well were incubated with the appropriate peptide overnight at 37°C in anti-murine IFN-γ-coated ELISPOT plates (Millipore, Billerica, MA) and developed using alkaline phosphatase detection reagents (Invitrogen). Cells were incubated without peptide as a negative control. Plates were scanned and quantified using the Immunospot plate reader and software (CTL Ltd., Cleveland, OH).

ICS. The frequencies of IFN-γ-secreting mouse $CD8^+$ and $CD4^+$ T lymphocytes were determined by intracellular cytokine staining (ICS) as described previously (20, 32). Briefly, 10^6 splenocytes were incubated with the appropriate peptide at 1 μM for 5 h with brefeldin A (BD Biosciences). Spleen cells incubated without peptide were used as a negative control. Cells were stained for cell surface markers with anti-$CD8^+$–α-peridinin chlorophyll protein (clone 53-6.7; BD Biosciences) and anti-$CD4^+$–fluorescein isothiocyanate (clone GK1.5; eBioscience, San Diego, CA), fixed in 2% formaldehyde, and incubated with anti-IFN-γ–allophycocyanin (clone XMG1.2; eBioscience). Samples were acquired on a FACSCalibur flow cytometer and data analyzed using CELLQuest software (BD Bioscience) or FlowJo software (Tree Star Inc, Ashland, OR). Human peripheral blood mononuclear cells (PBMCs) were stimulated at 10^6 cells/well with or without 1 μg/ml LLO peptide pool in the presence of brefeldin A for 6 h. Cells were fixed, frozen in FACSLyze solution, and permeabilized in Perm-2 solution (BD Biosciences). Cells were stained with antibodies to CD3 (clone SK7 [BD], allophycocyanin conjugate), CD4 (clone SK3 [BD], allophycocyanin-Cy7 conjugate), CD8 (clone T8 [Beckman Coulter], phycoerythrin-Cy7 conjugate), CD14 (clone MΦP9 [BD], peridinin chlorophyll protein conjugate, IFN-γ (clone 45.15 [Beckman Coulter], fluorescein isothiocyanate conjugate), and CD69 (clone TP1.55.3 [Beckman Coulter], phycoerythrin conjugate). Samples were acquired on a BD Canto flow cytometer and analyzed with FlowJo software.

IVS cultures. In vitro sensitization (IVS) cultures with human PBMCs were carried out as described previously (41). In brief, 3×10^6 to 5×10^6 PBMCs were resuspended in 1 ml complete RPMI (cRPMI) (RPMI plus 10% heat-inactivated human AB serum [Valley Biomedical, Winchester, VA], supplemented with 2 mM glutamine, 1× nonessential amino acids [Invitrogen], 1 mM sodium pyruvate, and 5 mM HEPES) and seeded in single wells of a 24-well plate in presence or absence of antigen; the LLO peptide pool was added at 0.5 μg/ml/peptide, and *L. monocytogenes* Δ*hly* and *L. monocytogenes* Δ*actA* Δ*inlB* were added at an MOI of 1. Infected cultures were washed extensively after 15 min, and PBMCs were resuspended in cRPMI containing 5 mM gentamicin (Sigma). After 3 days, 1 ml cRPMI containing 10 U/ml IL-2 (R&D Systems, Minneapolis, MN) was added. On day 6, an additional 10 U/ml IL-2 was added. Cultures were harvested and used in ELISPOT or ICS assays between days 9 and 10.

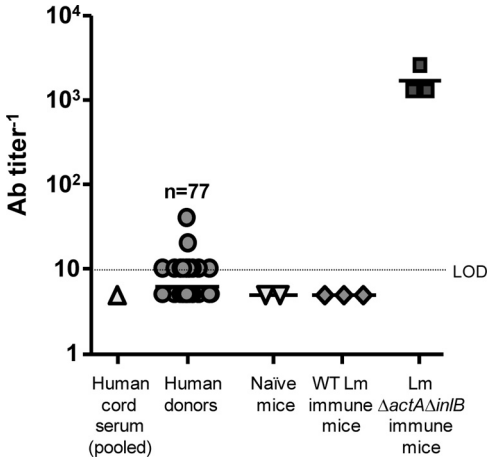

FIG. 1. *L. monocytogenes*-specific humoral immunity. Serum samples were collected from healthy human subjects and tested in an *L. monocytogenes*-specific complement fixation assay ($n = 77$). Pooled human cord serum was used as a control. Sera from naïve C57BL/6 mice ($n = 2$), and C57BL/6 mice vaccinated i.v. with WT *L. monocytogenes* and *L. monocytogenes* Δ*actA* Δ*inlB* ($n = 3$) were also tested in a complement fixation assay for comparison. The line indicates the mean response for each group.

Therapeutic in vivo tumor studies. Mice were injected i.v. with either HBSS or 5000 CFU of WT *L. monocytogenes*. After 44 days, they were implanted i.v. with 2×10^5 CT26 murine colon carcinoma cells. Three days later mice were vaccinated i.v. either with *L. monocytogenes* Δ*actA* Δ*inlB* encoding AH1/A5 or with HBSS as negative control. Lungs were harvested 20 days after tumor challenge and fixed in Bouin's solution, and the number of surface lung metastases was assessed. For survival studies, mice were sacrificed when they started to show signs of stress or labored breathing.

RESULTS

***L. monocytogenes*-specific humoral immunity.** To characterize the level of preexisting *L. monocytogenes*-specific immunity in healthy adult human subjects, we first assessed existing *L. monocytogenes*-specific humoral immunity by screening sera from 74 healthy volunteers for antibodies against intact bacteria, using complement fixation. In the individuals tested, the titers ranged from undetectable to 1:40 (Fig. 1). A total of 16.2% (12/74) had a measurable anti-*L. monocytogenes* antibody titer (titer of 1:10 or higher), while most (83.8%; 62/74) had no detectable *L. monocytogenes* antibodies (titer below 1:10). These results are consistent with the murine model of listeriosis where high levels of *L. monocytogenes*-specific humoral immunity are not induced following infection of mice with sublethal doses of WT *L. monocytogenes* (Table 1) (23). Since it has been shown that live-attenuated strains of *L. monocytogenes*, such as *L. monocytogenes* Δ*hly*, induce antilisterial antibodies in mice (23), we assessed whether *L. monocytogenes* Δ*actA* Δ*inlB*, a vaccine strain that unlike *L. monocy-*

TABLE 1. Anti-*L. monocytogenes* antibodies measured in C57BL/6 mice after multiple vaccinations with WT or attenuated *L. monocytogenes* vaccine strains

L. monocytogenes strain	Description	Titer^{-1}[a]	
		ELISA	Complement fixation
DP-L4056[b]	Wild type, 10403S	<10, <10, <10	<10, <10, <10
HKLM[c]	Wild type, 104030S, heat killed	81,920, 20,480, 2,560	80, 40, 20
DP-L4027[d]	Δ*hly*	10,240, 20,480, 20,480	80, 20, 20
DP-L4029[e]	Δ*actA*	640, 10,240	80, 160
ANZ-100[f]	Δ*actA* Δ*inlB*	163,840, 655,360, 163,840	1,280, 2,560, 1,280

[a] Sera from C57BL/6 mice were collected after the indicated vaccinations. Serial dilutions of serum samples were tested in *L. monocytogenes*-specific ELISAs or complement fixation assay. ELISAs and complement fixation assays were done in duplicate or triplicate. A titer of 10 was the limit of detection for both assays.
[b] 5×10^3 CFU i.v., four vaccinations 10 days apart.
[c] 3×10^8 CFU i.v., four vaccinations 10 days apart.
[d] 1×10^8 CFU i.v., four vaccinations 10 days apart.
[e] 2×10^7 CFU i.v., four vaccinations 10 days apart.
[f] 1×10^7 CFU i.v., four vaccinations 21 days apart.

togenes Δ*hly* establishes a cytosolic infection, induces antibodies and, if so, tested its biological relevance on in vitro infection and in vivo potency following repeated administration. C57BL/6 mice were vaccinated with live-attenuated *L. monocytogenes* Δ*actA* Δ*inlB*, *L. monocytogenes* Δ*hly*, *L. monocytogenes* Δ*actA*, or live or heat-killed WT *L. monocytogenes*, and sera from these mice were tested in *L. monocytogenes*-specific complement fixation assays and ELISAs. As shown previously (23), *L. monocytogenes* Δ*hly* induced significant *L. monocytogenes*-specific antibodies, with a minimum titer of 1:10,000 by ELISA or 1:20 by complement fixation. Vaccination with either *L. monocytogenes* Δ*actA* or *L. monocytogenes* Δ*actA* Δ*inlB* also induced robust anti-*L. monocytogenes* humoral immunity (Table 1; Fig. 1), with specificity to multiple *L. monocytogenes* proteins, including p60 and LLO (data not shown).

L. monocytogenes can infect both phagocytic cells, such as DC, and nonphagocytic cells (14, 24). While infection of professional antigen-presenting cells such as DC and macrophages is required for vaccine potency, infection of nonphagocytic cells likely is not, but it may contribute to vaccine toxicity. To investigate the effect of *L. monocytogenes*-specific antibodies on intracellular infection, in vitro infectivity studies were performed with phagocytic cell lines DC2.4 and J774 and with nonphagocytic cell lines B16 and Caco2. WT *L. monocytogenes* was first incubated with saline or high-titer anti-*L. monocytogenes* immune serum and then used to infect cells. Incubation with serum containing high-titer *L. monocytogenes*-specific antibodies reduced the infectivity of nonphagocytic cell lines tested by 60% and by greater than 90%, respectively; however, there was no effect on the infection of either phagocytic cell lines (Fig. 2A).

To test whether the presence of *L. monocytogenes*-specific antibodies affects the potency of recombinant *L. monocytogenes* vaccines, high-titer *L. monocytogenes*-specific serum was transferred to naïve mice 1 day before and 1 day after vaccination with *L. monocytogenes*-OVA, and the percentages of splenic $OVA_{257–264}$-specific and $LLO_{190–201}$-specific $CD8^+$ and $CD4^+$ T cells were measured 7 days later. Verification of anti-*L. monocytogenes* antibody titers in the serum was performed by *L. monocytogenes*-specific ELISA assays (see Fig. S1 in the supplemental material). As shown in Fig. 2B and C, mice given high-titer serum induced OVA- and LLO-specific T-cell immunity comparable to that in mice that received saline or serum with no measurable antibodies. Vaccination with live-attenuated *L. monocytogenes* induced a significant cellular immune response even in the presence of *L. monocytogenes*-specific antibodies (Table 1).

***L. monocytogenes*-specific cellular immunity in healthy adult subjects.** The level and frequency of *L. monocytogenes*-specific cellular immunity in the adult population are unknown; their characterization may be relevant to predicting the effectiveness of recombinant *L. monocytogenes* vaccines. To examine *L. monocytogenes*-specific cellular immunity, PBMCs from 95 healthy human adult donors were screened in an IFN-γ ELISPOT assay using an LLO peptide library as stimulators. Of the donors tested, 58% ($n = 55$) had measurable IFN-γ T-cell responses (Fig. 3A). Positive responses were defined as spot counts that were greater than those for unstimulated controls plus two standard deviations and at least 10 spot-forming cells (SFC) per million PBMCs. The responses were generally weak, with a median of 16.3 SFC/1 $\times$ 10^6 PBMCs and a range from undetectable to 289 SFC/1 $\times$ 10^6 PBMCs. When donor reactivity to LLO was assayed in an IL-2 ELISPOT assay for a subset of the donor PBMCs ($n = 78$), the reactivity pattern was largely replicated, with slightly higher SFC counts (Fig. 3B). Since only a subpopulation of $CD8^+$ T cells were able to produce IL-2 in response to antigen, we investigated whether $CD4^+$ or $CD8^+$ T cells were reactive to LLO using PBMCs from seven strong LLO responders and five nonresponders. All seven strong donors had low but detectable $CD4^+$ T-cell IFN-γ and IL-2 responses to LLO, ranging from 0.01 to 0.07% $CD4^+$ T cells, while $CD8^+$ T cells did not react (Fig. 4A). To increase the frequency of reactive T cells that might be present in the original PBMC samples, we carried out short-term in vitro cultures of PBMCs in the presence of antigen (IVS cultures). IVS cultures of PBMCs from approximately 15 donors confirmed that LLO reactivity resides in the $CD4^+$ T-cell compartment (Fig. 4B). LLO-specific $CD8^+$ T cells were either not present or below lower detection limits ($\leq$0.1% $CD8^+$ T cells after IVS), while $CD4^+$ LLO-specific T cells reached high frequencies. Under comparable IVS culture conditions, $CD8^+$ T cells specific for human disease antigens proliferated in the presence of cytomegalovirus, Epstein-Barr virus, and influenza virus peptides and were highly activated (data not shown). A fraction of T cells taken from the LLO IVS cultures were

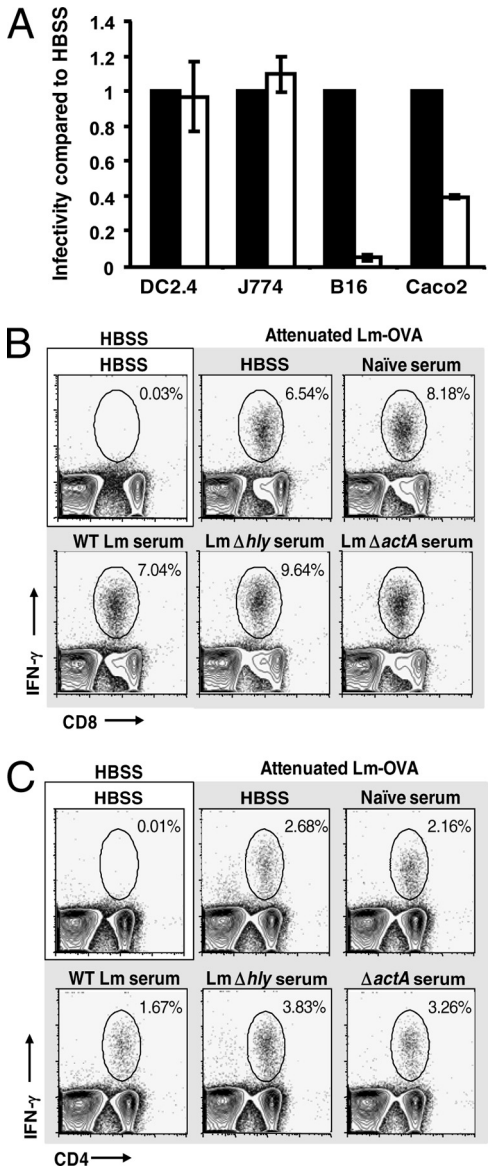

FIG. 2. Effect of preexisting humoral immunity on *L. monocytogenes*-based vaccines. (A) In vitro infections. WT *L. monocytogenes* was incubated with (white bars) or without (black bars) high-titer *L. monocytogenes*-immune serum for 1 h. DC2.4, J774, B16, and Caco2 cells were infected with WT *L. monocytogenes* at MOI of 0.1, 0.1, 1, and 10, respectively. Data are shown as fraction of infectivity compared to an HBSS control, which was normalized to 1. In vitro infections were performed in triplicate. Error bars indicate standard deviations. (B and C) C57BL/6 mice were vaccinated i.v. four times 1 week apart with HBSS, WT *L. monocytogenes* (1×10^4 CFU), *L. monocytogenes* Δ*hly* (1×10^8 CFU), or *L. monocytogenes* Δ*actA* (1×10^7 CFU), and serum was collected. One hundred microliters of high-titer *L. monocytogenes*-immune serum or HBSS was transferred into naïve mice 1 day before and 1 day after i.v. vaccination with 1×10^7 CFU of attenuated *L. monocytogenes*-OVA (shaded regions). Mice were vaccinated with HBSS as a control (boxed regions). $OVA_{257-264}$-specific (B) and $LLO_{190-201}$-specific (C) immune responses in three pooled spleens were assessed by IFN-γ ICS 1 week after vaccination. Unstimulated responses were less than 0.1%. Data are representative of two replicate studies.

highly activated even without restimulation in the ICS assays, resulting in unusually high background staining (Fig. 4B, left panel). Control IVS cultures without antigen or with peptide pools carrying endogenous (human) antigen did not exhibit such background (data not shown). IVS cultures of PBMCs infected with an attenuated strain of *L. monocytogenes* Δ*actA* Δ*inlB* (as the stimulus) showed that LLO-reactive $CD4^+$ but not $CD8^+$ T cells grew in the culture (Fig. 4C). An attenuated *L. monocytogenes* Δ*hly* strain, which does not produce LLO protein, served as negative control for the in vitro culture, suggesting that LLO produced endogenously by *L. monocytogenes* during the infection can serve as antigen for LLO-reactive T cells. Although it is unknown whether the T-cell responses measured against LLO are memory responses or primary responses due to a recent *L. monocytogenes* exposure, the data suggest that most people have been exposed to *L. monocytogenes* at some point in their life. Reactivity to one *L. monocytogenes* antigen, LLO, appears to be dominated by $CD4^+$ T cells in humans.

Effect of preexisting *L. monocytogenes*-specific cellular immunity on priming a response with *L. monocytogenes*-based vaccines. As a first step to evaluating the impact of preexisting immunity in humans on vaccine efficacy, we established a mouse model that recreated the levels of preexisting immunity observed in the humans. Although the natural route of *L. monocytogenes* infection in humans is oral, i.v. administration in mice provides a more robust and reproducible infection, due in part to the decreased affinity in mice of bacterial invasin InlA and host adhesion molecule E-cadherin, which is required for crossing the gut epithelial barrier (27). Increasing doses of WT *L. monocytogenes* were administered i.v. to BALB/c and C57BL/6 mice, and after 30 days, lymphocytes were isolated from peripheral blood of mice and LLO-specific responses were measured by IFN-γ ELISPOT assay using an overlapping LLO peptide library, analogous to the analysis of human samples. LLO-specific immune responses measured in mice were dose dependent. In C57BL/6 mice, vaccination with either 500 CFU or 5,000 CFU of WT *L. monocytogenes* generated detectable *L. monocytogenes*-specific cellular immunity, while lower doses of 5 or 50 CFU did not induce a measurable response (Fig. 5A). At the low doses of 50 or 500 CFU of *L. monocytogenes*, approximately half of the BALB/c mice induced a measurable immune response (two or three responders out of five mice at 50 or 500 CFU, respectively). LLO-specific immune responses were detected in all BALB/c mice vaccinated with the highest dose of *L. monocytogenes* (Fig. 5B). Mice vaccinated with 50 CFU and 500 CFU induced a level of *L. monocytogenes*-specific immunity most similar to the magnitude of responses measured in the human donors (Fig. 3A and 5). A dose-dependent increase in LLO-specific T-cell immunity was also observed in spleens of C57BL/6 and BALB/c mice by ELISPOT assay using the LLO peptide pool and by ICS using defined the $CD8^+$ epitope LLO_{91-99} for BALB/c mice and the defined $CD4^+$ epitope $LLO_{190-201}$ C57BL6 mice (data not shown).

To determine the effect of preexisting *L. monocytogenes* immunity on vaccine potency, cohorts of preimmune mice were vaccinated with an *L. monocytogenes* strain that expressed both OVA and the heteroclitic T-cell epitope for gp70, designated AH1/A5. Vaccination with AH1/A5 induces a T-cell response against the endogenous AH1 epitope of gp70 (35). An inverse correlation between the number of *L. monocyto-*

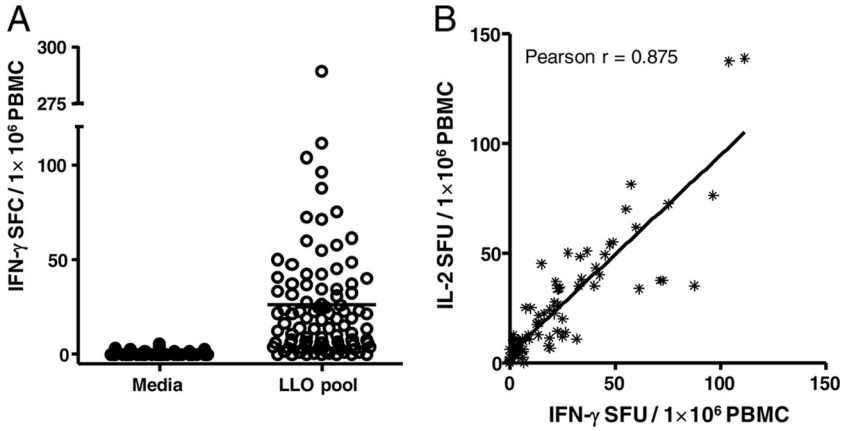

FIG. 3. *L. monocytogenes*-specific cellular immunity in a healthy human population. PBMCs were isolated from healthy human donors. LLO-specific responses were assessed with an LLO peptide pool (open symbols) compared to an unstimulated control (filled symbols) in IFN-γ ($n = 95$) (A) and IL-2 ($n = 78$) (B) ELISPOT assays. SFU, spot-forming units.

genes-specific T cells measured on the day of vaccination and the number of primed OVA- and AH1-specific T cells (Fig. 5 and 6A and B) was observed. Preexposure to 5 CFU of WT *L. monocytogenes* had no impact on the number of OVA-specific or AH1-specific T cells that were induced compared to that in the HBSS-vaccinated mice. Preexposure to 50 CFU of WT *L. monocytogenes* decreased the frequency of primed OVA-specific T cells, while no effect on the priming of AH1-specific T cells was observed in BALB/c mice. In groups that were preexposed to either 500 or 5,000 CFU of WT *L. monocytogenes*, there was a significant reduction in the number of OVA- and AH1-specific T cells compared to the saline controls (Fig. 6A and B). At all doses of preexisting immunity, a boost vaccination increased the OVA- and AH1-specific immune responses to levels similar to those induced by a single vaccination in a naïve animal (Fig. 6B and D). Boost vaccinations increased responses even where AH1-specific responses were not measurable after a primary vaccination, demonstrating that AH1-specific T cells were primed by a single vaccination but were below the limit of detection.

The period of time between WT *L. monocytogenes* exposure and vaccination with a recombinant *L. monocytogenes*-based vaccine may influence the impact of preexisting immunity on subsequent vaccinations with *L. monocytogenes*-based vectors. To address this possibility, BALB/c mice were injected i.v. with 50 or 5,000 CFU of WT *L. monocytogenes* and then after 28, 60, 90, or 120 days were vaccinated with *L. monocytogenes*-AH1/A5. While preexposure to 50 CFU of *L. monocytogenes* did not have a significant effect of AH1-specific responses, exposure to 5,000 CFU of WT *L. monocytogenes* markedly reduced the AH1 immune response after 28 days. AH1-specific responses were restored if preexposure occurred more than 90 days prior to vaccination (Fig. 6C). This demonstrates that previous exposure to *L. monocytogenes* does not necessarily diminish the potency of *L. monocytogenes*-based vaccine vectors; as the interval of time since exposure increases, the impact of *L. monocytogenes* preexisting immunity on vaccine potency declines.

It is well described that vector-specific immunity can dramatically reduce the ability of virus-based vectors to induce cellular immune responses to the encoded heterologous antigen (2, 11, 12, 38). To compare the effects of preexisting vector-specific immunity between recombinant virus- and *L. monocytogenes*-based vaccines, mice were first immunized with HBSS, empty VV, or WT *L. monocytogenes*. Twenty-eight days later, the mice were inoculated with 1/10 of the 50% lethal dose of either VV-OVA or *L. monocytogenes*-OVA, and the number of OVA-specific T cells induced was determined at the peak of the response. In mice vaccinated with *L. monocytogenes*-OVA, only preexposure to 5,000 CFU affected the number of induced OVA-specific T cells (Fig. 6D). Preexposure to VV or a low dose of WT *L. monocytogenes* did not impair the OVA-specific response induced by *L. monocytogenes*-OVA. However, in mice vaccinated with VV-OVA, the number of OVA-specific T cells induced was diminished at least 1 log unit after any amount of preexposure to VV tested (Fig. 6D). As expected, preexisting immunity to *L. monocytogenes* had no effect on induction of immune responses by the VV-based vaccine.

AH1-specific T cells can protect against a tumor challenge in the presence of *L. monocytogenes*-specific immunity. Since we determined that AH1-specific immunity could be induced in the presence of preexisting vector immunity (Fig. 6B and C), we next wanted to determine if these gp70-specific T cells were similar in function in a therapeutic lung metastasis tumor model to those elicited in naïve mice. CT26 tumor cells were chosen as a model for early clinical disease based on the expression of endogenous mouse antigen gp70. BALB/c mice were exposed to either HBSS or 5,000 CFU of WT *L. monocytogenes*, and 44 days later, the mice were implanted i.v. with 2×10^5 CT26 tumor cells, which express the endogenous retroviral gene product gp70. Three days later, mice were treated with *L. monocytogenes*-AH1/A5 or HBSS as a negative control. On day 20, lungs from three mice from each group were removed for the enumeration of metastases; the remaining mice were monitored for survival. As shown in Fig. 7, vehicle-treated control mice presented between 31 and 65 lung surface metastases on day 20 and succumbed to tumor by day 26. Mice without preexisting *L. monocytogenes* immunity vaccinated with *L. monocytogenes*-AH1/A5 had 10 or fewer lung metastases at day 20, and 60% of mice survived beyond day 125. In mice

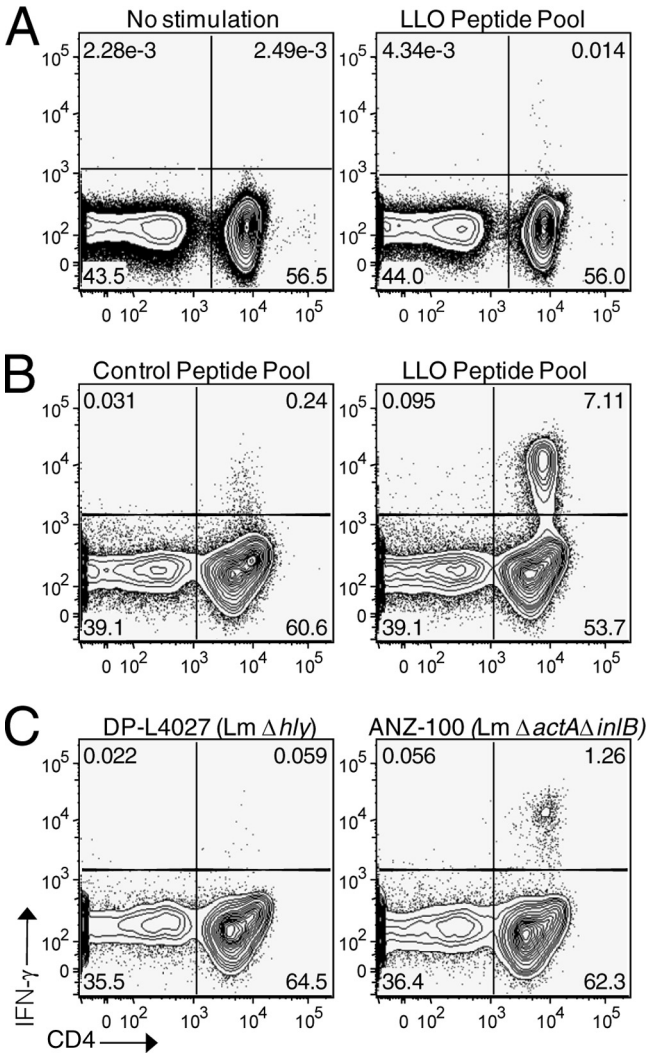

FIG. 4. Human LLO-specific cellular immunity is dominated by $CD4^+$ T cells. All plots are gated on $CD3^+$ lymphocytes, with $CD4^+$ on the *x* axis and IFN-γ on the *y* axis. (A) Ex vivo ICS assay using PBMCs from a healthy adult with a strong LLO ELISPOT response. PBMCs were stimulated either with (right plot) or without (left plot) the LLO peptide pool. Plots are gated on CD14-negative cells. (B) PBMCs from the same donor were cultured for 9 days with the LLO peptide pool (IVS cultures) before being restimulated in an IFN-γ ICS assay in either the presence (left plot) or absence (right plot) of the LLO peptide pool. (C) PBMCs from the same donor were infected with an LLO-negative *L. monocytogenes* strain (DP-L4027, *L. monocytogenes* Δ*hly*) (left plot) or an LLO-containing *L. monocytogenes* strain (Δ*actA* Δ*inlB*) (right plot) and cultured for 9 days. Cultured cells were restimulated in an IFN-γ ICS assay in the presence of the LLO peptide pool.

preexposed to *L. monocytogenes*, the number of lung metastases visible on day 20 was comparable to that measured in nonimmune mice (10 or fewer metastases per lung), with 30% long-term survival (Fig. 7). These data support the hypothesis that preexisting immunity does not preclude induction of immune responses or antitumor efficacy of *L. monocytogenes*-based vaccines.

DISCUSSION

There exists a significant global unmet medical need to prevent or treat acute and chronic infections. It is widely accepted that efficacious vaccines to address many of these diseases, such as human immunodeficiency virus disease, tuberculosis, and malaria, as well as cancer, must elicit functional cellular immunity specific for designated antigens. For more than two decades, multiple vaccine platforms based on recombinant viruses and bacteria have been developed. However, while many recombinant vectors are in various stages of evaluation in human clinical trials and there have been numerous publications demonstrating encouraging vaccine-induced cellular responses in humans, as yet none form the basis for a vaccine that has received an approved biologics license from the FDA (1, 6–9, 15, 19, 36, 42).

Both preexisting and induced vector-specific neutralizing immunity have severely limited the development and success of diverse recombinant vector platforms (2, 11, 12, 38). While the use of adenovirus vectors based on rare or chimeric viral serotypes may be useful for priming in a population commonly having neutralizing immunity against serotype 5 virus (2, 33), such a strategy does not circumvent a likely requirement for repeated immunization or a desired application to multiple diseases with immunologically distinct antigens. Prime-and-boost immunization regimens consisting of different vector platforms (e.g., plasmid DNA prime and viral vector boost) or serial immunization with vectors based on distinct viral serotypes (e.g., adenovirus) or distinct genera within a viral family (e.g., modified VV Ankara and canarypox) are attractive in that both vector-neutralizing antibody responses and avoidance of possible immunodominance of complex vectors limiting boosting of heterologous antigen-specific immune responses are addressed by this strategy (18, 28, 29). Nevertheless, such approaches are complex and expensive, and they do not address the need to develop vaccines targeting diverse antigens. Thus, development of recombinant vaccine platforms that retain potency in the face of preexisting immunity or with repeated immunization is a major challenge for the biomedical community.

Recombinant vaccine platforms based on *L. monocytogenes* are being developed and evaluated clinically (clinicaltrials.gov identifier NCT00327652 and NCT00585845), based upon a rationale of potent recombinant *L. monocytogenes* vaccine-induced innate and adaptive cellular immunity specific for encoded heterologous antigens. Here we found that the level of *L. monocytogenes*-specific antibodies in healthy adults was generally low and that cellular immunity to LLO was predominantly class II restricted. To explore how *L. monocytogenes*-specific immunity in humans might affect vaccine efficacy in ongoing clinical studies, we sought to model and characterize in mice vaccine potency with established levels of humoral or cellular *L. monocytogenes*-specific immunity. For the first time, we showed that, unlike the case for viruses, the presence of *L. monocytogenes*-specific antibodies has no impact on vaccine potency. Extending prior work, we found that while preexisting cellular immunity did diminish the ability of recombinant strains to elicit $CD8^+$ T-cell responses specific for an encoded antigen, this was dependent on the dose and time of WT exposure, and repeated immunization overcame the reduction in vaccine potency. Viewed in the context of *L. monocytogenes*-specific immunity measured in healthy adults, the results of these modeling studies suggest that neither cellular nor hu-

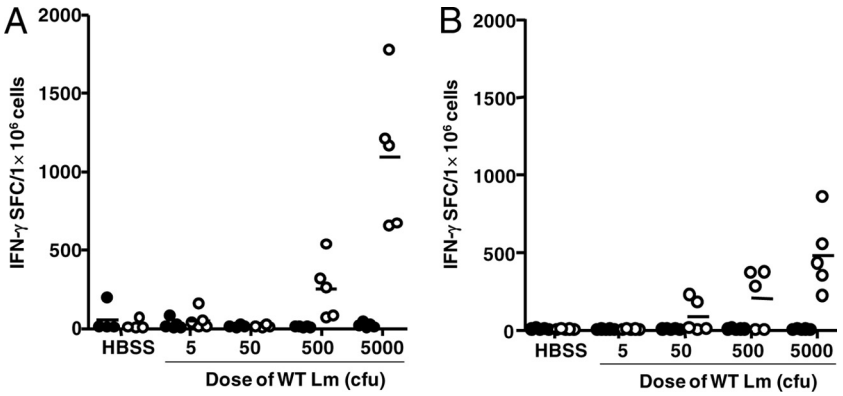

FIG. 5. Modeling of *L. monocytogenes*-specific cellular immunity for a healthy human population in mice. C57BL/6 (A) and BALB/c (B) mice were vaccinated i.v. with HBSS or with 5, 50, 500, or 5,000 CFU of WT *L. monocytogenes*. LLO peptide pool (open circles) and unstimulated (filled circles) immune responses were assessed in an IFN-γ ELISPOT assay using PBMCs isolated from peripheral blood 4 weeks after vaccination. ELISPOT data represent means from triplicate wells. Data are representative of those from two experiments.

moral preexisting *L. monocytogenes*-specific immunity would affect vaccine potency in humans.

Epidemiologic and serologic studies suggest that the majority of humans have been exposed to *L. monocytogenes* sometime during their life, and most often people are exposed several times each year (17a). A study by Gentschev et al. showed that the majority of sera from healthy individuals tested positive for p60-specific antibodies. Interestingly, anti-

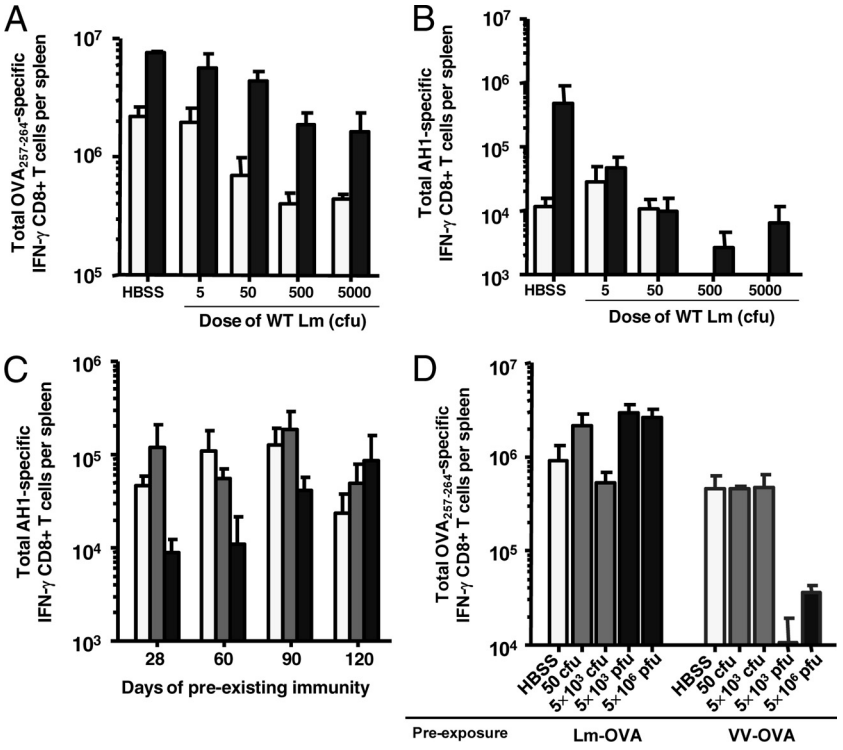

FIG. 6. Effect of *L. monocytogenes*-specific preexisting cellular immunity on OVA- and AH1-specific immunity. (A and B) C57BL/6 (A) and BALB/c (B) mice ($n = 3$) were vaccinated i.v. with HBSS or with 5, 50, 500, or 5,000 CFU of WT *L. monocytogenes*. After 28 days, *L. monocytogenes*-immune mice were next vaccinated i.v. with 5×10^6 CFU of attenuated *L. monocytogenes*-AH1/A5-OVA (open bars). Separate cohorts of mice ($n = 3$) were boosted i.v. with 5×10^6 CFU of attenuated *L. monocytogenes*-AH1/A5-OVA (black bars) after 3 weeks. $OVA_{257–264}$-specific (A) and AH1-specific (B) immune responses in spleens were assessed by IFN-γ ICS 7 days after the first vaccination or 5 days after the second vaccination. (C) Duration of preimmunity. BALB/c mice ($n = 3$) were vaccinated i.v. with HBSS (open bars) or with 50 CFU (gray bars) or 5,000 CFU (black bars) of WT *L. monocytogenes*. After 28, 60, 90, and 120 days, mice were vaccinated i.v. with 5×10^6 CFU of attenuated *L. monocytogenes*-AH1/A5. AH1-specific immune responses in spleens were assessed by IFN-γ ICS after 7 days. (D) Comparison of VV and *L. monocytogenes*. C57BL/6 mice ($n = 3$) were vaccinated i.v. with HBSS (open bars), 50 or 5×10^3 CFU of WT *L. monocytogenes* (gray bars), or 5×10^3 or 5×10^6 PFU of empty VV (black bars). After 41 days, mice were vaccinated i.v. with 1/10 of the 50% lethal dose of either attenuated *L. monocytogenes*-OVA (5×10^6 CFU) or VV-OVA (5×10^6 PFU). $OVA_{257–264}$-specific responses in spleens were measured by IFN-γ ICS after 7 days. Error bars indicate standard deviations.

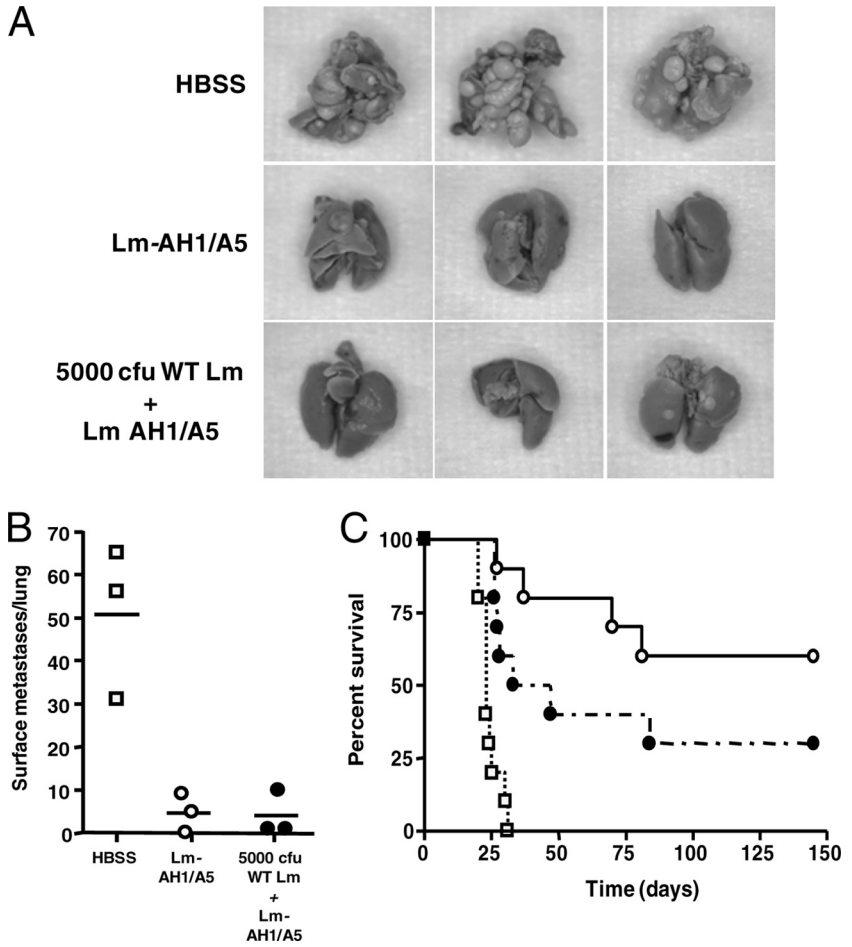

FIG. 7. Antitumor efficacy in the presence of preexisting immunity. Three days after tumor implant, naïve (filled circles) and WT *L. monocytogenes* preimmune (open circles) mice were vaccinated i.v. with attenuated *L. monocytogenes*-AH1/A5. Unvaccinated controls (open squares) were given tumor on same day. At 20 days after tumor implant, lungs were harvested from all groups ($n = 3$) and stained with Bouin's solution (A), and surface metastases were enumerated (B). The remaining mice ($n = 10$) were monitored for survival for 145 days (C).

bodies to other proteins, such as LLO, were not detected (21). In contrast, we observed a lower frequency of healthy individuals (~16%) with detectable *L. monocytogenes*-specific humoral immunity. The difference between our results and those of Gentschev et al. may be due to the methods used to detect *L. monocytogenes*-specific antibodies. Whereas we applied a complement fixation assay and ELISA using intact bacteria as the antigen, Gentschev et al. utilized an immunoblot to LLO and p60 for the detection of *L. monocytogenes*-specific antibody responses. Our result of low-level humoral immunity in a minority of healthy humans is analogous to the observations with mice, where sublethal doses of WT *L. monocytogenes* do not induce a significant antibody response. In contrast, robust humoral responses were observed in mice following i.v. administration of high doses of live-attenuated strains of *L. monocytogenes*. We have observed similar results with cynomolgus monkeys given high i.v. doses of vaccine strains based on *L. monocytogenes* Δ*actA* Δ*inlB* (unpublished data). Significantly, the results of our modeling studies with mice suggest that even if high-titer bacterium-specific antibodies are induced in humans, the potency of the *L. monocytogenes*-based vaccine will not be diminished.

Although much has been learned about the adaptive response to *L. monocytogenes* in mice, prior to this study little was known about the level of *L. monocytogenes*-specific cellular immunity in healthy individuals or people with known exposure to *L. monocytogenes*. We characterized *L. monocytogenes*-specific cellular immunity in healthy adults and modeled this situation in mice to determine the impact of preexisting immunity on the potency of *L. monocytogenes*-based vaccines. Over 60% of the adult subjects tested presented with a measurable level of LLO-specific IFN-γ- and IL-2-secreting T cells in PBMCs. Further analyses demonstrated that the response was almost exclusively confined to the $CD4^+$ T-cell population (unpublished data), which is strikingly similar to observations made in the mouse model of listeriosis, including diverse syngeneic mouse strains such as C57BL/6, CBA, FVB/n, and C3H/HeJ (see Fig. S2 in the supplemental material). BALB/c mice are exceptional in that the *L. monocytogenes*-specific T-cell response is largely confined to the $CD8^+$ T-cell population specific for $LLO_{91–99}$. In the mouse model, cellular immunity induced by WT *L. monocytogenes* exposure reduced the magnitude of the cellular response to the encoded antigen following immunization with a recombinant *L. monocytogenes* vac-

cine in a WT *L. monocytogenes* dose-dependent manner. Consistent with previous reports by Tvinnereim et al., the antigen-specific T-cell response was attenuated (39) but was not completely abrogated as suggested by Vijh et al. (40). The negative impact of preexisting cellular immunity was dependent on the dose and the time of previous exposure to WT *L. monocytogenes* (Fig. 4). In the comparative studies shown here, the impact of vector-specific immunity on vaccine potency was substantially greater for a recombinant VV than for a recombinant *L. monocytogenes* vaccine. Repeated immunization with *L. monocytogenes* (with both the same vaccine and dose) could overcome the limitation of preexisting immunity, demonstrating that sufficient numbers of naïve T cells were primed. Significantly, in the presence of vector-specific immunity, recombinant *L. monocytogenes* vaccines provided therapeutic efficacy in the CT26 colorectal tumor model in the presence of vector-specific immunity. However, the impact of existing *L. monocytogenes* immunity on the immune responses induced in humans is unknown.

The impact of preexisting *L. monocytogenes*-specific cellular immunity on the potency of *L. monocytogenes*-based vaccines likely depends on both the genetic attenuation of the vaccine strain and the target antigen. The attenuated *L. monocytogenes* Δ*actA* Δ*inlB* strain used in this study is rapidly cleared following limited expansion in vivo, in contrast to vaccine strains based on WT *L. monocytogenes*, which undergo extensive in vivo proliferation. The higher input dose of antigen due to higher vaccine doses with attenuated strains may allow more efficient priming of naïve T cells in the presence of *L. monocytogenes*-specific immunity, resulting in a diminished effect of preexisting immunity.

The results of modeling studies conducted here strongly suggest that unlike recombinant viruses, either preexisting or induced bacterium-specific antibodies will not affect the potency of recombinant *L. monocytogenes* in humans. However, consistent with the mouse model of listeriosis, cellular immunity clearly can affect vaccine potency. Our survey of human donors indicated that a majority (60%) had a low level of cellular immunity against the tested *L. monocytogenes* protein, LLO, and our modeling studies demonstrated that when it was sufficient, cellular immunity diminished vaccine potency. The fact that boosting could overcome inhibition is encouraging but also underlines the importance of monitoring the level of *L. monocytogenes*-specific cellular immunity during clinical trials to be able to draw possible correlations between vaccine efficacy and vector-specific immunity (either preexisting or induced upon repeated dosing). Additionally, the kinetics of vaccine-induced *L. monocytogenes*-specific immunity may be important in guiding the clinical development of an efficacious immunization regimen. Significantly, however, the results reported here support the continued evaluation of recombinant *L. monocytogenes* vaccines in humans without the presence of *L. monocytogenes*-specific immunity being a criterion for exclusion from trial enrollment and ongoing immunization regimens utilizing repeated dosing.

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