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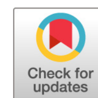

Listeria-Vectored Multiantigenic Tuberculosis Vaccine Enhances Protective Immunity against Aerosol Challenge with Virulent *Mycobacterium tuberculosis* in BCG-Immunized C57BL/6 and BALB/c Mice

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ABSTRACT *Mycobacterium tuberculosis* infects approximately one-third of the world's population, causing active tuberculosis (TB) in ~10 million people and death in ~1.5 million people annually. A potent vaccine is needed to boost the level of immunity conferred by the current *Mycobacterium bovis* BCG vaccine that provides moderate protection against childhood TB but variable protection against adult pulmonary TB. Previously, we developed a recombinant attenuated *Listeria monocytogenes* (rLm)-vectored *M. tuberculosis* vaccine expressing the *M. tuberculosis* 30-kDa major secretory protein (r30/Ag85B), recombinant attenuated *L. monocytogenes* Δ*actA* Δ*inlB prfA**30 (rLm30), and showed that boosting BCG-primed mice and guinea pigs with rLm30 enhances immunoprotection against challenge with aerosolized *M. tuberculosis* Erdman strain. To broaden the antigen repertoire and robustness of rLm30, we constructed 16 recombinant attenuated *L. monocytogenes* vaccine candidates expressing 3, 4, or 5 among 15 selected *M. tuberculosis* antigens, verified their protein expression, genetic stability, and growth kinetics in macrophages, and evaluated them for capacity to boost protective efficacy in BCG-primed mice. We found that boosting BCG-primed C57BL/6 and BALB/c mice with recombinant attenuated *L. monocytogenes* multiantigenic *M. tuberculosis* vaccines, especially the rLm5Ag(30) vaccine expressing a fusion protein of 23.5/Mpt64, TB10.4/EsxH, ESAT6/EsxA, CFP10/EsxB, and r30, enhances BCG-induced protective immunity against *M. tuberculosis* aerosol challenge. In immunogenicity studies, rLm5Ag(30) strongly boosts *M. tuberculosis* antigen-specific CD4-positive ($CD4^+$) and $CD8^+$ T cell-mediated TH1-type immune responses in the spleens and lungs of BCG-primed C57BL/6 mice but does so only weakly in BCG-primed BALB/c mice. Hence, rLm5Ag(30) boosts BCG-primed immunoprotection against *M. tuberculosis* aerosol challenge in both C57BL/6 and BALB/c mice despite major differences in the magnitude of the vaccine-induced Th1 response in these mouse strains. Given the consistency with which recombinant attenuated *L. monocytogenes* vaccines expressing the 5 *M. tuberculosis* antigens in rLm5Ag(30) are able to boost the already high level of protection conferred by BCG alone in two rigorous mouse models of pulmonary TB and the broad $CD4^+$ and $CD8^+$ T cell immunity induced by rLm5Ag(30), this vaccine holds considerable promise as a new vaccine to combat the TB pandemic, especially for the majority of the world's population immunized with BCG in infancy.

IMPORTANCE TB, one of the world's most important infectious diseases, afflicts approximately 10 million people and kills approximately 1.5 million people annually. The current vaccine, BCG, developed over a century ago, has been administered to about 5 billion people, mostly in infancy, but is only modestly protective. Hence, a vaccine is urgently needed to boost the level of protection afforded by BCG. Herein, we describe a safe potent live vaccine that utilizes as a vector an attenuated strain of *Listeria*

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monocytogenes, a bacterium that mimics the intracellular lifestyle of *Mycobacterium tuberculosis*, the causative agent of TB. The vaccine produces multiple immunologically protective proteins of *M. tuberculosis*. In two mouse models of pulmonary TB, the vaccine boosts the level of protection afforded by BCG. Thus, this vaccine holds considerable promise as a new vaccine to combat the TB pandemic, especially for the majority of the world's population immunized with BCG.

KEYWORDS *Listeria monocytogenes*, *Listeria* vector, *Mycobacterium tuberculosis*, live vector vaccines, tuberculosis, tuberculosis vaccines

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a deadly global disease. It is estimated that one-quarter of the world's population has been infected with *M. tuberculosis*, most of whom develop latent TB infection, and that 10 million people develop active TB and 1.5 million people die of TB annually. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), developed more than 100 years ago and the only licensed vaccine against TB, has been used to vaccinate infants and to protect young children against severe forms of TB; however, BCG has shown variable efficacy in preventing pulmonary TB in adolescents and adults, the most prevalent form (1). As BCG has been widely used worldwide to vaccinate 88% of infants within the first year of their life (1, 2), booster vaccines that improve upon the efficacy of BCG, even to a small extent, could have a significant impact on the TB pandemic.

Several strategies have been employed to develop replacement and booster vaccines for BCG against TB. BCG replacement vaccines include recombinant BCG (rBCG30) overexpressing the *M. tuberculosis* 30-kDa major secretory protein (r30/Ag85B) (3, 4), an iron-limited version of rBCG30 [rBCG(*mbtB*)30] with an inability to acquire iron but which can be preloaded with iron-siderophore and thereby multiply for several generations in the host (5), a recombinant BCG (BCG::Δ*ureC hly*$^+$) engineered to acidify the phagosome and secrete listeriolysin (6), a live genetically attenuated *M. tuberculosis* vaccine (MTBVAC) (7); and killed whole-cell *Mycobacterium* vaccines, among others (8). BCG booster vaccines include primarily protein/adjuvant vaccines comprising fusion proteins of selected *M. tuberculosis* antigens administered with a strong T cell-stimulating adjuvant and viral-vectored vaccines, wherein viruses, including adenovirus, modified vaccinia Ankara virus, and cytomegalovirus, express recombinant proteins (9–15). Recent developments include substantial protection against *M. tuberculosis* challenge in rhesus macaques vaccinated intravenously with BCG (16), 50% protection against progression to pulmonary TB in humans vaccinated intramuscularly with a subunit vaccine (M72/ASO1$_E$) (11), and 45% prevention of *M. tuberculosis* infection (defined as sustained conversion by QuantiFERON-TB Gold In-Tube assay) in adolescents revaccinated intradermally with BCG (12).

Previously, in our design of a novel TB booster vaccine, we employed a highly attenuated replicating bacterium as a vaccine vector, *Lm* Δ*actA* Δ*inlB prfA**, a *Listeria monocytogenes* with deletions in two major virulence genes (*actA* and *inlB*) and a single amino acid substitution (G155S) in PrfA (positive regulatory factor A) resulting in constitutive overexpression of PrfA and PrfA-dependent genes, a modification exploited to enhanced vaccine efficacy (17). *Listeria monocytogenes* is an intracellular bacterium that invades mononuclear phagocytes, resides in a membrane-bound phagosome, and, ultimately, escapes the phagosome to reside and multiply in the cytoplasm (18). Its intraphagosomal and intracytoplasmic locations favor antigen presentation via both MHC class I and II, respectively, allowing induction of both CD4-positive (CD4$^+$) and CD8$^+$ antigen-specific T cells, both important to immunity against TB. A *Listeria* vector also has other immunologic advantages, including the capacity to carry and express a large amount of recombinant protein cargo; the ability to disseminate to organs that are impacted by *M. tuberculosis*, such as the lung and spleen, before being cleared by the immune system, thereby promoting local immunity at sites of *M. tuberculosis* infection; and the fact that preexisting immunity does not negatively affect efficacy (19, 20). Additional practical advantages of a *Listeria*-vectored vaccine are an established safety

TABLE 1 recombinant attenuated *L. monocytogenes* vaccine candidates

Vaccine	Antigen expression cassette	Estimated pI/MW[a] (Da)	Integration locus
rLm23.5	ActAN-Mpt64(Δ1V-23A)	4.54/30,560	$tRNA^{arg}$
rLm3Ag	ActAN-Ag85B(Δ2Q-43A)-TB10.4-ESAT6	4.65/59,691	$tRNA^{arg}$
rLm4Ag	ActAN-Mpt64(Δ1V-23A)-TB10.4-ESAT6-CFP10	4.57/62,683	$tRNA^{arg}$

[a]pI, isoelectric point; MW, molecular weight.

profile (21), as the vector has been used safely in cancer vaccines, and low cost of manufacture in simple broth culture without the need for extensive purification as in the case of protein/adjuvant and viral-vectored vaccines.

In a previous study, we developed a recombinant *Listeria monocytogenes* Δ*actA* Δ*inlB prfA**-vectored vaccine candidate (rLm30) expressing the *M. tuberculosis* 30-kDa major secretory protein (r30/Ag85B/Rv1886) driven by the *hly* promoter and leader sequence or the *actA* promoter and leader sequence to facilitate the expression and secretion of r30 by recombinant attenuated *L. monocytogenes*. We found that rLm30 significantly enhances BCG-primed protective efficacy against aerosol challenge with the virulent *M. tuberculosis* Erdman strain in mice and guinea pigs (22). Boosting BCG-primed C57BL/6 mice with rLm30 induces strong antigen-specific T cell-mediated immune responses, including greater frequencies of antigen-specific polyfunctional $CD4^+$ and $CD8^+$ T cells expressing interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and/or interleukin 2 (IL-2) in the spleens and lungs.

To expand the *M. tuberculosis* antigen repertoire of this recombinant attenuated *L. monocytogenes* vaccine, we evaluated 14 proteins in addition to r30 for potential inclusion in a multiantigenic vaccine. We constructed a total of 16 new recombinant attenuated *L. monocytogenes* multiantigenic vaccine candidates, including 11 vaccines expressing a fusion protein of 5 *M. tuberculosis* antigens, and evaluated them for efficacy against *M. tuberculosis* aerosol challenge in C57BL/6 and/or BALB/c mice. We identified rLm5Ag(30), expressing the fusion protein 23.5(Mpt64)-TB10.4(EsxH)-ESAT6 (EsxA)-CFP10(EsxB)-r30(Ag85B), as one of the most promising vaccine candidates. We then studied the immunogenicity of rLm5Ag(30) in BCG-primed C57BL/6 and BALB/c mice. We found that while the recombinant attenuated *L. monocytogenes*-vectored multiantigenic vaccines boost BCG-primed protection in both C57BL/6 and BALB/c mice, the rLm5Ag(30) vaccine induces strong T cell-mediated immune responses, evidenced by enhanced antigen-specific frequencies of splenic and lung $CD4^+$ and $CD8^+$ T cells expressing IFN-γ, TNF-α, and/or IL-2, in BCG-primed C57BL/6 mice but not in BALB/c mice, where such responses are markedly limited. Thus, while the multiantigenic recombinant attenuated *L. monocytogenes* vaccines enhance protective immunity in both BCG-immunized mouse strains, they do so via disparate immune responses.

RESULTS

Construction and verification of new recombinant attenuated *L. monocytogenes* vaccines expressing 1, 3, or 4 recombinant *M. tuberculosis* proteins. Previously we have shown that rLm30, expressing the r30/Ag85B downstream of the Lm *actA* promoter and ligated to the N-terminal 100 amino acids of ActA (ActAN) as a fusion protein, boosts BCG-primed efficacy against TB (22). To expand the *M. tuberculosis* antigen repertoire of the recombinant attenuated *L. monocytogenes* vaccine platform, we initially constructed 3 new recombinant attenuated *L. monocytogenes* vaccine candidates, rLm23.5, expressing the mature peptide of 23.5/Mpt64(Δ1V-23A); rLm3Ag, expressing the fusion protein of Ag85B(Δ2Q-43A)-RP-TB10.4-GGSG-ESAT6 (RP; a dipeptide encoded by EagI restriction enzyme site [*CGGCCG*] for cloning purposes; GGSG, a flexible fusion protein linker); and rLm4Ag, expressing the fusion protein of Mpt64(Δ1V-23A)-RP-TB10.4-GGSG-ESAT6-GSSGGSSG-CFP10 (GSSGGSSG, a flexible linker) (Table 1). The *M. tuberculosis* antigens in each vaccine construct were expressed as a C-terminal

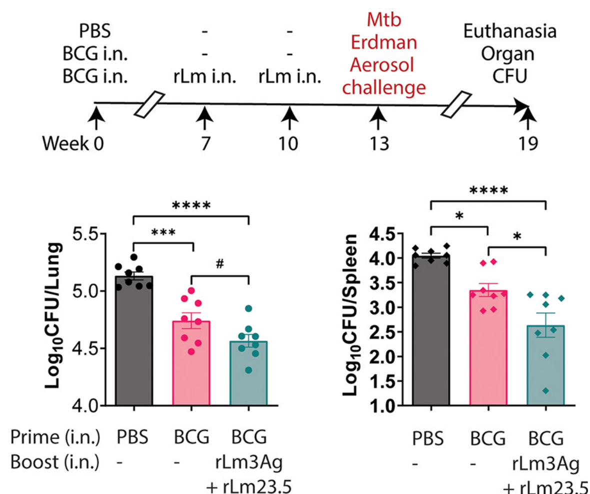


FIG 1 Efficacy against *M. tuberculosis* aerosol challenge of boosting BCG-primed mice with a combination of recombinant attenuated *L. monocytogenes* vaccines expressing 4 *M. tuberculosis* antigens. Female C57BL/6 mice (n = 8/group) were immunized intranasally (i.n.) with PBS (sham) or BCG at week 0; not boosted or boosted i.n. twice with 10^6 CFU total of combined recombinant attenuated *L. monocytogenes* expressing 4 *M. tuberculosis* proteins, rLm3Ag (expressing Ag85B, TB10.4, and ESAT6) and rLm23.5 at weeks 7 and 10; challenged with aerosolized *M. tuberculosis* at week 13; and euthanized at week 19 (top). Lungs (bottom left) and spleens (bottom right) of mice were assayed for organ bacterial burden. One symbol represents one animal. Values are mean $\log_{10}$ CFU ± SEM. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$ by one-way ANOVA with Tukey's multiple-comparison test (Prism v9.2.0); #, $P < 0.05$, by one-way ANOVA and Fisher's LSD criteria (Prism v9.2.0).

fusion protein to Lm ActAN; the *M. tuberculosis* protein expression cassette was driven by the Lm *actA* promoter and integrated at the *tRNA^arg^* locus of the *Lm* Δ*actA* Δ*inlB prfA** chromosome.

We verified the expression of the heterologous protein ActAN-Mpt64 by rLm23.5 as a 31-kDa protein band detected by a polyclonal antibody to a peptide comprising 18 amino acids (A30-K47) of ActAN (AK18) (courtesy of J. Skoble and P. Lauer) (see Fig. S1a in the supplemental material), expression of ActAN-Ag85B-TB10.4-ESAT6 by rLm3Ag as a 59-kDa protein band detected by a rabbit polyclonal antibody to r30 (Fig. S1b, top) or a polyclonal antibody to TB10.4 (Fig. S1b, bottom), and expression of ActAN-Mpt64-TB10.4-ESAT6-CFP10 by rLm4Ag as a 63-kDa protein band detected by AK18 (Fig. S1c).

Boosting BCG-primed C57BL/6 mice with the combined rLm3Ag and rLm23.5 vaccines enhances protection against aerosolized *M. tuberculosis*. To determine the efficacy of these multiantigenic vaccine candidates as a booster vaccine in protecting BCG-immunized mice against aerosolized *M. tuberculosis* challenge, we immunized C57BL/6 mice, 8/group, intranasally (i.n.) with phosphate-buffered saline (PBS) (sham) or BCG at week 0 and boosted one group of BCG-immunized mice i.n. with a combination of recombinant attenuated *L. monocytogenes* vaccines expressing 4 *M. tuberculosis* antigens, rLm3Ag (expressing Ag85B-10.4-ESAT6) plus rLm23.5 at weeks 7 and 10. The mice were then challenged with aerosolized *M. tuberculosis* Erdman (2.6×10^5 CFU for 30 min, resulting in an average of 21 CFU in the lungs at day 1 postchallenge) at week 13, euthanized at week 19, and their lungs and spleens assayed for *M. tuberculosis* CFU. As shown in Fig. 1, mice primed-boosted with BCG with rLm3Ag and rLm23.5 had a significantly lower bacterial burden in their lungs and spleens than sham-immunized mice and mice immunized with BCG. This result indicates that boosting BCG-primed C57BL/6 mice with an recombinant attenuated *L. monocytogenes* multiantigenic vaccine enhances immunoprotection against *M. tuberculosis* aerosol challenge.

Boosting BCG-primed BALB/c mice with the combined rLm4Ag and rLm30 vaccines enhances protection against aerosolized *M. tuberculosis*. To further verify the immunoprotection against *M. tuberculosis* challenge of multiantigenic recombinant attenuated *L. monocytogenes* vaccine candidates as a booster vaccine in BCG-immunized

mice, we immunized and challenged a different strain of mice, BALB/c mice. We immunized BALB/c mice, 8/group, intradermally (i.d.) with PBS (sham) or BCG at week 0 and did not boost or boosted BCG-immunized mice intramuscularly (i.m.) with *L. monocytogenes* vector or with a combination of recombinant attenuated *L. monocytogenes* vaccines now expressing 5 *M. tuberculosis* antigens—rLm4Ag (expressing Mpt64-TB10.4-ESAT6-CFP10) with rLm30 (the combined vaccines are abbreviated as rLm5Ag*)—at weeks 14 and 18. The mice were then challenged with aerosolized *M. tuberculosis* Erdman (2.6×10^5 CFU for 30 min, resulting in an average of 19 CFU in the lungs at day 1 postchallenge) at week 22, euthanized at week 32, and their lungs and spleens assayed for *M. tuberculosis* CFU (Fig. 2a, top). As shown in Fig. 2a, bottom panels, immunizing with BCG alone was highly effective, reducing CFU in the lung band spleen by 1.1- and 2.2-$\log_{10}$ CFU, respectively, compared with sham immunization. Mice primed-boosted with BCG i.d.-rLm5Ag* i.m. once or twice had significantly lower $\log_{10}$ CFU in their lungs ($P < 0.0001$) and spleens ($P < 0.0001$) than the sham-immunized mice; in mice boosted twice, CFU in the lungs were 1.75-$\log_{10}$ CFU lower and, in the spleens, 2.5 $\log_{10}$ lower than in sham-immunized animals, thus attaining a $\geq$1.75-$\log_{10}$ CFU threshold of protection achieved by only a small minority of TB booster vaccines administered to BCG-immunized animals (23). Of note, mice primed with BCG i.d. and boosted with rLm5Ag* i.m. twice had significantly lower CFU in their lungs than mice immunized i.d. with BCG alone ($P < 0.05$) or primed with BCG and immunized with *L. monocytogenes* vector ($P < 0.05$) despite the high level of protection conferred by BCG alone. These mice also had significantly fewer CFU in their spleen than BCG-immunized mice boosted with the *L. monocytogenes* vector ($P < 0.05$) despite the high level of protection (1.5-$\log_{10}$ CFU) conferred by the BCG-*L. monocytogenes* vector prime-boost. These results verify in BALB/c mice that boosting BCG-primed mice with an recombinant attenuated *L. monocytogenes* multiantigenic vaccine enhances immunoprotection against *M. tuberculosis* aerosol challenge.

In a companion experiment challenged at the same time as the experiment described in Fig. 2a, we compared different delivery routes for the recombinant attenuated *L. monocytogenes* multiantigenic booster vaccines. We immunized BALB/c mice, 8/group, i.d. with 5×10^5 CFU of BCG at week 0 and boosted them once at week 18 with rLm5Ag* (a combination of recombinant attenuated *L. monocytogenes* Mpt64-TB10.4-ESAT6-CFP10 and rLm30) via the i.d., intravenous (i.v.), subcutaneous (s.c.), or intramuscular (i.m.) route. Mice immunized i.d. with PBS (Sham) or BCG at week 0 and not boosted served as controls. At week 22, we challenged the mice with aerosolized *M. tuberculosis* Erdman (as described in the companion experiment above). At week 26, we euthanized the mice and assayed bacillus burdens in their lungs and spleens (Fig. 2b, top).

As shown in Fig. 2b, bottom panels, immunizing with BCG i.d. alone was highly effective in comparison with sham immunization, reducing CFU by 1.3 $\log_{10}$ in the lung and 1.8 $\log_{10}$ in the spleen. Despite the especially high efficacy of BCG alone, BCG prime recombinant attenuated *L. monocytogenes* boosting further reduced CFU in the lungs and spleens after aerosol challenge with *M. tuberculosis*; the differences in CFU between boosted and nonboosted mice were statistically significant in the lungs for boosting by the s.c. ($P < 0.05$) or i.v. route ($P < 0.05$) and in the spleens for boosting via the s.c. route ($P < 0.05$), i.d. route ($P < 0.05$), or i.v. route ($P < 0.001$). Of note, boosting BCG-primed mice via the i.d., s.c., or i.v. routes reduced CFU compared with sham-immunized mice by 1.6- to 1.7-$\log_{10}$ CFU in the lungs and 2.4- to 2.8-$\log_{10}$ CFU in the spleen.

Among the 4 boosting routes tested, the effectiveness in reducing lung CFU by route was s.c. > i.v. > i.d. > i.m., lowering $\log_{10}$ CFU by 0.43, 0.37, 0.29, and 0.13 log, respectively, versus BCG alone, and the effectiveness in reducing spleen CFU by route was i.v. > s.c. > i.d. > i.m., lowering $\log_{10}$ CFU by 1.07, 0.63, 0.61, and 0.18 log, respectively, versus BCG alone. Thus, the most effective routes were s.c. and i.v., and s.c. was the superior route in the lung. Surprisingly, the i.m. route was the least efficacious in both the lung and spleen. However, in the companion experiment described above (Fig. 2a), boosting i.m. twice with rLm5Ag* (rLm4Ag with rLm30) provided improved protection in the lung ($P < 0.05$) and spleen compared

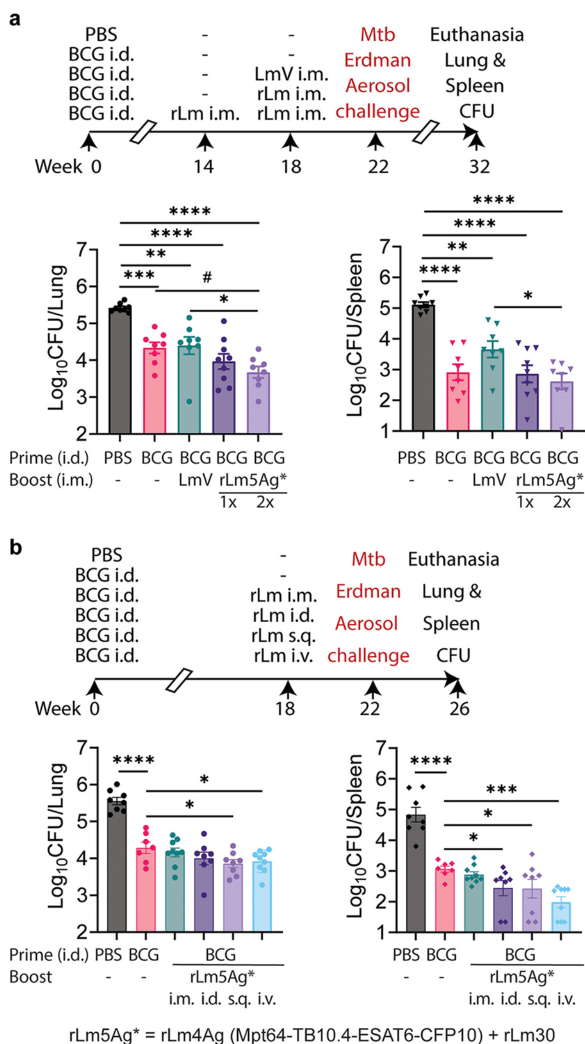


FIG 2 Efficacy against *M. tuberculosis* aerosol challenge of boosting BCG-primed mice with a combination of recombinant attenuated *L. monocytogenes* vaccines expressing 5 *M. tuberculosis* antigens. (a) Efficacy of boosting with 5Ag recombinant attenuated *L. monocytogenes* vaccines i.m. once versus twice. BALB/c mice (n = 8/group) were immunized i.d. with PBS or with BCG at week 0. BCG-primed mice were either not boosted or boosted intramuscularly (i.m.) or subcutaneously (s.c.) once (×1) at week 18 with *L. monocytogenes* vector (LmV) or the combination of rLm4Ag (expressing *M. tuberculosis* fusion protein Mpt64-TB10.4-ESAT6-CFP10) and rLm30. An additional group was boosted twice (×2) at weeks 14 and 18 with rLm4Ag plus rLm30. The mice were then challenged with aerosolized *M. tuberculosis* Erdman (average of 19 CFU delivered to the lungs of each animal, as assayed at day 1 postchallenge to 2 mice) at week 22 and euthanized at week 32 (top). Lungs (bottom left) and spleens (bottom right) of mice were assayed for organ bacterial burden. Shown are means ± SEM. One symbol represents one animal. Organ $\log_{10}$ CFU were analyzed by one-way ANOVA. *, $P < 0.05$; ****, $P < 0.0001$ by Tukey's multiple-comparison test; #, $P < 0.05$ by Fisher's LSD test (Prism 9.2.0). (b) Efficacy of 5Ag recombinant attenuated *L. monocytogenes* vaccines by route of administration. BALB/c mice (8/group) were immunized i.d. with PBS (sham) or 5×10^5 CFU BCG at week 0. Mice immunized i.d. with BCG were either not boosted or boosted i.m., i.d., subcutaneously (s.c.), or intravenously (i.v.) once at week 18 with 2×10^6 CFU of the combination of rLm4Ag (expressing Mpt64-TB10.4-ESAT6-CFP10) and rLm30. The mice were then challenged at week 22 with aerosolized *M. tuberculosis* Erdman at the same time as the companion experiment shown in panel a (average of 19 CFU delivered to the lungs of each animal) and euthanized at week 26 (top). Afterward, lungs (bottom left) and spleens (bottom right) were removed and assayed for bacillus burdens. Shown are means ± SEM. Each symbol represents one mouse. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$ by one-way ANOVA with Fisher's LSD test (Prism 9.2.0).

with boosting once. Given the safety and practical advantage of the s.c. route of administration and the fact that the s.c. route was superior to other routes in the lung, the major site of *M. tuberculosis* pathology, we selected the s.c. route for future studies.

TABLE 2 Fifteen *M. tuberculosis* proteins selected as vaccine candidates

Gene Rv no.	Product (62)	Length (aa)	Protection in animal models[a] (reference[s])	Absence in BCG	Homolog in BCG[b,c]
0288	TB10.4/EsxH/CFP-7, ESAT6 family	96	31	No	0328
1174c	TB8.4, low-molecular-wt T-cell antigen	110	27	No	1237c
1793	EsxN, putative ESAT-6-like protein	94		No	1825
1860	Apa, alanine and proline rich secreted glycoprotein	325	28	No	1896
1886c	r30/Ag85B/FbpB, 30-kDa major secreted protein	325	4, 9, 41	No	1923c
1980c	23.5/antigen Mpt64	228	25, 26	Yes	
2031	HspX, heat shock protein HspX (α-crystallin homolog)	144	35, 36	No	2050c
2431c	PE25, Esx-5, secreted with PPE41 and EspG5	99		No	2450c
2626c	HrpI, hypoxic response protein 1	143	37	No	2653c
3407	Antitoxin VapB47, part of the toxin-antitoxin operon with Rv3408	99	37, 38	No	3477
3615c	EspC, ESX-1 secretion-associated protein C	103	24	No	3679c
3616c	EspA, ESX-1 secretion-associated protein A	392	24	No	3680c
3873	PPE68, interacts with ESAT6, CFP10, and TB10.4	368	24	Yes	
3874	CFP-10/EsxB, cotranscribed with Rv3875	100	30	Yes	
3875	ESAT-6/EsxA, early secretory antigen target	95	24, 29	Yes	

[a]As an individual protein.
[b]Blasted using nucleotide BLAST tool via https://blast.ncbi.nlm.nih.gov/Blast.cgi.
[c]*M. bovis* BCG reference strain, Pasteur 1173P2, GenBank accession number AM408590.1.

Selection of *M. tuberculosis* antigens and construction of 13 new rLm5Ag vaccine candidates. To further expand the *M. tuberculosis* antigen repertoire, we selected 15 *M. tuberculosis* proteins (including r30) as potential vaccine candidates for further investigation (Table 2), including (i) secreted proteins r30 (4), Mpt64 (24–26), TB8.4 (27), and Apa (28); (ii) ESAT6 and associated proteins secreted by the Esx/type VII secretion system, ESAT6 (24, 29), CFP10 (30), TB10.4 (31, 32), EspA (24), EspC (24), and EsxN; (iii) antigenic PE/PPE proteins PE25 (33) and PPE68 (24, 34); and (iv) latency-associated proteins α-crystallin/hspX (35, 36), Hrp1 (37), and VapB47 (37, 38). Among the 15 selected proteins, all but two (EsxN and PE25) have been shown by us or others to be immunoprotective antigens when incorporated into various vaccines, including protein/adjuvant, DNA, *Listeria*-vectored, or virus-vectored vaccines, and 4 proteins, Mpt64, PPE68, CFP-10, and ESAT-6, are absent either from all BCG strains or the modern BCG strain (Mpt64) (39, 40) (Table 2).

We constructed 11 new rLm5Ag vaccine candidates carrying a single copy of an ActAN-*M. tuberculosis* 5-antigen fusion protein expression cassette downstream of the Lm *actA* promoter integrated at the 3′ end of the *tRNA^{arg}* locus in the recombinant attenuated *L. monocytogenes* chromosome; in all such cases, the first four proteins in the fusion protein were Mpt64-EsxH-EsxA-EsxB followed by a GSSGGSSG flexible linker, and the fifth protein was 1 of the 11 other selected *M. tuberculosis* proteins in Table 2. In addition, we constructed 2 recombinant attenuated *L. monocytogenes* vaccine candidates, rLm4Ag(*comK*) expressing the *M. tuberculosis* 4Ag (Mpt64-EsxH-EsxA-EsxB) fusion protein, and rLm30(*comK*) expressing Ag85B from the *comK* locus of the recombinant attenuated *L. monocytogenes* chromosome, respectively, to allow a comparison of vaccines expressing proteins at this locus versus the *tRNA^{arg}* locus and to explore the possibility of later expressing proteins from both loci in the same vaccine (Table 3).

We examined *M. tuberculosis* fusion protein expression by rLm5Ag vaccine candidates grown in broth medium. As shown in Fig. 3, one major protein band (indicated by red asterisks to the right of the protein band) at the estimated molecular weight (MW) (Table 3, 3rd column from the left) of the fusion protein and multiple minor protein bands (possible N-terminal protein breakdown products) were detected in the lysates of 10 recombinant attenuated *L. monocytogenes* vaccine candidates (lanes 4 to 12 and 14) by the rabbit Ak18 polyclonal antibody to ActAN. No specific protein band was detected from the lysate of the *L. monocytogenes* vector (LmV, lane 3) as expected. One recombinant attenuated *L. monocytogenes* vaccine candidate (lane 13), rLm5Ag (VapB47), also showed no protein expression in the lysate; however, two other clones of the same construct did express a protein of the approximately expected MW (lanes

TABLE 3 Thirteen new recombinant attenuated *L. monocytogenes* multiantigenic vaccine candidates

Vaccine	*M. tuberculosis* antigen expression cassette[a]	Estimated pI/MW (Da)[b]	Integration locus
rLm5Ag(30)	ActAN-Mpt64-EsxH-EsxA-EsxB-r30(Δ2Q-43A)	4.63/94,106	*tRNA*^{arg}
rLm5Ag(EspA)	ActAN-Mpt64-EsxH-EsxA-EsxB-EspA(Δ111F-193L)	4.69/94,889	*tRNA*^{arg}
rLm5Ag(EspC)	ActAN-Mpt64-EsxH-EsxA-EsxB-EspC	4.64/74,239	*tRNA*^{arg}
rLm5Ag(EsxN)	ActAN-Mpt64-EsxH-EsxA-EsxB-EsxN	4.59/73,386	*tRNA*^{arg}
rLM5Ag(PPE68)	ActAN-Mpt64-EsxH-EsxA-EsxB-PPE68	4.46/100,774	*tRNA*^{arg}
rLm5Ag(PE25)	ActAN-Mpt64-EsxH-EsxA-EsxB-PE25(Δ66I-73L)	4.67/73,422	*tRNA*^{arg}
rLm5Ag(Apa)	ActAN-Mpt64-EsxH-EsxA-EsxB-APA(Δ2H-39A)	4.55/92,355	*tRNA*^{arg}
rLm5Ag(HspX)	ActAN-Mpt64-EsxH-EsxA-EsxB-HspX(Δ121I-126V)	4.66/78,888	*tRNA*^{arg}
rLm5Ag(TB8.4)	ActAN-Mpt64-EsxH-EsxA-EsxB-TB8.4(Δ2R-28A)	4.55/71,912	*tRNA*^{arg}
rLm5Ag(VapB47)	ActAN-Mpt64-EsxH-EsxA-EsxB-VapB47	4.80/74,453	*tRNA*^{arg}
rLm5Ag(Hrp1)	ActAN-Mpt64-EsxH-EsxA-EsxB-Hrp1	4.65/78,962	*tRNA*^{arg}
rLm4Ag(*comK*)	ActAN-Mpt64-EsxH-EsxA-EsxB	4.57/62,683	*comK*
rLm30(*comK*)	ActAN-Ag85B(Δ2Q-43A)	4.67/38,789	*comK*

[a]Mpt64 refers to Mpt64(Δ1V-23A).
[b]The estimated M_w of each fusion protein is calculated as a secreted form without the signal peptide for ActA (1V-29A, 3.3 kDa).

17 and 18). Similar protein bands were detected by the ActA AK18 antibody in the lysates of J774A.1 cells infected with the relevant recombinant attenuated *L. monocytogenes* vaccine candidates (data not shown). We also detected the *M. tuberculosis* protein expression by rLm4Ag(*comK*) and rLm30(*comK*) grown in broth medium (data not shown). Thus, we have verified that 13 new recombinant attenuated *L. monocytogenes* vaccine candidates express a fusion protein of the expected size when grown in broth and macrophages.

We also verified the *M. tuberculosis* protein expression cassette integrated at the *tRNA*^{arg} locus by PCR and nucleotide sequencing of the resultant PCR products. As shown in Fig. S2a, amplification across the bacterial attachment site *tRNA*^{arg}*-attBB′* with primers NC16 and PL95 resulted in a 548-bp fragment in each selected clone of the 10 recombinant attenuated *L. monocytogenes* candidates (lanes 3 to 10, 12, and 14); with regard to rLm5Ag(VapB47), 2 out of 3 clones selected tested positive (lanes 13, 15, and 16). Amplification with primers 319 and 327 across the antigen expression cassette resulted in various sizes of the PCR product, as shown in Fig. S2b. Consistent with the

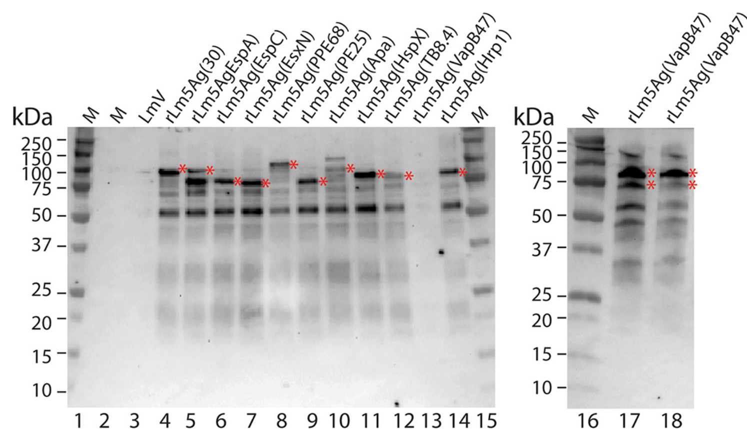


FIG 3 Expression of fusion proteins comprising 5 *M. tuberculosis* antigens by 11 new recombinant attenuated *L. monocytogenes* vaccine candidates grown in broth. Glycerol stocks of the *L. monocytogenes* vector and each of 11 new recombinant attenuated *L. monocytogenes* vaccine candidates expressing 5 *M. tuberculosis* antigens were grown in brain heart infusion medium supplemented with streptomycin (200 μg/mL) overnight at 37°C. Cells were collected by centrifugation and lysed in SDS buffer, and the cell lysate was processed by standard SDS-PAGE and Western blotting using a polyclonal antibody to ActA AK18. In both panels, the expected major protein bands are indicated by red asterisks to the right of each protein band. The MWs of the protein standards are labeled on the left of each panel. The *M. tuberculosis* fusion protein expression cassette and the estimated MW of the fusion protein for each of the vaccines listed at the top of the gels are described in Table 3. Note that lane 13 shows one clone, and lanes 17 and 18 show two different clones of the same construct, expressing the secreted (74-kDa) and nonsecreted (78-kDa) fusion proteins.

PCR result using primers NC16 and PL95, an ~2,154-bp DNA fragment was amplified with primers 319 and 327 from 2 out of 3 selected clones of rLm5Ag(VapB47) (Fig. S2b, lanes 13, 17, and 18).

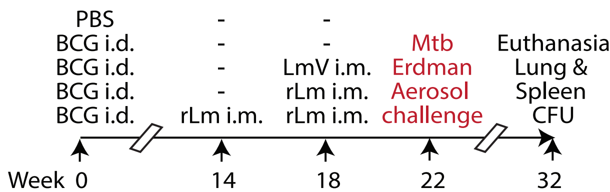
We tested the genetic stability of the pPL2e-vectored *M. tuberculosis* 5Ag expression cassette integrated at the *tRNA*^{arg} locus of the recombinant attenuated *L. monocytogenes* chromosome by culturing the vaccine candidates in the presence and absence of erythromycin, a marker used for selection of the recombinant attenuated *L. monocytogenes* constructs. We observed that the *M. tuberculosis* 5Ag antigen expression cassettes were stable after passage *in vitro* in the absence of antibiotic selection, except for one clone of the rLm5Ag(VapB47) expressing Rv3407 as the fifth protein (Fig. S3). Rv3407 encodes an antitoxin virulence-associated protein, B47, that is part of the toxin-antitoxin operon with Rv3408. Expressing Rv3407 independently of Rv3408 may have resulted in instability of this recombinant attenuated *L. monocytogenes* fusion protein. However, when we grew the vaccine candidates carrying the antigen expression cassette, including Rv3407 in the presence of antibiotic selection, these vaccines expressed the *M. tuberculosis* fusion proteins abundantly with two major bands of ~74 (secreted form) and ~78 kDa (nonsecreted form) (Fig. 3, lanes 17 and 18).

Growth kinetics of rLm5Ag vaccine candidates in broth medium and in infected murine and human macrophages. We examined the growth kinetics in brain heart infusion (BHI) broth of the 11 new rLm5Ag vaccine candidates. As shown in Fig. S4a to c, all of the rLm5Ag vaccine candidates, except rLm5Ag(VapB47) (this clone was subsequently discarded and replaced by a new clone shown in Fig. 3, lane 17), grew similarly in broth to the *L. monocytogenes* vector. rLm4Ag(comK) and rLm30(comK) also grew similarly to the *L. monocytogenes* vector in broth medium (data not shown).

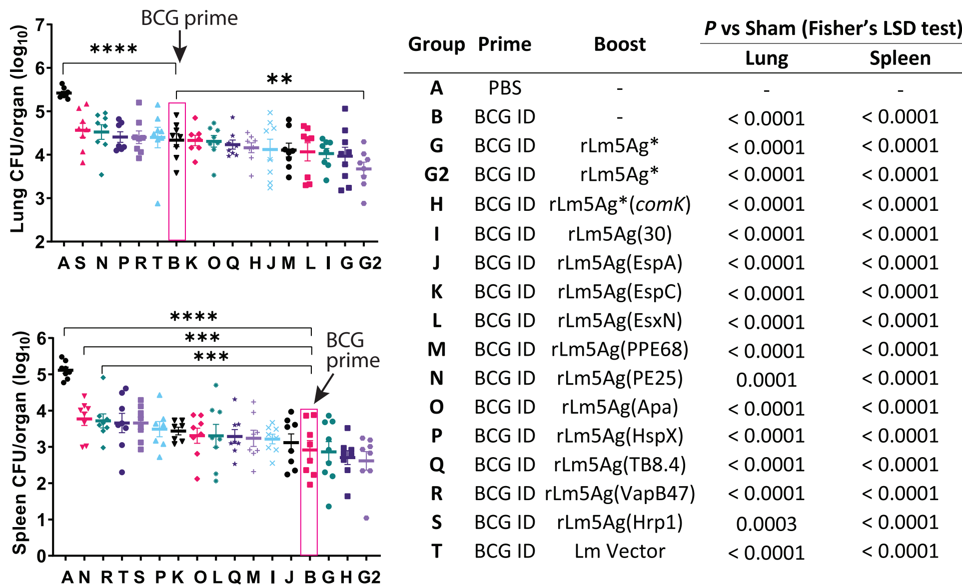
To examine the growth kinetics of rLm5Ag vaccine candidates in macrophage-like cells, we infected monolayers of murine J774A.1 cells or monolayers of human THP-1 cells differentiated by phorbol 12-myristate 13-acetate (PMA) with the *L. monocytogenes* vector or with the recombinant attenuated *L. monocytogenes* vaccine candidates at a multiplicity of infection (MOI) of 10. In general, rLm5Ag vaccine candidates expressing fusion proteins comprising *M. tuberculosis* 4Ag (Mpt64-TB10.4-ESAT6-CFP10) ligated with a 5th antigen grew similarly to the *L. monocytogenes* vector in both murine (Fig. S4d to f) and human (Fig. S4g to i) macrophage-like cells.

Protective immunity of rLm5Ag vaccine candidates against aerosol challenge with virulent *M. tuberculosis* Erdman strain in BALB/c mice. To screen for optimal *M. tuberculosis* antigens, we examined the protective efficacy against aerosolized *M. tuberculosis* by priming mice i.d. with BCG and boosting them i.m. (this experiment was initiated prior to our obtaining results of the experiment described above that determined that the optimal route was s.c.) with the 11 new rLm5Ag vaccine candidates, each expressing the *M. tuberculosis* 4Ag fusion protein (Mpt64-TB10.4-ESAT6-CFP10) ligated at its C terminus with a new 5th antigen. We immunized BALB/c mice, 8 per group, i.d. with PBS (sham) or i.d. with 5×10^5 CFU of BCG at week 0 and boosted them i.m. once at week 18 with 2×10^6 CFU each of the *L. monocytogenes* vector, 11 rLm5Ag candidates, or with 1 of 2 recombinant attenuated *L. monocytogenes* vaccine combinations expressing the same 5 *M. tuberculosis* antigens as rLm5Ag(30). At week 22, we challenged the mice with aerosolized *M. tuberculosis* (average of 19 CFU delivered to the lungs of each animal). At week 32 (10 weeks postchallenge), we euthanized the mice and assayed bacillus burdens in their lungs and spleens (Fig. 4a). Among the groups tested (Fig. 4b, right), group G (rLm4Ag with rLm30) served as a control for group I [rLm5Ag(30)] for comparison of the combination of two recombinant attenuated *L. monocytogenes* vaccines (rLm4Ag plus rLm30) with a single vaccine, rLm5Ag(30), expressing *M. tuberculosis* 4Ag fused with r30; group G2 served as a control for group G for comparison of 1 (week 18) versus 2 (week 14 and week 18) boosts with this same combination of recombinant attenuated *L. monocytogenes* vaccines; group H served as a control for group G for comparison of *M. tuberculosis* antigens expressed from the *comK* locus [rLm4Ag(*comK*) plus rLm30(*comK*)] versus the *tRNA*^{arg} locus (rLm4Ag plus rLm30) in the Lm chromosome. As shown in Fig. 4b, BCG immunization alone gave a

a. Experiment schedule



b. Organ bacterial burden



Group	Prime	Boost	*P* vs Sham (Fisher's LSD test)	
			Lung	Spleen
A	PBS	-	-	-
B	BCG ID	-	< 0.0001	< 0.0001
G	BCG ID	rLm5Ag*	< 0.0001	< 0.0001
G2	BCG ID	rLm5Ag*	< 0.0001	< 0.0001
H	BCG ID	rLm5Ag*(*comK*)	< 0.0001	< 0.0001
I	BCG ID	rLm5Ag(30)	< 0.0001	< 0.0001
J	BCG ID	rLm5Ag(EspA)	< 0.0001	< 0.0001
K	BCG ID	rLm5Ag(EspC)	< 0.0001	< 0.0001
L	BCG ID	rLm5Ag(EsxN)	< 0.0001	< 0.0001
M	BCG ID	rLm5Ag(PPE68)	< 0.0001	< 0.0001
N	BCG ID	rLm5Ag(PE25)	0.0001	< 0.0001
O	BCG ID	rLm5Ag(Apa)	< 0.0001	< 0.0001
P	BCG ID	rLm5Ag(HspX)	< 0.0001	< 0.0001
Q	BCG ID	rLm5Ag(TB8.4)	< 0.0001	< 0.0001
R	BCG ID	rLm5Ag(VapB47)	< 0.0001	< 0.0001
S	BCG ID	rLm5Ag(Hrp1)	0.0003	< 0.0001
T	BCG ID	Lm Vector	< 0.0001	< 0.0001

FIG 4 Efficacy against *M. tuberculosis* aerosol challenge of priming mice with BCG and boosting them with recombinant attenuated *L. monocytogenes* expressing 5 *M. tuberculosis* antigens. (a) Experimental schedule. BALB/c mice (8/group) were immunized i.d. with PBS (sham) or 5×10^5 CFU of BCG at week 0. Mice immunized i.d. with BCG were either not boosted or boosted once at week 18 or twice at weeks 14 and 18 (group G2 only) with 2×10^6 CFU of rLm5Ag vaccine candidates. At week 22, the mice were challenged with aerosolized *M. tuberculosis* Erdman strain (average of 19 CFU delivered to the lungs of each animal, as assayed at day 1 postchallenge to mice), and at week 32, 10 weeks postchallenge, mice were euthanized. (b) Organ bacterial burden. Lungs (top) and spleens (bottom) of mice in the vaccinated and challenge groups (described in the table to the right of the graphs) were removed and assayed for bacillus burdens. Each symbol represents one mouse, and the means $\pm$ SEM are shown as bars. Group designations are listed beneath the horizontal axis. The pink-colored boxes indicate group B, BCG-primed only. The $\log_{10}$ CFU in the lungs and spleens were compared to group A (sham) and group B (BCG i.d.) by one-way ANOVA with Fisher's LSD test. The significant *P* values to group B (BCG i.d.) in the lungs and spleens are shown in the graph; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$. The significant *P* values between sham group (A) and all other groups are listed in the table to the right.

high level of protection, 1.1-$\log_{10}$ CFU in the lung ($P < 0.0001$) and 2.2-$\log_{10}$ CFU in the spleen ($P < 0.0001$) versus sham-immunized mice. Also as shown in Fig. 4b, (i) the *L. monocytogenes* vector (group T) does not boost protective immunity induced by BCG alone; (ii) among the 13 recombinant attenuated *L. monocytogenes* vaccines screened, the recombinant attenuated *L. monocytogenes* vaccine candidates expressing 4Ag plus r30 in various forms, i.e., rLm4Ag plus rLm30, rLm4Ag(*comK*) plus rLm30(*comK*), and rLm5Ag(30) in groups G, G2, H, and I, are the best booster vaccines; the lung CFU in the mice boosted with these vaccines are lower than those in mice primed i.d. with BCG only, although the difference did not reach statistical significance for a single booster immunization (however, boosting twice with rLm4Ag plus rLm30 induced immunoprotection significantly more greatly than BCG alone in the lung [$P < 0.01$]); (iii) immunity induced by *M. tuberculosis* 5Ag expressed from a single recombinant attenuated *L. monocytogenes* vaccine [rLm5Ag(30); group I] is comparable to that induced by the same *M. tuberculosis* 5Ags expressed by two recombinant attenuated *L. monocytogenes* vaccines administered together (rLm4Ag plus rLm30; group G), as

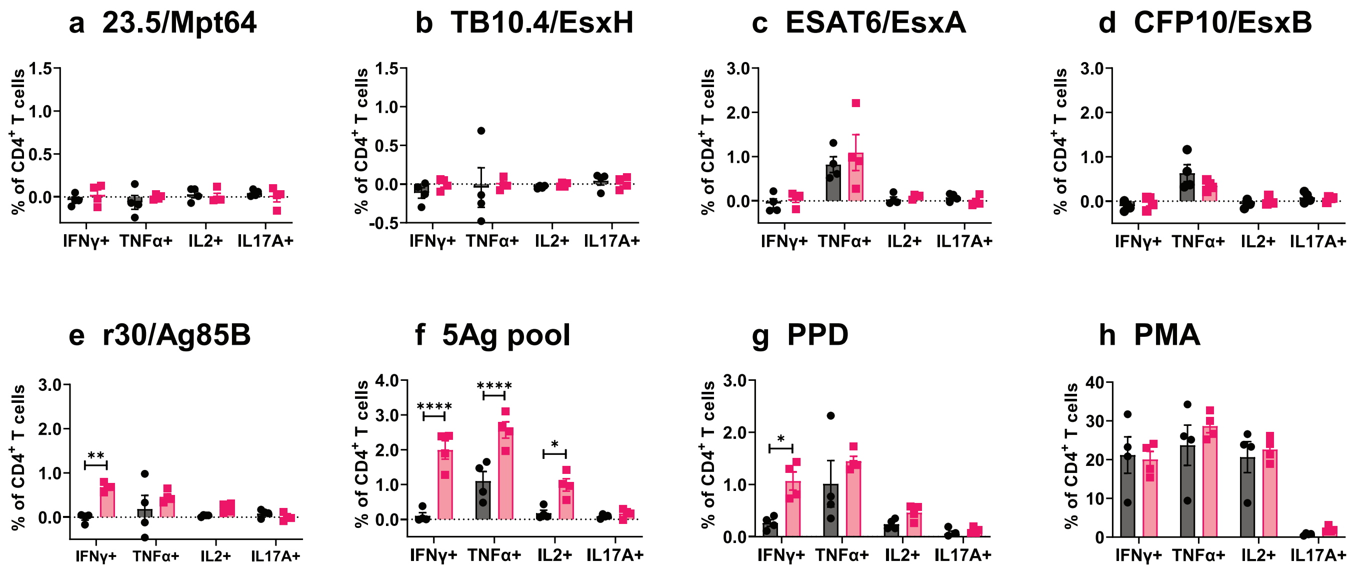
evidenced by the equivalent CFU in the lungs of mice in these two groups; (iv) immunity induced by the combination of two vaccines (rLm4Ag plus rLm30) expressing *M. tuberculosis* 5Ag from the *comK* locus (group H) is comparable to that induced by the combination of two parallel vaccines expressing *M. tuberculosis* 5Ag from the $tRNA^{arg}$ locus (group G); and (v) as noted, two boosts with rLm5Ag (rLm4Ag plus rLm30) (group G2) are more efficacious than one boost (group G), as was similarly demonstrated in the experiment shown in Fig. 2a. Of note, boosting BCG twice with rLm5Ag* reduced CFU in the lung by 1.75-$\log_{10}$ CFU ($P < 0.0001$) and in the spleen by 2.2-$\log_{10}$ CFU ($P < 0.0001$) compared with sham-immunized mice.

Overall, of the single vaccines expressing 5 *M. tuberculosis* antigens, we considered rLm5Ag(30) as the most efficacious, as it had the lowest CFU count in the lung, the major site of TB pathology, and the second-lowest CFU count in the spleen. Moreover, the 5 antigens expressed by the vaccine were shown to significantly enhance the level of protective immunity conferred by BCG alone in three independent experiments. Hence, this vaccine was evaluated further for immunogenicity.

Boosting BCG-primed mice with rLm5Ag(30) induces disparate antigen-specific CD4$^+$ and CD8$^+$ T-cell responses and serum antibody responses in C57BL/6 and BALB/c mice. To determine the immunogenicity of rLm5Ag(30) as a booster vaccine in BCG-primed mice, we primed C57BL/6 and BALB/c mice, 4/group, i.d. with 5×10^5 CFU of BCG at week 0 and boosted them s.c. twice at weeks 14 and 18 with *L. monocytogenes* vector or rLm5Ag(30). Six days after the last immunization, we anesthetized the mice, bled and euthanized them, prepared single-cell suspensions of spleen and lung cells, seeded the cells in 96-well cell culture plates, stimulated the cells with various *M. tuberculosis* antigens, and assayed T-cell immunity by intracellular cytokine staining (ICS). Immune sera were assayed for serum IgG to *M. tuberculosis* proteins and formalin-killed rLm5Ag(30) (FK-rLm5Ag).

C57BL/6 mice, but not BALB/c mice, primed-boosted with BCG and rLm5Ag(30), produced a lower frequency of CD4$^+$ T cells, greater frequency of CD8$^+$ T cells, and lower CD4$^+$/CD8$^+$ T cell ratio than mice primed-boosted with BCG-*L. monocytogenes* vector in their lungs after *in vitro* stimulation without (medium control) or with *M. tuberculosis* antigens (Fig. S5). There are no significant differences in the frequencies of CD4$^+$ and CD8$^+$ T cells in the spleens of C57BL/6 and BALB/c mice (Fig. S6). With respect to CD4$^+$ T cells in the lungs and spleens of C57BL/6 mice, as shown in Fig. 5 and 6, mice primed with BCG and boosted with rLm5Ag(30) produce significantly greater frequencies of CD4$^+$ T cells expressing intracellular cytokines IFN-γ, TNF-α, and/or IL-2 (Fig. 5) and polyfunctional CD4$^+$ T cells expressing IFN-γ and TNF-α, or IFN-γ, TNF-α, and IL-2 (Fig. 6) in response to *in vitro* stimulation with r30/Ag85B, 5Ag pool, purified protein derivative (PPD), or TB10.4/EsxH than mice immunized with the *L. monocytogenes* vector. No significant differences in the frequencies of CD4$^+$ T cells expressing any of the cytokines were detected after *in vitro* stimulation with ESAT6/EsxA, CFP10/EsxB, and 23.5/Mpt64. As expected, mice immunized with the *L. monocytogenes* vector and rLm5Ag(30) produced mostly comparable amounts of cytokines after *in vitro* stimulation with PMA (Fig. 5 and 6).

With respect to CD8$^+$ T cells in lungs and spleens of C57BL/6 mice (Fig. 7), mice primed-boosted with BCG-rLm5Ag(30) produced significantly greater frequencies of lung and spleen CD8$^+$ T cells expressing IFN-γ and TNF-α in response to *in vitro* stimulation with the TB10.4/EsxH ($P < 0.0001$ for both cytokines in both organs) (Fig. 7b and j) and the 5Ag pool ($P < 0.0001$ for both cytokines in both organs) (Fig. 7f and n) than mice immunized with the *L. monocytogenes* vector. Two notable differences between lungs and spleens were that mice primed-boosted with BCG-rLm5Ag(30) produced significantly greater frequencies of CD8$^+$ T cells expressing IFN-γ and TNF-α in response to *in vitro* stimulation with the ESAT6/EsxA ($P < 0.05$ and $P < 0.001$, respectively) (Fig. 7c) and PPD ($P < 0.0001$) (Fig. 7g) for cells from their lungs but not their spleens (Fig. 7k and o), while mice primed-boosted with BCG-rLm5Ag(30) produced significantly greater frequencies of CD8$^+$ T cells expressing IFN-γ and TNF-α for cells from their spleens ($P < 0.001$ and $P < 0.05$, respectively) (Fig. 7p), but not their lungs (Fig. 7h), in response to *in vitro*



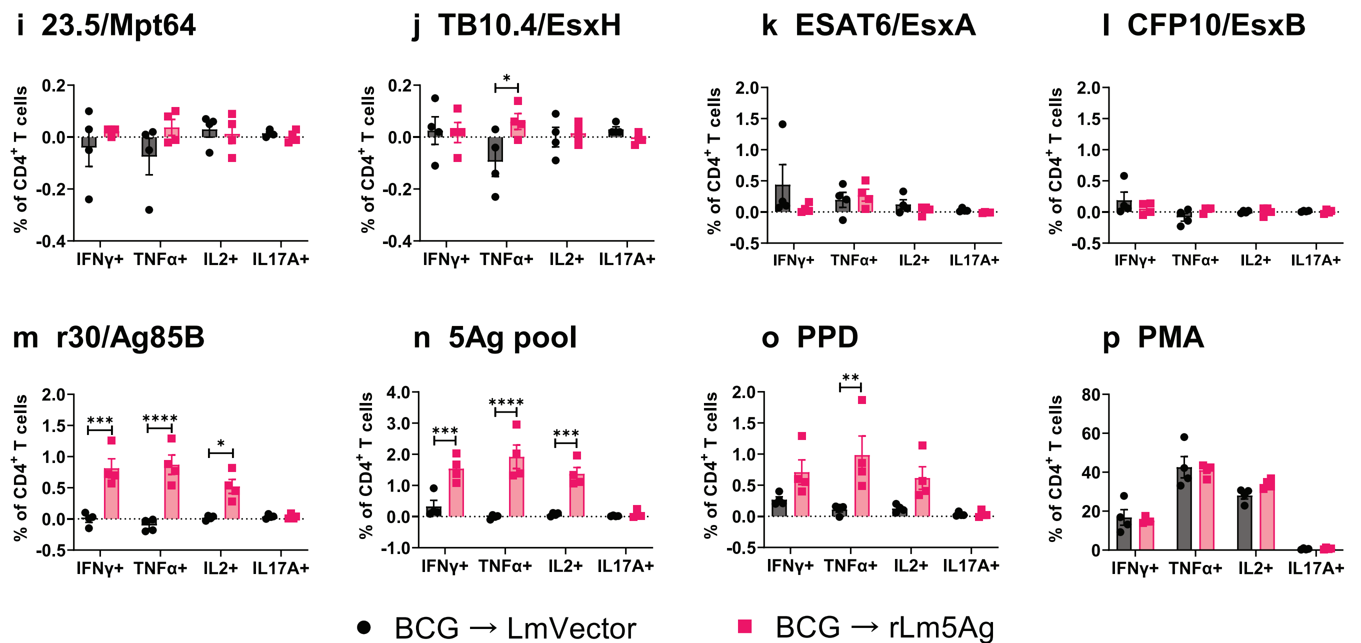
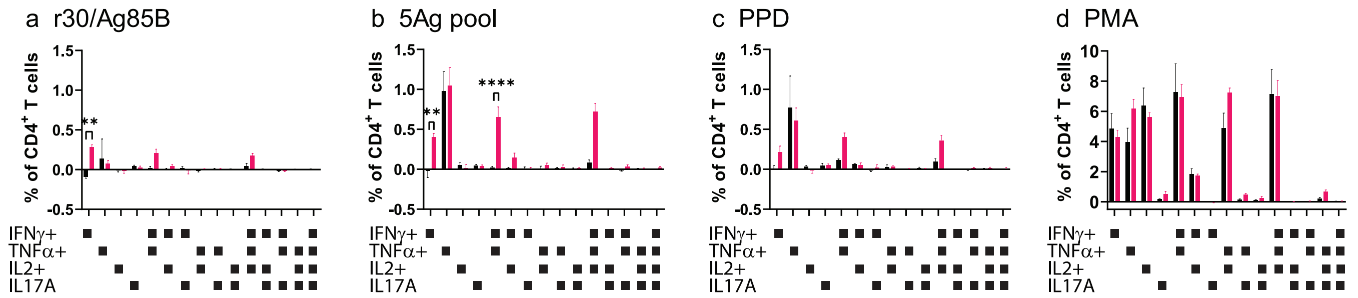


FIG 5 Frequency of cytokine-expressing $CD4^+$ T cells in the lungs and spleens of C57BL/6 primed with BCG and boosted twice with *L. monocytogenes* vector or rLm5Ag(30). Mice (n = 4/group) were primed with BCG at week 0 and boosted twice at weeks 14 and 18 with *L. monocytogenes* vector (black bars and symbols) or rLm5Ag(30) (rLm5Ag) (pink bars and symbols) expressing Mpt64-TB10.4-ESAT6-CFP10-r30. Six days after the last immunization, mice were euthanized, their lungs and spleens removed, and single-cell suspensions prepared and stimulated with recombinant proteins 23.5/Mpt64 (a, i), TB10.4/EsxH (b, j), ESAT6/EsxA (c, k), CFP10/EsxB (d, l), r30/Ag85B (e, m), pool of the 5Ags (f, n), PPD (g, o), or PMA (positive control) (h, p) in the presence of anti-CD28 monoclonal antibody for 6 h (except in the case of PMA [positive control] for 4 h), and the cells were assayed by intracellular cytokine staining (ICS) for surface markers of CD4 and intracellular markers of IFN-γ, TNF-α, IL-2, and IL-17A, as indicated below each panel. Each symbol represents one animal. Values are the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by two-way ANOVA with Sidak's post-multiple-comparison test.

stimulation with PMA than mice immunized with the *L. monocytogenes* vector; the relative differences in frequency and in statistical significance were much less than for 5Ag and TB10.4/EsxH (Fig. 7j and n). No significant differences in the frequencies of lung and spleen $CD8^+$ T cells expressing any of the cytokines were detected after *in vitro*

Lung



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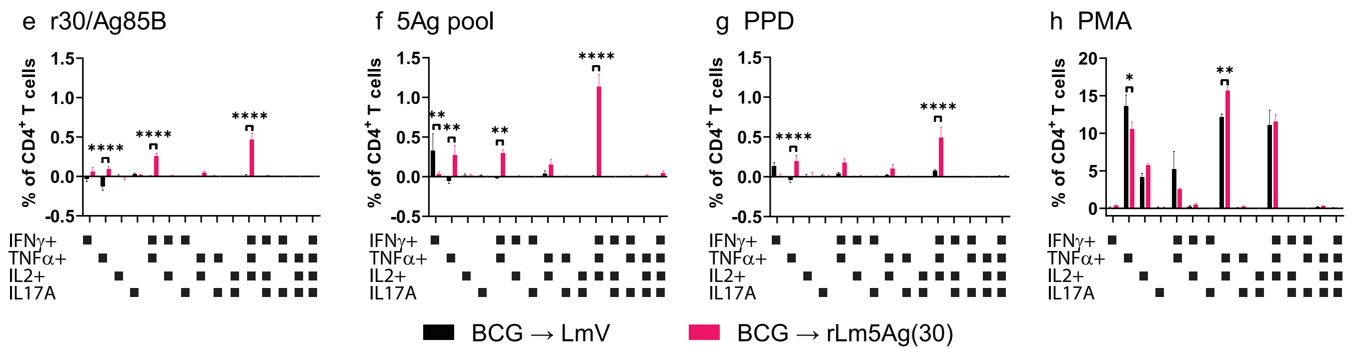


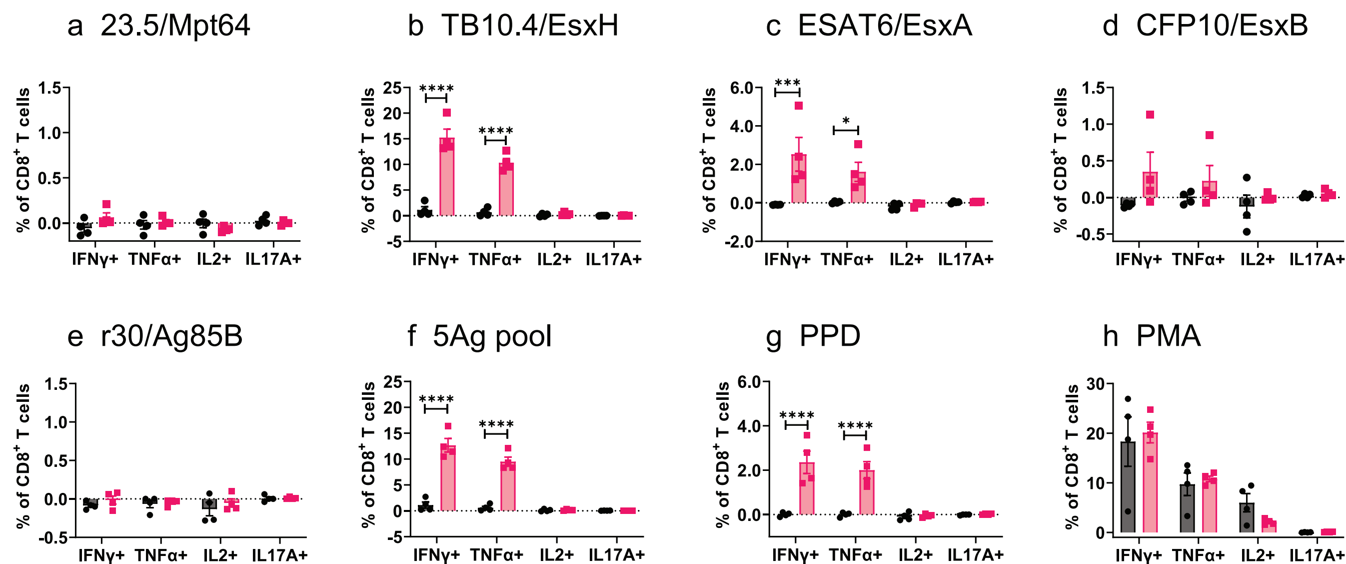
FIG 6 Frequency of polyfunctional cytokine-expressing CD4$^+$ T cells in the lungs and spleens of C57BL/6 mice primed with BCG and boosted twice with *L. monocytogenes* vector or rLm5Ag(30). Mice were immunized, and lung and spleen cells were prepared and stimulated with recombinant proteins as described in the legend to Fig. 5. The cells were assayed by ICS for surface markers of CD4 and intracellular markers of IFN-γ, TNF-α, IL-2, and IL-17A. The frequencies of CD4$^+$ T cells expressing 1 or combinations of 2, 3, or 4 of the four cytokines assayed in response to stimulation with r30/Ag85B (a, e), pool of the 5Ags (b, f), PPD (c, g), or PMA (positive control) (d, h) are shown, as indicated below each panel. Values are the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$ by two-way ANOVA with Sidak's post-multiple-comparison test.

stimulation with 23.5/Mpt64 (Fig. 7a and i), r30/Ag85B (Fig. 7e and m), or CFP10/EsxB (Fig. 7d and l). These results show that boosting BCG-primed C57BL/6 mice with rLm5Ag (30) induces *M. tuberculosis* antigen-specific CD4$^+$ and CD8$^+$ T cell-mediated immune responses.

In a similar experiment performed in BALB/c mice (Fig. 8), in contrast to C57BL/6 mice, there were no significant differences between mice primed-boosted with BCG-*L. monocytogenes* vector and BCG-rLm5Ag(30) in frequencies of lung and splenic CD4$^+$ and CD8$^+$ T cells expressing IFN-γ, TNF-α, IL-2, or IL-17 in response to *in vitro* stimulation with 23.5, TB10.4, ESAT6, r30/Ag85B, PPD, and PMA; the only exceptions were that mice primed-boosted with BCG-rLm5Ag(30) had significantly greater frequencies of lung CD4$^+$ T cells expressing IFN-γ in response to stimulation with the 5Ag pool ($P < 0.01$) (Fig. 8f), and mice primed-boosted with BCG-rLm5Ag(30) had significantly greater frequencies of lung CD4$^+$ T cells expressing TNF-α in response to stimulation with CFP10 ($P < 0.01$) (Fig. 8d). With respect to CD8$^+$ T cells (Fig. 9), compared with mice primed-boosted with BCG-*L. monocytogenes* vector, BALB/c mice primed-boosted with BCG-rLm5Ag(30) produced significantly greater frequencies of lung and spleen CD8$^+$ T cells expressing IFN-γ or TNF-α in response to TB10.4 ($P < 0.01$ for lung cells [Fig. 9b] and $P < 0.05$ for spleen cells [Fig. 9j]), but not to other *M. tuberculosis* antigens expressed by the rLm5Ag(30) vaccine (Fig. 9). These results indicate the rLm5Ag(30) multiantigenic vaccine candidate induces a qualitatively and quantitatively different immune response in BCG-primed C57BL/6 and BALB/c mice.

With respect to the humoral immune response induced by BCG-rLm5Ag primed-boost vaccination, as shown in Fig. S7, C57BL/6 mice primed-boosted with BCG-rLm5Ag(30) produced serum IgG antibodies specific to FK-rLm5Ag(30), but not to individual *M. tuberculosis* protein antigens (Ag85B, EsxA, EsxH, Mpt64, and PPD), at a level significantly

Lung



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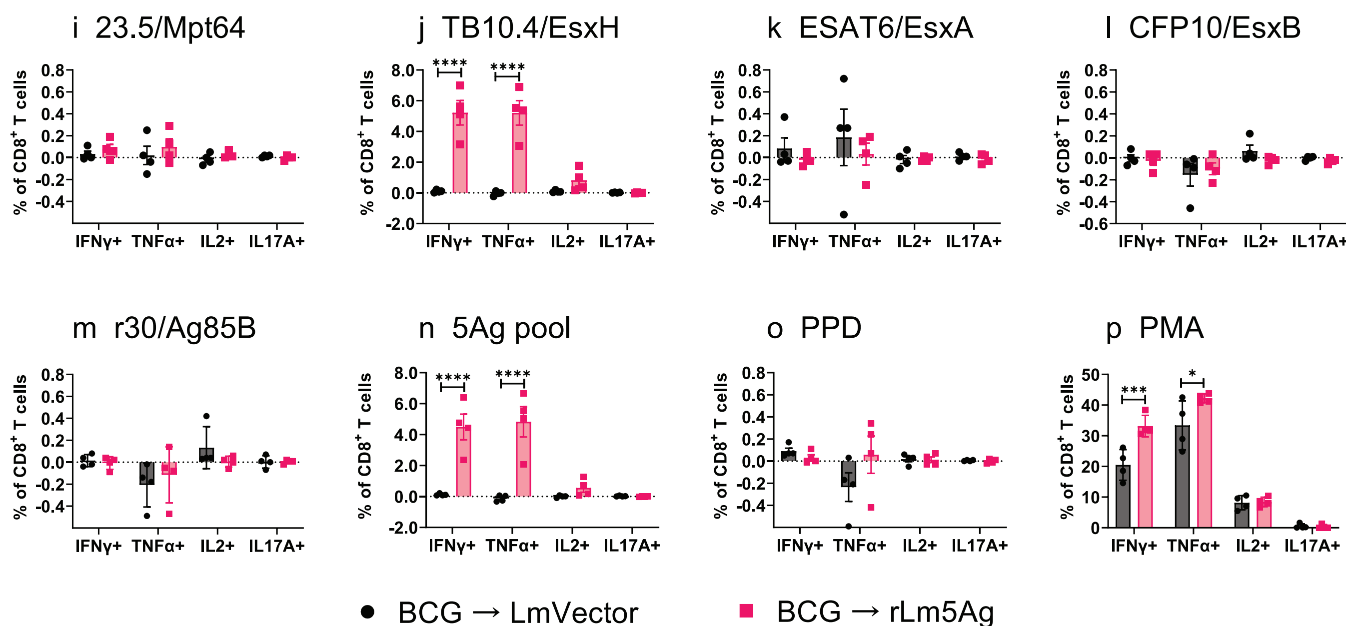
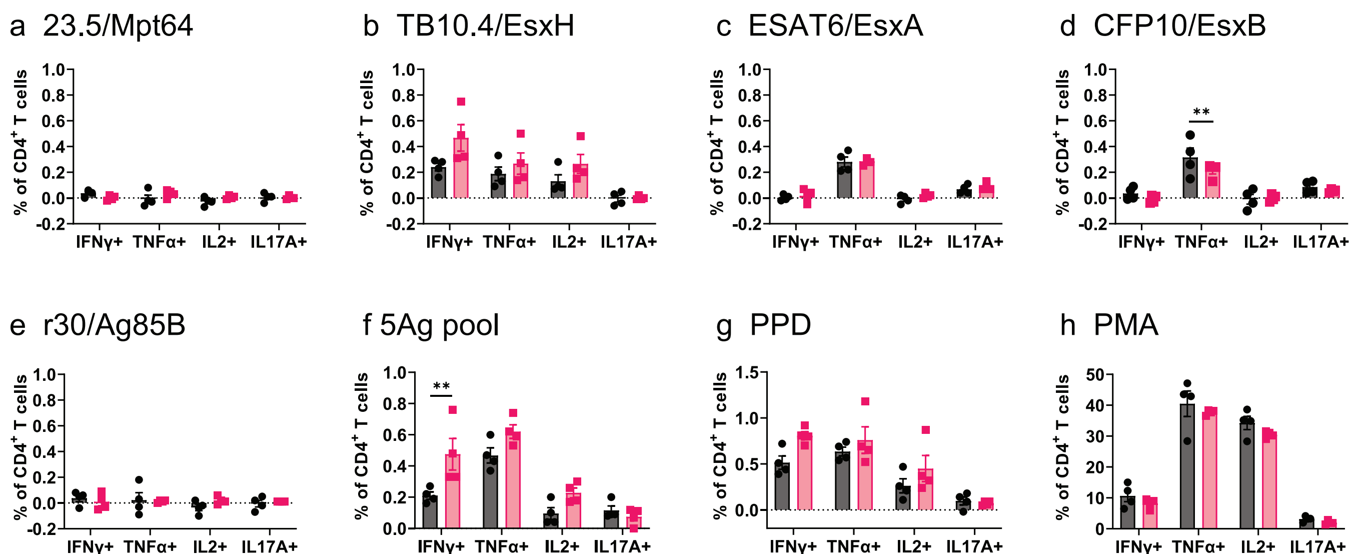


FIG 7 Frequency of cytokine-expressing $CD8^+$ T cells in the lungs and spleens of C57BL/6 mice primed with BCG and boosted twice with *L. monocytogenes* vector or rLm5Ag(30). Mice were immunized, and lung and spleen cells were prepared and stimulated with recombinant proteins as described in the legend to Fig. 5. The cells were assayed by ICS for surface markers of CD8 and intracellular markers of IFN-γ, TNF-α, IL-2, and IL-17A. The frequencies of $CD8^+$ T cells expressing IFN-γ, TNF-α, IL-2, and IL-17A in response to stimulation with recombinant proteins 23.5/Mpt64 (a, i), TB10.4/EsxH (b, j), ESAT6/EsxA (c, k), CFP10/EsxB (d, l), r30/Ag85B (e, m), pool of the 5Ags (f, n), PPD (g, o), and PMA (positive control) (h, p) are shown. Each symbol represents one animal. Black bars and symbols, primed-boosted with BCG and *L. monocytogenes* vector; pink bars and symbols, primed-boosted with BCG and rLm5Ag(30). Values are the mean ± SEM. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$ by two-way ANOVA with Sidak's post-multiple-comparison test.

higher than mice primed-boosted with BCG-*L. monocytogenes* vector (Fig. S7a). There were no significant differences in serum antibody titers to any of the antigens between BALB/c mice primed-boosted with BCG-rLm5Ag(30) or BCG-*L. monocytogenes* vector (Fig. S7b). These results indicate that boosting BCG-immunized mice with rLm5Ag(30) has a negligible or very limited impact on the humoral immune response to *M. tuberculosis* antigens.

Lung



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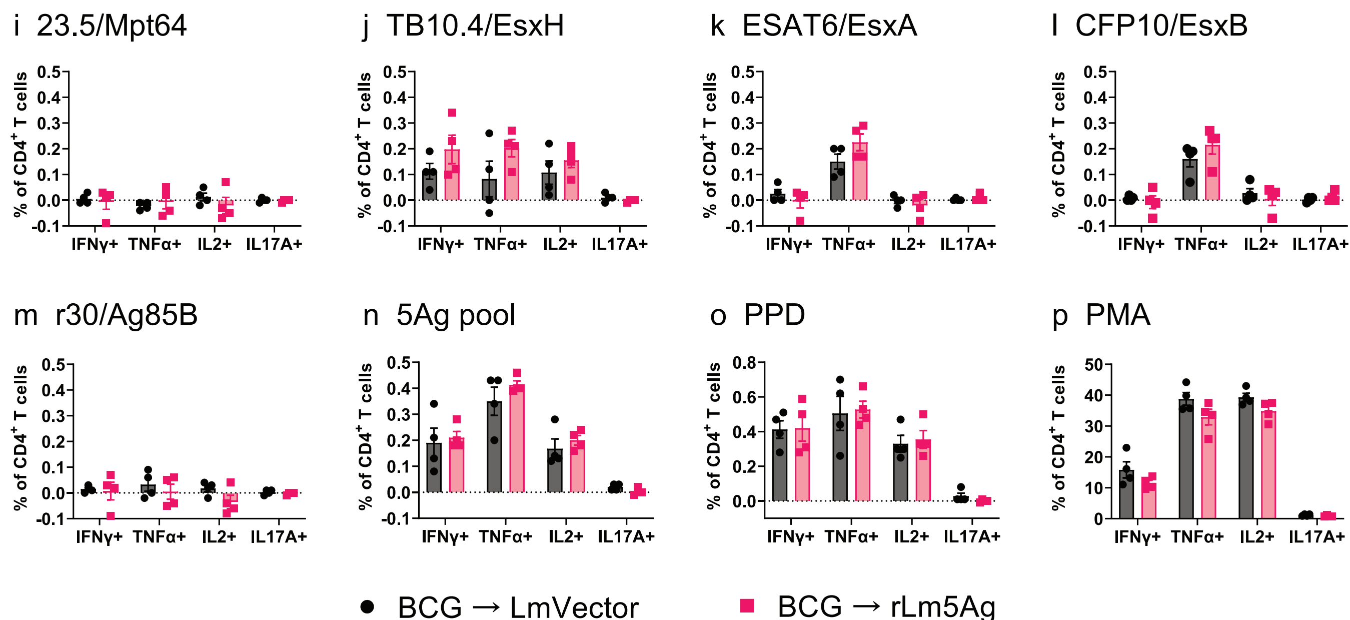
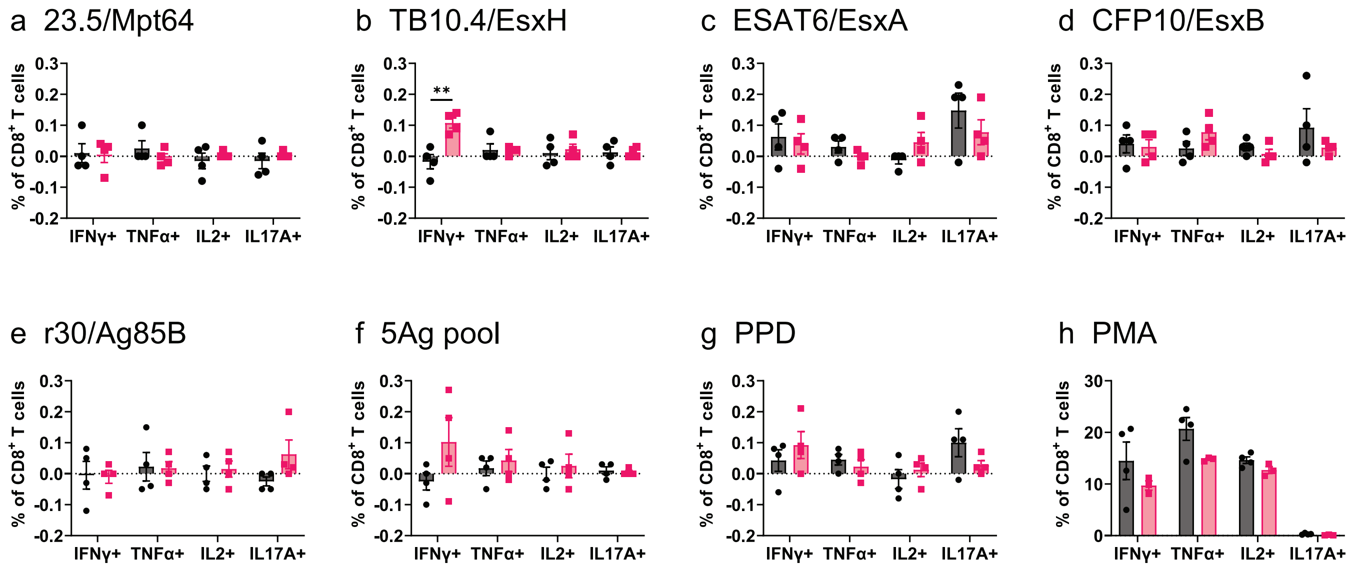


FIG 8 Frequency of cytokine-expressing $CD4^+$ T cells in the lungs and spleens of BALB/c mice primed with BCG and boosted twice with *L. monocytogenes* vector or rLm5Ag(30). BALB/c mice ($n = 4$/group) were primed with BCG at week 0 and boosted twice at weeks 14 and 18 with *L. monocytogenes* vector (black bars and symbols) or rLm5Ag(30) (rLm5Ag) (pink bars and symbols) expressing 23.5-TB10.4-ESAT6-CFP10-r30. Six days after the last immunization, mice were euthanized, their spleens and lungs were removed, and single-cell suspensions were prepared and stimulated with recombinant proteins 23.5/Mpt64 (a, i), TB10.4/EsxH (b, j), ESAT6/EsxA (c, k), and CFP10/EsxB (d, l), r30/Ag85B (e, m), pool of the 5Ags (f, n), PPD (g, o), or PMA (positive control) (h, p) and in the presence of anti-CD28 monoclonal antibody for 6 h (except in the case of PMA [positive control] for 4 h), and the cells were assayed by intracellular cytokine staining (ICS) for surface markers of CD4 and intracellular markers of IFN-γ, TNF-α, IL-2, and IL-17A, as indicated below each panel. Each symbol represents one animal. Values are the mean ± SEM. **, $P < 0.01$ by two-way ANOVA with Sidak's post-multiple-comparison test.

DISCUSSION

Our study shows that boosting BCG-primed C57BL/6 and BALB/c mice with an Lm-vectored multiantigenic *M. tuberculosis* vaccine candidate expressing combinations of *M. tuberculosis* proteins, especially rLm5Ag(30), expressing a fusion protein of r30/Ag85B, TB10.4/EsxH, ESAT6/EsxA, CFP10/EsxB, and 23.5/Mpt64, enhances the immunoprotection conferred by BCG against aerosol challenge with the virulent *M. tuberculosis* Erdman



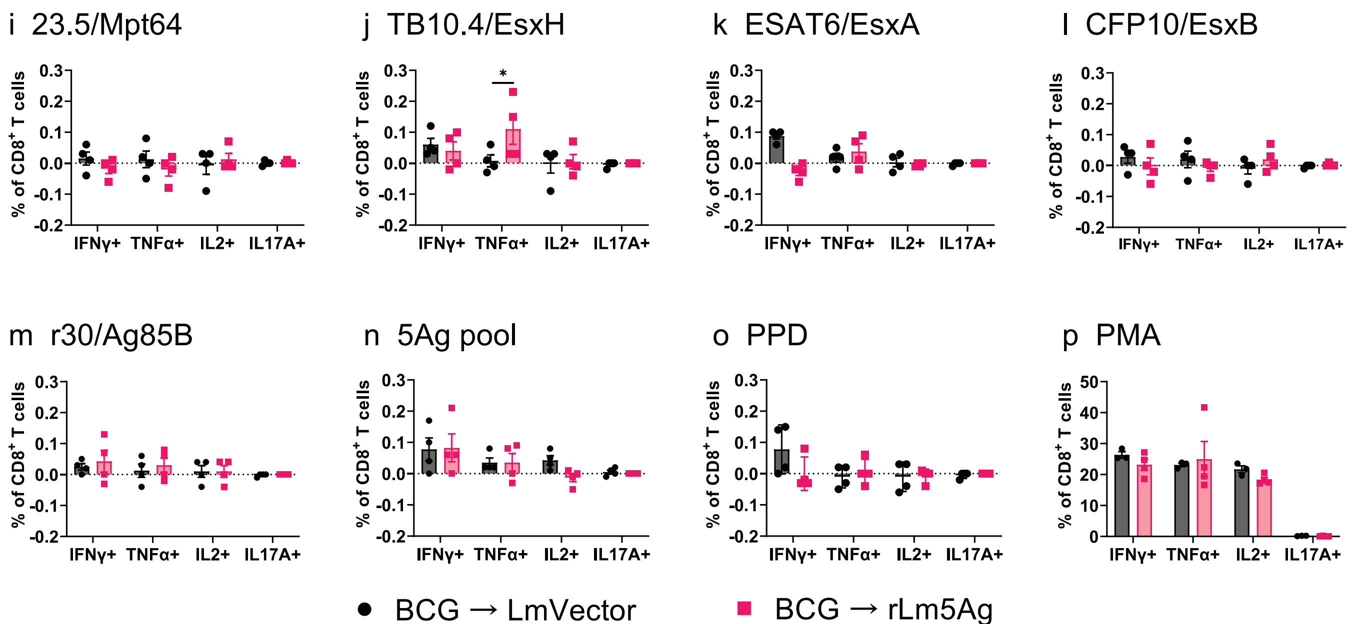


FIG 9 Frequency of cytokine-expressing CD8$^+$ T cells in the lungs and spleens of BALB/c mice primed with BCG and boosted twice with *L. monocytogenes* vector or rLm5Ag(30). BALB/c mice were immunized, and spleen and lung cells were prepared and stimulated with recombinant proteins as described in the legend to Fig. 8. The cells were assayed by ICS for surface markers of CD8 and intracellular markers of IFN-γ, TNF-α, IL-2, and IL-17A. The frequencies of CD8$^+$ T cells expressing IFN-γ, TNF-α, IL-2, and IL-17A in response to stimulation with recombinant proteins 23.5/Mpt64 (a, i), TB10.4/EsxH (b, j), ESAT6/EsxA (c, k), and CFP10/EsxB (d, l), r30/Ag85B (e, m), pool of the 5Ags (f, n), PPD (g, o), or PMA (positive control) (h, p) are shown. Each symbol represents one animal. Black bars and symbols, primed-boosted with BCG and *L. monocytogenes* vector; pink bars and symbols, primed-boosted with BCG and rLm5Ag(30). Values are the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ by two-way ANOVA with Sidak's post-multiple-comparison test.

strain in both mouse strains. Boosting C57BL/6 mice with rLm5Ag(30) significantly enhances the level of CD8$^+$ T cell expression in the lungs and spleen, the frequency of polyfunctional splenic CD4$^+$ T cells expressing IFN-γ, TNF-α, and IL-2 in response to r30/Ag85B, PPD, and the 5Ag pool, and the frequency of lung and spleen CD8$^+$ T cells expressing IFN-γ and TNF-α in response to TB10.4/EsxH, ESAT6/EsxA, the 5Ag pool, and PPD.

Although boosting BCG-primed BALB/c mice with rLm5Ag(30) also enhances the frequency of some cytokine-secreting lymphocytes, specifically lung $CD4^+$ T cells expressing IFN-γ or TNF-α in response to the 5 Ag pool or CFP10 and splenic $CD8^+$ T cells expressing IFN-γ or TNF-α in response to TB10.4/EsxH antigen, the response is much more limited than in BCG-primed C57BL/6 mice.

Of the five recombinant *M. tuberculosis* antigens expressed by rLm5Ag(30), all have previously been demonstrated to be immunoprotective individually as well as in combination with other *M. tuberculosis* antigens. r30/Ag85B has been demonstrated to be highly protective when administered as an adjuvanted recombinant protein (9) or when expressed by recombinant BCG (rBCG30) (4, 41) or an *L. monocytogenes* vector (22) in guinea pigs and mice. TB10.4 alone or as part of an Ag85B-TB10.4 fusion protein in adjuvant has been shown to induce protection in mice (31) and guinea pigs (42, 43). ESAT6, alone or in combination with antigen 85B, administered with the adjuvant monophosphoryl lipid A, has been shown to induce protective immunity in mice (29, 32). CFP10 delivered via a DNA vaccine induces protection against aerosolized *M. tuberculosis* Erdman in C3H/HeJ mice (30), and a *Salmonella*-vectored vaccine expressing an ESAT6-CFP10 fusion protein protects C57BL/6 mice against aerosolized *M. tuberculosis* H37Rv (44). Finally, the 23.5/Mpt64 protein expressed by a DNA vaccine (25) or surface expressed by recombinant BCG (26) has been found to induce protective immunity in C57BL/6 mice challenged intravenously with H37Rv (25) or by aerosol with *M. tuberculosis* Erdman (26).

Notably, of the five antigens in rLm5Ag(30), three are absent from BCG entirely (ESAT6/EsxA and CFP10/EsxB) or from modern strains of BCG (23.5/Mpt64). Hence, boosting BCG with rLm5Ag(30) not only potentially enhances the level of immunity to immunoprotective proteins present in BCG but additionally broadens the potential immune response to encompass antigens present in *M. tuberculosis* but absent from BCG.

Also of note, the five proteins comprising rLm5Ag(30) are all secreted or extracellularly released proteins. Such extracellular proteins have been demonstrated to be especially important immunoprotective antigens of intracellular pathogens and hypothesized early on to play a central role in vaccines against such pathogens, including *Legionella pneumophila* and *Mycobacterium tuberculosis* (45, 46).

Our screen of 11 *Listeria*-vectored vaccines expressing 5 *M. tuberculosis* antigens, all comprising a fusion protein of Mpt64-TB10.4-ESAT6-CFP10 plus 1 of 11 additional antigens, revealed several vaccine candidates that induced protection better than BCG and almost comparable to rLm5Ag(30). The most potent alternative "fifth" antigens were EsxN, PPE68, EspA, and TB8.4. Of these, one antigen, PPE68, is absent from BCG. We have subsequently constructed a 9-antigen *Listeria*-vectored vaccine incorporating these additional four *M. tuberculosis* antigens, and in ongoing studies, we are evaluating it for protective efficacy in mice, guinea pigs, and nonhuman primates.

The rLm5Ag(30) vaccine induced significantly enhanced levels of antigen-specific cytokine-secreting $CD4^+$ and $CD8^+$ T cells in BCG-immunized C57BL/6, but not in BCG-immunized BALB/c, mice, where such responses were weak and sporadic, reflecting the well-established Th1 bias of C57BL/6 mice versus the Th2 bias of BALB/c mice. Similarly, BCG immunization alone has been found to induce a greater Th1-type response in C57BL/6 than BALB/c mice (47, 48), although this has not been observed universally (49). In any case, despite the disparate immune responses induced by the rLm5Ag(30) vaccine in BCG-immunized C57BL/6 and BALB/c mice, recombinant attenuated *L. monocytogenes* vaccines expressing these five antigens boosted protection against *M. tuberculosis* aerosol challenge in both mouse strains. This result mirrors previous observations that differences in the ability of these two mouse strains to generate Th1 helper cells are not reflected by differences in their ability to resist *M. tuberculosis* infection (50, 51). In an interesting twist on the subject, Garcia-Pelayo et al. found that in mice immunized i.d. with BCG Danish, BALB/c mice developed a stronger TH1 response than C57BL/6 mice in both lung and spleen cells after stimulation with a cocktail of *M. tuberculosis*-secreted proteins; nevertheless, both mouse strains showed equivalent protection against virulent *Mycobacterium bovis* challenge (49). Clearly, TH1 type responses are important for

immunoprotection against *M. tuberculosis*, as evidenced by the increased susceptibility to *M. tuberculosis* challenge of IFN-γ knockout mice and the enhancement in protection against *M. tuberculosis* challenge in BALB/c mice administered IL-12 (52, 53). However, taken together, the aforementioned studies showing comparable resistance of BALB/c and C57BL/6 mice to virulent mycobacterial challenge, albeit conducted under different experimental conditions, indicate that additional immune mechanisms, aside from the strength of the TH1 response, are also important.

Of note, in all three experiments in which they were evaluated, recombinant attenuated *L. monocytogenes* vaccines expressing the 5 *M. tuberculosis* antigens in rLm5Ag (30) significantly boosted the level of protection conferred by BCG despite the already high level of protection conferred by BCG alone (1.1- to 1.3-$\log_{10}$ CFU in the lungs and 1.8- to 2.2-$\log_{10}$ CFU in the spleen). Many TB booster vaccines are able to boost the level of protection afforded by BCG alone only if that level is very small, e.g., $<$0.3-$\log_{10}$ CFU (23). Moreover, in all three of these protection experiments, the reduction in $\log_{10}$ CFU versus sham-immunized mice conferred by the BCG prime-recombinant attenuated *L. monocytogenes* boost was $\geq$1.75-$\log_{10}$ CFU in the lung and/or spleen (1.70- to 1.75-$\log_{10}$ CFU in the lung and 2.5- to 2.8-$\log_{10}$ CFU in the spleen), a threshold of protection achieved by relatively few TB booster vaccines in BCG-immunized animals (23).

A potential major advantage of a *Listeria*-vectored vaccine, particularly with respect to protein/adjuvant vaccines, is enhanced capacity to induce $CD8^+$ T cells. $CD8^+$ T cells are required to resist *M. tuberculosis* infection, as demonstrated by studies in mice employing antibody depletion or TAP1 knockout of $CD8^+$ T cells (54–57). Consistent with these observations, adoptive transfer of $CD8^+$ T cells enhances resistance to TB (58). Of note, $CD8^+$ T cells appear to play a more important role in primates than in rodents (59); hence, efficacy studies in rodents may underestimate the efficacy of *Listeria*-vectored vaccines in nonhuman primates and humans. In current studies, we are evaluating the efficacy of a multiantigenic *Listeria*-vectored vaccine in nonhuman primates.

In conclusion, recombinant attenuated *L. monocytogenes* multiantigenic vaccines, including the rLm5Ag(30) vaccine, abundantly express *M. tuberculosis* recombinant proteins in broth and macrophages, stably retain the antigen expression cassettes in the absence of antibiotic selection, and display growth kinetics equivalent to the vector in murine and human macrophages. recombinant attenuated *L. monocytogenes* vaccines expressing the 5 *M. tuberculosis* antigens 23.5/Mpt64, TB10.4/EsxH, ESAT6/EsxA, CFP10/EsxB, and r30/antigen 85B consistently and significantly boost the high level of protection conferred by BCG alone in both C57BL/6 and BALB/c mouse models of pulmonary TB. The single rLm5Ag(30) vaccine induces broad $CD4^+$ and $CD8^+$ T cell responses in the lungs and spleens of mice. Thus, the rLm5Ag(30) vaccine holds substantial promise for future development as a vaccine capable of enhancing the level of protective immunity in the majority of the world's population, who reside in areas of TB endemicity and are immunized during infancy with BCG.

MATERIALS AND METHODS

Ethics statement. All animals were maintained in a specific-pathogen-free animal facility and used according to protocols approved by the UCLA Institutional Animal Care and Use Committee.

Cell lines, bacteria, animals, and proteins. Murine (J774A.1, ATCC TIB-67) and human (THP-1, ATCC TIB-202) monocytes were differentiated into macrophage-like cells and cultured in Dulbecco's modified Eagle's medium (DMEM) and RMPI 1640 (RPMI) medium, respectively, containing penicillin (100 μg/mL) and streptomycin (100 U/mL) and supplemented with 10% fetal bovine serum (FBS). *Mycobacterium bovis* BCG Tice was purchased from Organon. *M. tuberculosis* Erdman strain (ATCC 35801) was harvested from infected outbred guinea pigs to verify virulence, cultured on 7H11 agar, subjected to gentle sonication to obtain a single-cell suspension, and frozen at -80°C for use in animal challenge experiments. All *Listeria* vector and recombinant *Listeria*-vectored vaccine stocks were grown to mid-log phase in yeast extract broth medium, and the bacteria were collected by centrifugation, resuspended in phosphate-buffered saline (PBS), titrated, and stored in 20% glycerol/PBS at -80°C until use. Six- to 8-week-old female C57BL/6 mice were purchased from Harlan (currently Envigo, Livermore, CA, USA) or Jackson Laboratory (Bar Harbor, ME, USA), and BALB/c mice were purchased from Jackson Laboratory.

The following *M. tuberculosis* protein reagents were obtained through BEI Resources, NIAID, NIH: Ag85B (gene Rv1886c), purified native protein from strain H37Rv, NR-14857; ESAT-6, recombinant protein reference standard, NR-49424; CFP-10, recombinant protein reference standard, NR-49425; and

Mpt64, recombinant protein reference standard, NR-44102. The *M. tuberculosis* protein TB10.4 (gene Rv0288) was obtained from Aeras (formerly in Rockville, MD, USA).

Construction and verification of Lm-vectored multiantigenic vaccines. We constructed *Listeria*-vectored multiantigenic recombinant attenuated *L. monocytogenes* vaccine candidates using the *Lm* Δ*actA* Δ*inlB prfA** vector, as we previously described (22). Briefly, to construct recombinant attenuated *L. monocytogenes* vaccine candidates expressing *M. tuberculosis* multiantigenic proteins, we analyzed the protein sequences of the selected 15 *M. tuberculosis* proteins, removed the predicted signal peptides of TB8.4 (2R-28A) (gene Rv1174c), Apa (2H-39A) (gene Rv1860), r30/Ag85B (2Q-43A) (gene Rv1886c), and 23.5/Mpt64 (1V-23A) (gene Rv1980c) and the internal regions of HspX (121I-128V) (gene Rv2031c), PE25 (66I-73L) (gene Rv2431c), and EspA (111F-193L) (geneRv3616c) that might interfere with protein secretion from the recombinant attenuated *L. monocytogenes* vaccine constructs; we kept the full-length sequences for TB10.4 (gene Rv0288), EsxN (gene Rv1793), Hrp1 (gene Rv2626c), VapB47 (gene RV3407), EspC (gene 3615c), PPE68 (gene Rv3873), CFP-10 (gene Rv3874), and ESAT-6 (gene Rv3875). We optimized the coding sequence for each of the selected proteins for expression in *Listeria*, purchased them from DNA2.0 (Newark, CA), and assembled the optimized DNAs encoding the indicated multiantigenic proteins with or without a spacer encoding a GGSG or GSSGGSSG flexible linker by traditional molecular cloning methods. We cloned the final assembled DNAs into a phage-based *Listeria* site-specific integration vector derived from pPL1 (kindly provided by P. Lauer) or pPL2e (kindly provided by J. Skoble) (60) downstream of the Lm *actA* promoter and ligated in-frame to the C terminus of the N-terminal 100 amino acids (aa) of ActA (ActAN). Subsequently, we integrated the *M. tuberculosis* antigen expression cassette vectored by pPL1 and pPL2e into the *comK* and the 3′ end of the *tRNA^{arg}* locus, respectively, on the recipient *Listeria* chromosome, as described previously by us (22) and Lauer et al. (17, 60). All molecular plasmid constructs were confirmed by restriction enzyme digestion and nucleotide sequencing. The final *M. tuberculosis* antigen expression cassette in the recombinant attenuated *L. monocytogenes* chromosome was verified by PCR using primers NC16 (GTCAAAACATACGCTTCTTATC) and PL95 (ACATAATCAGTCCAAAGTAGATGC) (60), specifically amplifying a unified 548-bp PCR product in strains that contain an integration vector at the bacterial attachment site *tRNA^{arg}-attBB*′, and using primers 319 (ACCGACTGGAAACAGGCAAA) and 327 (ACCAAGATACGAAACTGCACG), specifically amplifying a PCR product across the inserted gene with various sizes in different strains; the PCR products were further confirmed by nucleotide sequencing.

Growth kinetics of recombinant attenuated *L. monocytogenes* multiantigenic vaccines in broth culture and in murine and human macrophage-like cells. The growth kinetics of recombinant attenuated *L. monocytogenes* vaccine candidates in broth culture and macrophage-like cells were examined as described by us previously with modifications (22). Glycerol stocks of the *L. monocytogenes* vector and recombinant attenuated *L. monocytogenes* vaccine candidates were inoculated into BHI medium supplemented with streptomycin (200 μg/mL) (the *L. monocytogenes* vector is streptomycin resistant) to prevent any contamination and grown overnight under stationary conditions in a 37°C incubator with 5% CO_2. The overnight culture was inoculated into 5 mL fresh BHI with streptomycin at an initial optical density at 540 nm (OD_{540}) of ~0.05 and incubated at 37°C with shaking at 180 rpm. At 0, 3, 5, and 7 h postinoculation, a 1-mL aliquot of each culture was removed and measured for OD_{540}.

The growth kinetics in macrophage-like cells was assayed by infecting monolayers of murine macrophage-like cells (J774A.1) or phorbol 12-myristate 13-acetate (PMA)-differentiated monolayers of human macrophage-like cells (THP-1) with the *L. monocytogenes* vector or recombinant attenuated *L. monocytogenes* candidates cultured overnight to stationary phase at a multiplicity of infection of 1:10 for 90 min in DMEM (J774A.1) or RPMI (THP-1) medium supplemented with 10% heat-inactivated FBS (HI-FBS). After 90 min infection, cells were washed three times with PBS supplemented with 2% HI-FBS. The infected cells were cultured for an additional 4.5 h in DMEM or RPMI medium supplemented with 10% HI-FBS and gentamicin (10 μg/mL). At 0, 2, 4, and 6 h postinfection, the medium was removed; the monolayers were lysed with 0.1% saponin-PBS, and the cell lysates were serially diluted in PBS and plated on BHI agar plates supplemented with streptomycin (200 μg/mL). The plates were incubated at 37°C for 2 days, and colonies were enumerated.

Immunization and aerosol challenge of mice with virulent *M. tuberculosis* Erdman strain. Groups of BALB/c or C57BL/6 mice, 8/group, were primed with BCG intradermally (i.d.) or intranasally (i.n.). BCG-primed mice were either not boosted or boosted once or twice with 2×10^6 CFU of a single recombinant attenuated *L. monocytogenes* vaccine or a combination of two recombinant attenuated *L. monocytogenes* vaccine candidates expressing multiple *M. tuberculosis* antigens and challenged 3 or 4 weeks later by exposure to aerosolized *M. tuberculosis* Erdman strain generated by a Collison type 6 jet nebulizer (CH Technologies USA, Waltham, MA) from 10 mL of *M. tuberculosis* bacterial suspension (1.6×10^5 to 2.6×10^5 CFU/mL) for 30 min followed by 5 min to allow for settling of bacteria. The challenge dose was verified by euthanizing two animals and assaying CFU in their entire lungs at day 1 postchallenge. The mice were euthanized at various times postchallenge, and the spleens and lungs were removed and assayed for bacillus burden as described by us previously (22).

Immunization of mice and assay for intracellular cytokine staining of mouse spleen and lung cells. To determine the immunogenicity of rLm5Ag(30) expressing the fusion protein of 5 *M. tuberculosis* antigens (23.5-10.4-ESAT6-CFP10-r30) as a booster vaccine, we immunized C57BL/6 and BALB/c mice, 4/group, subcutaneously (s.c.) with BCG at week 0; boosted them at weeks 14 and 18 with 2×10^6 CFU of the *L. monocytogenes* vector or rLm5Ag(30); anesthetized, bled, and euthanized the mice at 6 days after the last immunization; prepared single-cell suspensions of spleen and lung cells; stimulated the single-cell suspensions without protein antigen (medium alone, negative control) or with a single *M. tuberculosis* protein, pool of multiple *M. tuberculosis* proteins, purified protein derivative (PPD), or PMA (positive

control); and assayed T-cell immunity by intracellular cytokine staining (ICS) using methods that we published previously (22, 61) with modifications as described below.

We conducted ICS by using an eight-color flow cytometry panel to simultaneously analyze multiple cytokines at the single-cell level. Specifically, a single-cell suspension of 5×10^5 lung cells per well or 1.0×10^6 splenocytes per well was seeded in U-bottom 96-well plates and stimulated with medium alone (negative control), 5 μg/mL of recombinant proteins r30/Ag85B (our lab stock, isolated from recombinant *Mycobacterium smegmatis*); ESAT6/EsxA (BEI Resources); CFP10/EsxB (BEI Resources); TB10.4/EsxH (Aeras); 23.5/Mpt64 (BEI Resources); pool of 5 antigens (5Ag) comprising r30, ESAT6, CFP10, TB10.4, and 23.5, each at 2 μg/mL; or PPD (5 μg/mL) in the presence of anti-CD28 monoclonal antibody (clone 37.51) for a total of 6 h. Cells stimulated with PMA served as a positive control. Four hours prior to harvest, GolgiPlug (protein transport inhibitor containing brefeldin A) diluted in T-cell medium was added to all wells; PMA was additionally added to positive-control wells. Following *in vitro* stimulation, cells were harvested, washed with PBS, incubated with Live/Dead Fixable Near-IR cell stain (Invitrogen) for 10 min at room temperature to identify dead cells, and surface stained with antibodies against CD4 (clone RM4-5, conjugated with Brilliant Violet 510) and CD8 (clone 53-6.7, conjugated with Brilliant Violet 605). Cells were then fixed/permeabilized with Cytofix/Cytoperm (BD BioSciences) and stained for CD3 (clone 17A2, conjugated with Alexa Fluor 488 [AF488]), IFN-γ (clone XMG1.2, conjugated with Brilliant Violet 650), IL-2 (clone JES6-5H4, conjugated with PE), TNF-α (clone MP6-XT22, conjugated with PerCPCy5.5), and IL-17A (clone TC11-18H10.1, conjugated with Alexa Fluor 647). Note that due to the internalization of CD3 in responding CD4$^+$ T cells, cells were stained for CD3 after fixing/permeabilization. The fluorochrome-conjugated antibodies were purchased from BioLegend. For flow cytometry analysis, a minimum of 100,000 lymphocytes per sample were acquired with an HTLSRII (BD) flow cytometer. The data were analyzed by using FlowJo software. The initial gating of total events included a lymphocyte gate, followed by selection for singlet cells and live CD3$^+$ T cells (near-infrared AF488$^+$); CD4$^+$ and CD8$^+$ T cells were identified by CD4$^+$ (BV510$^+$ BV605$^-$) and CD8$^+$ (BV605$^+$ BV510$^-$) expression, respectively. The gates for frequencies of antigen-specific IFN-γ-, IL-2-, TNF-α-, and IL-17A-producing CD4$^+$ and CD8$^+$ T cells were determined by using the unstimulated cells; Boolean combinations of the four intracellular cytokine gates were used to uniquely discriminate responding cells based on their frequency with respect to cytokine production. Each cytokine-positive cell was assigned to 1 of the 15 possible combinations. In some cases, background frequencies of CD4$^+$ and CD8$^+$ T cells producing cytokines without antigen stimulation were subtracted.

Enzyme-linked immunosorbent assay for serum antibody specific for *M. tuberculosis* antigens in mouse sera. Mouse sera were assayed for IgG antibodies specific to *M. tuberculosis* protein antigens of Ag85B, EsxA, EsxH, Mpt64, and PPD and to formalin-killed rLm5Ag(30) (FK-rLm5Ag). Briefly, high-binding 96-well plates (Costar) were coated with carbonate/bicarbonate buffer without antigen (control), *M. tuberculosis* protein antigens (1 μg/mL), or FK-rLm5Ag (1×10^8/mL) overnight at 4°C, blocked in 3% bovine serum albumin (BSA) for 3 h at room temperature, washed 4 times, incubated with sera at 2-fold serial dilutions starting at 1:50 or with PBS overnight at 4°C, washed 4 times, and incubated for 90 min with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) at a dilution of 1:2,500 at ambient temperature. After the plates were washed 3 times, 100 μL of NPP (*p*-nitrophenylphosphate) substrate in diethanolamine buffer (phosphatase substrate kit; Bio-Rad, Hercules, CA) was added to each well and incubated for 20 min. The reaction was stopped by adding 100 μL of 0.1 N sodium hydroxide, and the solutions were read at 415 nm for absorbance. The endpoint antibody titer was calculated as the reciprocal of the highest immune serum dilution that gave a minimum difference of 0.05 optical density units when comparing the test (with antigen) and control (without antigen) wells.

Statistical analyses. Two-way analysis of variance (ANOVA) with Sidak's multiple-comparison test was performed using GraphPad Prism v9.2.0 (San Diego, CA) to determine significance in comparisons of mean frequencies of lymphocytes, T cells and cytokine-producing CD4$^+$ and CD8$^+$ T cells, and serum IgG antibody titer between mice vaccinated with the *L. monocytogenes* vector and mice vaccinated with rLm5Ag (30). One-way ANOVA with Tukey's multiple-comparison test and/or with Fisher's least significant difference (LSD) test was performed using GraphPad Prism v9.2.0 (San Diego, CA) to determine significance in comparisons of means of $\log_{10}$ CFU in spleens and lungs among mice in vaccinated and control groups.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, EPS file, 2.4 MB.

FIG S2, EPS file, 2.2 MB.

FIG S3, EPS file, 1.5 MB.

FIG S4, EPS file, 1.9 MB.

FIG S5, EPS file, 2.5 MB.

FIG S6, EPS file, 2.4 MB.

FIG S7, EPS file, 1.7 MB.

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