Models Derived from *In Vitro* Analyses of Spleen, Liver, and Lung Leukocyte Functions Predict Vaccine Efficacy against the *Francisella tularensis* Live Vaccine Strain (LVS)

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ABSTRACT **Currently, there are no licensed vaccines and no correlates of protection against *Francisella tularensis*, which causes tularemia. We recently demonstrated that measuring *in vitro* control of intramacrophage bacterial growth by murine *F. tularensis*-immune splenocytes, as well as transcriptional analyses, discriminated *Francisella* vaccines of different efficacies. Further, we identified potential correlates of protection against systemic challenge. Here, we extended this approach by studying leukocytes derived from lungs and livers of mice immunized by parenteral and respiratory routes with *F. tularensis* vaccines. Liver and lung leukocytes derived from intradermally and intranasally vaccinated mice controlled *in vitro Francisella* Live Vaccine Strain (LVS) intramacrophage replication in patterns similar to those of splenocytes. Gene expression analyses of potential correlates also revealed similar patterns in liver cells and splenocytes. In some cases (e.g., tumor necrosis factor alpha [TNF-α], interleukin 22 [IL-22], and granulocyte-macrophage colony-stimulating factor [GM-CSF]), liver cells exhibited even higher relative gene expression, whereas fewer genes exhibited differential expression in lung cells. In contrast with their strong ability to control LVS replication, splenocytes from intranasally vaccinated mice expressed few genes with a hierarchy of expression similar to that of splenocytes from intradermally vaccinated mice. Thus, the relative levels of gene expression vary between cell types from different organs and by vaccination route. Most importantly, because studies comparing cell sources and routes of vaccination supported the predictive validity of this coculture and gene quantification approach, we combined *in vitro* LVS replication with gene expression data to develop analytical models that discriminated between vaccine groups and successfully predicted the degree of vaccine efficacy. Thus, this strategy remains a promising means of identifying and quantifying correlative T cell responses.**

IMPORTANCE **Identifying and quantifying correlates of protection is especially challenging for intracellular bacteria, including *Francisella tularensis*. *F. tularensis* is classified as a category A bioterrorism agent, and no vaccines have been licensed in the United States, but tularemia is a rare disease. Therefore, clinical trials to test promising vaccines are impractical. In this report, we further evaluated a novel approach to developing correlates by assessing T cell immune responses in lungs and livers of differentially vaccinated mice; these nonprofessional immune tissues are colonized by *Francisella*. The relative degree of vaccine efficacy against systemic challenge was reflected by the ability of immune T cells, particularly liver T cells, to control the intramacrophage replication of bacteria *in vitro* and by relative gene expression of several immunological mediators. We therefore developed analytical models that combined bacterial replication data and gene expression data. Several resulting models provided excellent discrimination between vaccines of different efficacies.**

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Francisella tularensis causes tularemia, a zoonosis, with sporadic human infections. Although tularemia does not represent a significant public health problem in the United States, *F. tularensis* is considered a category A bioterrorism agent due to high rates of infectivity and mortality following pulmonary infection (1). An investigational vaccine, denoted Live Vaccine Strain (LVS) (2), was derived from *F. tularensis* subsp. *holarctica* (also known as type B [1]), a less virulent strain of *Francisella* present in Europe and Asia. Vaccination with LVS ameliorates disease to some degree in professional workers (1, 3), and vaccination with other attenuated *Francisella* strains has resulted in some protection against tularemia in Russia (4, 5), where type B *Francisella* is endemic. However, the extent of protection provided to humans by LVS against *F. tularensis* subsp. *tularensis* (also denoted type A),

the most virulent strain, is not well known in humans. Partial protection is obtained when LVS-vaccinated mice are challenged parenterally with a low dose of type A *F. tularensis* (6–9) and when LVS-vaccinated humans are challenged by aerosol (8).

Currently, other new vaccines against tularemia are being developed, and some appear to be comparable to LVS in protecting against type A *Francisella* in animal models (10, 11). However, it remains difficult to extrapolate results obtained in animal models to predict vaccine efficacy in humans without a thorough understanding of the immune response against *F. tularensis*. For instance, for infection with bacteria such as *F. tularensis*, for which human clinical trials are unpractical or unethical, regulatory approval of new vaccines might be pursued via the "Animal Rule" (11). Application of this option implies the need to evaluate vaccine efficacy in animal studies and then bridge efficacy and doses to humans without exposing human subjects to the bacteria. A limiting factor in extrapolating the results of animal studies to humans is the lack of knowledge about correlates of protection against *F. tularensis* (12). Many infections caused by encapsulated bacteria or toxins induce humoral immune responses that correlate with protection (13). In contrast, measurement of serum antibodies alone has not correlated with protection against *Francisella* infection. As with other intracellular pathogens, resistance to *Francisella* infection appears to depend heavily on T cell-based immune responses (6, 12, 14).

An issue in determining correlates of protection against intracellular bacterial infections is the nature of methodologies that are used to quantify T cell activities. In general, T cell functions are elicited by *in vitro* activation, followed by measurement of activities such as proliferation, production of cytokines, or cytotoxicity. Proliferation assays, enzyme-linked immunosorbent spot assays (ELISPOT), and enzyme-linked immunosorbent assays (ELISA) for cytokine production and killing assays are commonly accepted methodologies, especially for experimental group comparisons, but these assays are complex, and different variables may strongly influence the results. To date, no mediator measured after T cell stimulation, such as gamma interferon (IFN-γ), has proven to be a useful correlate. Thus, the identification of factors that may be quantified and correlated with immune status remains problematic.

Recently, we developed an *in vitro* tissue culture methodology that couples *in vitro* stimulation of T cells obtained after vaccination with a panel of LVS-related vaccines, control of intramacrophage LVS growth, and quantitation of relative gene expression. In this *in vitro* coculture method, control of intramacrophage bacterial growth depends on immune T cells (15). We demonstrated that the ability of immune T cells to control *in vitro* intramacrophage bacterial replication correlated with *in vivo* vaccine efficacy against systemic challenge (15). Further, transcriptional analyses of recovered splenocytes allowed the identification of immune mediators that were differentially expressed, and that also correlated with *in vivo* vaccine efficacy. At least some of these mediators were likely not just biomarkers but also involved in controlling bacterial replication *in vitro* as well as protection *in vivo*. The mediators detected by this approach included IFN-γ and tumor necrosis factor alpha (TNF-α), whose important roles in controlling *F. tularensis* infections have already been described (6, 12). Additional studies have now validated the biological functions of other potential correlates of protection that were identified, including interleukin 6 (IL-6) (16), T-bet (48), and IL-12Rβ_2 (17).

TABLE 1 Effects of different vaccination routes on survival against lethal challenge with LVS[a]

Vaccine	% survival after: i.d. vaccine, i.p. challenge	% survival after: i.n. vaccine, i.p. challenge
None (naive mice)	0	0
LVS	100	100
HK-LVS	5	4
LVS-R	50	80

[a] Five mice per group were vaccinated i.d or i.n. with the indicated vaccines and then challenged 6 weeks later with a lethal dose of 10^6 LVS i.p. Results represent the combined outcomes from five independent experiments, using a total of 20 to 25 mice per group.

In mice, LVS introduced intraperitoneally (i.p.) or intravenously is lethal (6). In contrast, LVS introduced via skin sites or by respiratory administration establishes a sublethal vaccinating infection, with dissemination of bacteria to spleens, livers, lungs, and lymph nodes. In the present report, we built on previous studies to validate these correlate approaches by examining the relative functions of cells from other involved organs, namely, liver and lung. We further assessed the role of vaccination route in detecting correlates. Finally, using both *in vitro* data on the control of intramacrophage LVS replication and gene expression analyses of selected correlates of protection, we identified mathematical approaches to quantitatively model correlates of protection. The best models combined both types of measurements, and thereby yielded excellent discrimination between vaccinated mice which were protected to different degrees.

RESULTS

Control of intramacrophage LVS growth during coculture by *Francisella*-immune liver and lung cells after differential vaccination. To determine whether lymphocytes derived from livers and lungs exhibit differential control of *in vitro* bacterial replication, similar to splenocytes (15), we vaccinated C57BL/6J mice with LVS-derived vaccines that provide different degrees of protection. Mice were vaccinated (i) with 10^4 LVS, the most-protective vaccine, (ii) with 10^4 of the moderately protective opacity variant LVS-R, or (iii) with the equivalent of 10^8 heat-killed LVS (HK-LVS), which is poorly protective. Four to 6 weeks after vaccination, mice were challenged with a lethal dose of 10^6 LVS i.p. As expected, mice vaccinated with LVS survived lethal LVS challenge, but nonvaccinated mice did not. Mice vaccinated with LVS-R and HK-LVS exhibited on average 50% and 5% survival, respectively (Table 1). These results confirmed the relative hierarchy of protection provided by these vaccines against lethal i.p. LVS challenge, which in turn reflected the hierarchy of protection against subcutaneous (s.c.) challenge with fully virulent type A *Francisella* (15). In parallel with survival studies, single-cell suspensions from spleens, livers, and lungs were prepared from mice vaccinated with LVS, LVS-R, or HK-LVS or from naive mice. In evaluating the yield and proportions of lymphocyte subpopulations obtained from each organ, there were no differences between those in naive mice and those from any of the vaccinated groups. Figure 1 therefore shows the distribution of lymphocytes obtained from the three organs, using results pooled from naive mice and

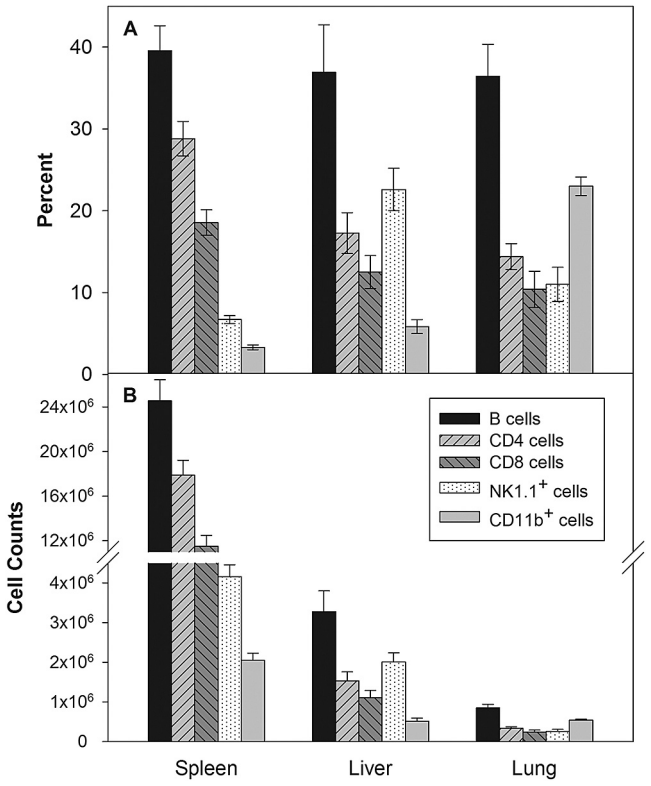

FIG 1 Spleen cell populations differ from liver and lung lymphocyte preparations. Single-cell preparations obtained from naive and immune spleens, livers, and lungs from i.d. vaccinated mice were quantitated and then stained with a panel of fluorescent antibodies to cell surface markers and with a fluorescent viability dye. After exclusion of fragments and aggregates by side scatter (SSC-A) versus forward scatter (FSC-A) and by FSC-W versus FSC-H, cells were initially analyzed for viable lymphocytes (live $CD45^+$ cells). Subpopulations were then quantified. B and T cells were identified as $B220^+$ $CD19^+$ B cells or $TCR\beta^+$ $B220^-$ $CD19^-$ T cells. T cells were further discriminated according to CD4 and CD8a markers. The remaining non-B and non-T cells were analyzed using NK1.1, CD11b, CD11c, and Gr-1 markers. $NK1.1^+$ cells represent both natural killer and NK T cells. $CD11b^+$ cells include neutrophils ($CD11b^+$ $Gr\text{-}1^+$), dendritic cells ($CD11c^+$ $CD11b^+$ $Gr\text{-}1^-$), and macrophages ($CD11b^+$ $Gr\text{-}1^-$ $CD11c^-$). The percentages (A) and numbers (calculated using the total numbers of viable cells obtained per mouse) (B) of leukocytes identified are shown; error bars indicate the standard deviations (SD) of the means of results pooled from six experiments that included both naive cells and cells derived from mice vaccinated with LVS, LVS-R, and HK-LVS.

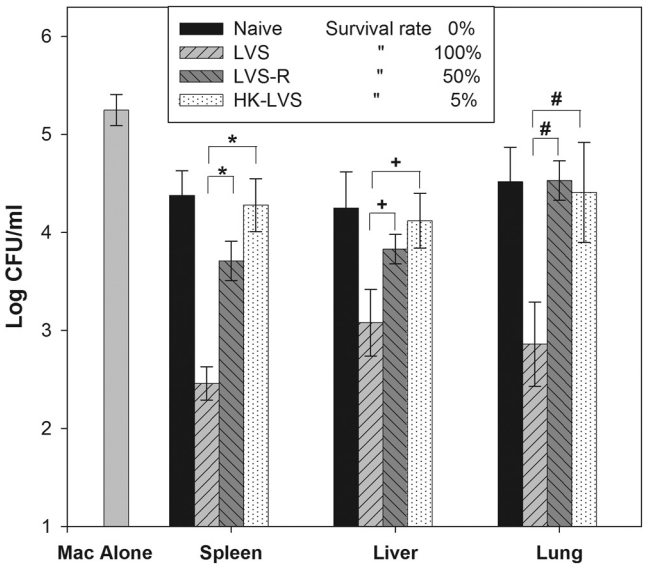

FIG 2 Spleen, liver, and lung lymphocytes from mice vaccinated with LVS-related vaccines exhibit similar hierarchies of control of intramacrophage LVS growth. Bone marrow-derived macrophages from C57BL/6J mice were infected with LVS (Mac alone) and cocultured with lymphocytes obtained from spleens, livers, or lungs of naive or i.d. vaccinated C57BL/6J mice, as indicated. After 2 days of coculture, macrophages were washed, lysed, and plated to evaluate the recovery of intracellular bacteria. Values shown are the mean numbers of CFU/ml of viable bacteria ± SD for triplicate samples. Results shown are from 1 representative experiment of 3 to 7 independent experiments of similar designs and outcomes. *, +, and # indicate, respectively, significant differences ($P < 0.05$) within spleen, liver, or lung cell groups between the recovery of bacteria in cocultures using LVS-immune cells and the recovery of bacteria from either LVS-R-immune cells or HK-LVS-immune cells, as indicated by the brackets. Compared to that from naive splenocytes, the recovery of bacteria from LVS-immune splenocytes is significantly different ($P < 0.05$) from the recovery of bacteria from LVS-R-immune splenocytes, but that from HK-LVS-immune splenocytes is not. Compared to the number of bacteria recovered from naive liver lymphocytes, the number of bacteria recovered from LVS-immune liver lymphocytes or from LVS-R-immune liver lymphocytes is significantly different ($P < 0.05$), but the number recovered from HK-LVS-immune splenocytes is not. Compared to that from naive lung lymphocytes, the recovery of bacteria from LVS-immune lung lymphocytes is significantly different ($P < 0.05$), but that from LVS-R-immune lung lymphocytes or HK-LVS-immune splenocytes is not.

from mice vaccinated with LVS, LVS-R, and HK-LVS. $NK1.1^+$ cells, including both natural killer (NK) cells and NK T cells, comprised a higher proportion of liver leukocytes compared to splenocytes (Fig. 1A). In contrast, $CD11b^+$ cells, comprised of macrophages and, to a lesser extent, dendritic cells and neutrophils, comprised a higher proportion of lung preparations compared to spleen and liver leukocytes (Fig. 1A). The total numbers of lymphocytes obtained from livers or lungs were much lower than those obtained from spleens; yields from livers averaged about 5×10^6 per mouse, and those from lungs were about 1×10^6 to 2×10^6 per mouse, with correspondingly smaller amounts of each subpopulation (Fig. 1B). Lymphocytes from immune mice were then compared for *in vitro* activities by adding them to LVS-infected macrophages, and the impact on replication of LVS bacteria over time in *in vitro* coculture was determined. As shown in Fig. 2, cells derived from livers and lungs of mice vaccinated with different vaccines exhibited patterns of control of intramacrophage bacterial replication similar to those exhibited by splenocytes: spleen and liver lymphocytes derived from LVS-vaccinated mice were the most efficient, followed by those from LVS-R-vaccinated mice and then those derived from HK-LVS-vaccinated mice, which were the least efficient. As illustrated by Fig. 2, lung lymphocytes from some groups (e.g., LVS-R) were usually less efficient in controlling intramacrophage LVS growth than either splenocytes or liver lymphocytes. This is in accordance with a lower proportion of T cells (Fig. 1A), as well as with the reduced viability of recovered lung cells, as observed using both vital dye staining and flow cytometry-based analyses (data not shown).

To further analyze interactions, supernatants and cells were recovered after 2 days of *in vitro* coculture. Supernatants were analyzed for cytokine production by ELISA, recovered cells were characterized by flow cytometry, and mRNA was prepared from recovered cells and analyzed for relative gene expression. The relative amounts of TNF-α, IFN-γ, IL-12, and IL-6 produced by

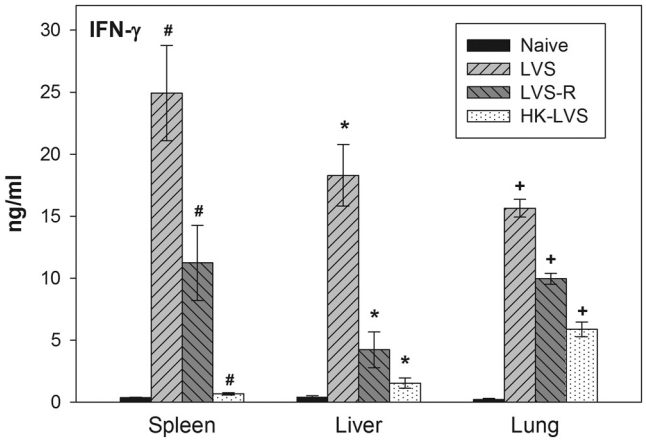

FIG 3 The pattern of IFN-γ production by liver and lung lymphocytes is similar to that of splenocytes. Supernatants from the experiment shown in Fig. 2, using cells obtained from i.d. vaccinated mice, were collected after 2 days of coculture and separated from cells for analyses of cytokines by ELISA. Concentrations were calculated using a standard curve as a reference. Values shown are the mean concentrations in ng/ml ± SD of results from triplicate samples. Results shown are from one representative experiment of five independent experiments of similar designs and outcomes. *, +, and # indicate, respectively, significant differences ($P < 0.05$) within spleen, liver, or lung cell groups between amounts of IFN-γ produced in cocultures using LVS-immune cells, LVS-R-immune cells, or HK-LVS-immune cells; within each tissue, all possible combinations were significantly different from each other.

splenocytes in relation to vaccine efficacy were consistent with the results of previous studies (15). Relative cytokine production by cocultures with liver and lung cells, particularly IFN-γ production, exhibited patterns similar to those observed using splenocytes (Fig. 3). Lymphocytes derived from LVS-vaccinated mice exhibited the highest production of IFN-γ (Fig. 3) and other cytokines (data not shown); smaller amounts were produced by cells obtained from mice vaccinated with the less effective vaccines. Flow cytometry analyses of cells recovered after 2 days of coculture revealed a relative enrichment of T cells in each organ and vaccine group (data not shown) compared to T cell levels in cells initially added to cocultures (Fig. 1A). However, as noted above, recovered lung cells had poor viability in comparison to liver and spleen cells (data not shown). Taken together, these data reveal that liver and lung lymphocytes possess abilities similar to those exhibited by splenocytes to control intramacrophage bacterial growth and to produce cytokines in patterns that reflect relative vaccine efficacy.

Relative gene expression during coculture by *Francisella*-immune liver and lung cells after differential vaccination. Using splenocytes recovered from coculture, we previously identified 12 genes whose relative expression best reflected the hierarchy of *in vivo* vaccine efficacy (15). To evaluate whether relative gene expression of potential correlates of protection was reflected during *in vitro* immune responses by liver and lung lymphocytes, we evaluated the expression of this group of genes in liver and lung cells recovered from cocultures and compared their relative gene expression to that of splenocytes (Table 2). Spleen, liver, and lung cells were cultured for 2 days with LVS-infected macrophages. Cells were then recovered and analyzed for gene expression by real-time PCR. Most of genes exhibited similar expression patterns in liver lymphocytes and splenocytes, which correlated with vaccine efficacy. As observed previously using immune splenocytes, expression of most of these genes was higher in liver and lung cells derived from LVS-vaccinated mice than in cells from LVS-R and HK-LVS-vaccinated mice, and their expression was higher than in either cell type obtained from nonvaccinated mice. In particular, in immune liver cells, the expression profiles of IFN-γ, Il-18bp, IL-27, IL-6, and SOCS-1 was related to the vaccines' hierarchy. In addition, a few genes, including those for TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-22, were more strongly upregulated in liver lymphocytes than in splenocytes, particularly in cells from the LVS-vaccinated group. T-bet was also strongly upregulated in spleen and liver cells from LVS-vaccinated mice, compared to its levels in cells from LVS-R and HK-LVS-vaccinated mice. The degree of differences between vaccine groups was smaller for the remaining genes in all 3 cell types. Further, in contrast to liver lymphocytes, a few genes such as IFN-γ in lung lymphocytes were strongly upregulated only in the LVS group.

Effect of vaccination routes on activities during coculture by *Francisella*-immune liver and lung cells after differential vacci-

TABLE 2 Relative gene expression of potential correlates of protection in cocultures using immune lymphocytes derived from different organs

	Fold change in expression in cells from the indicated organs following the indicated vaccinations[a]								
	Spleen			Liver			Lung		
Gene	LVS	LVS-R	HK-LVS	LVS	LVS-R	HK-LVS	LVS	LVS-R	HK-LVS
IFN-γ	52.3	34.4	1.7	78.0	3.1	0.9	418	7.1	7.1
TNF-α	1.9	1.6	1.0	3.3	1.4	1.2	3.8	2.3	2.0
IL-6	5.6	3.3	1.1	3.0	1.2	0.7	2.4	0.4	0.8
IL-12rβ2	4.2	3.1	1.2	4.8	1.1	1.3	2.2	0.9	2.0
IL-18bp	1.6	2.1	0.9	2.4	1.9	0.7	4.2	1.7	1.2
IL-27	2.3	2.7	1.2	3.8	2.5	1.2	3.0	1.7	3.4
GM-CSF	5.1	6.3	1.4	13.1	2.8	1.3	2.8	0.9	1.2
T-bet	4.2	1.8	1.2	4.6	1.3	1.2	3.2	1.3	2.5
IL-22	3.5	4.9	1.5	39.2	8.6	2.0	3.2	1.1	1.7
CCL7	0.8	0.6	0.5	0.6	1.3	0.8	0.8	0.8	0.5
Irf-1	1.7	1.4	1.3	2.1	1.5	0.9	1.8	1.0	0.6
SOCS-1	1.4	1.9	2.0	3.9	2.2	0.6	3.7	3.3	1.7

[a] Mice were vaccinated i.d. with each of the indicated vaccines. Six weeks later, lymphocytes from spleens, livers, and lungs were recovered from vaccinated mice and cocultured with LVS-infected macrophages. Nonadherent cells were recovered after 2 days of coculture, RNA was prepared, and gene expression was analyzed as described in Materials and Methods. The median fold change in the expression of each gene compared to its expression in naive cells (calculated from 4 to 7 independent experiments) is shown.

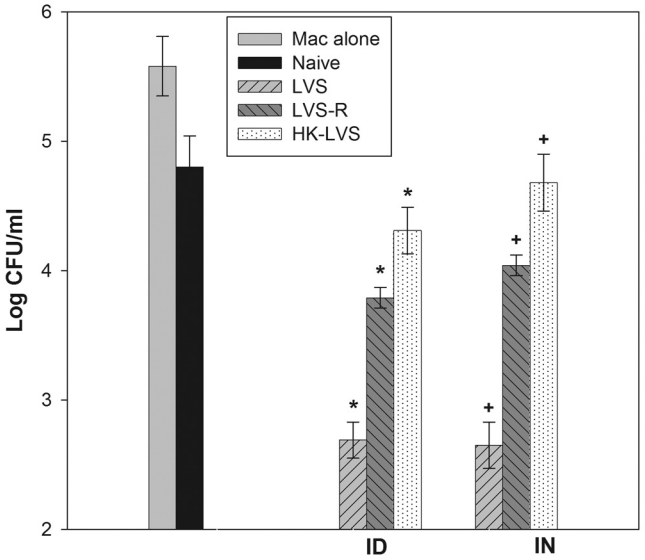

FIG 4 Splenocytes from mice vaccinated i.d. or i.n. with a panel of different vaccines exhibit similar patterns of intramacrophage LVS control. Bone marrow-derived macrophages from C57BL/6J mice were infected with LVS (Mac alone) and cocultured with lymphocytes obtained from spleens of naive C57BL/6J mice or C57BL/6J mice vaccinated i.d. or i.n. with the indicated LVS-related vaccines. After 2 days of coculture, macrophages were washed, lysed, and plated to evaluate the recovery of intracellular bacteria. Values shown are the mean numbers of CFU/ml of viable bacteria ± SD from triplicate samples. Significant differences ($P < 0.05$) between CFU recovered from cocultures using LVS-immune cells, LVS-R-immune cells, or HK-LVS-immune cells obtained from mice vaccinated either i.d. (*) or i.n. (+) are indicated; within each route of vaccination, all possible combinations were significantly different from each other. Results shown are from one representative experiment of four independent experiments of similar designs and outcomes.

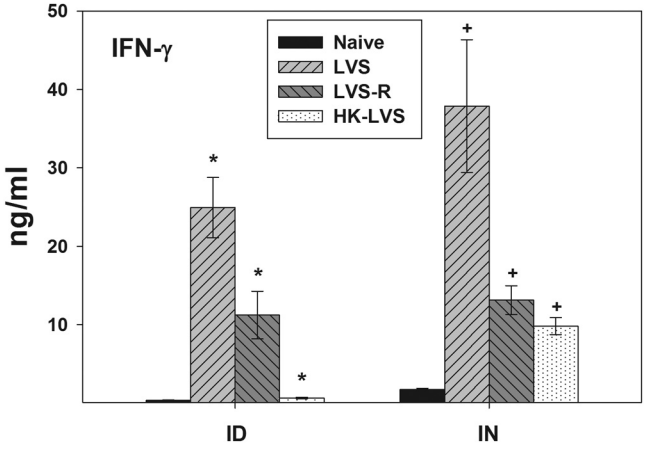

FIG 5 Splenocytes from mice vaccinated i.d. or i.n. with a panel of different vaccines exhibit similar patterns of IFN-γ production. Supernatants collected after 2 days of coculture were analyzed for IFN-γ production by ELISA. Values shown are the mean concentrations (in ng/ml) ± standard deviations of results from of five independent experiments of similar designs and outcomes. Significant differences ($P < 0.05$) between amounts of IFN-γ recovered from cocultures using LVS-immune cells, LVS-R-immune cells, or HK-LVS-immune cells obtained from mice vaccinated either i.d. (*) or i.n. (+) are indicated; within each route of vaccination, all possible combinations were significantly different from each other. Results shown are combined from 2 to 3 experiments of similar designs and outcomes.

nation. To evaluate whether vaccination route impacts the activities of cells from any of the target organs, C57BL/6J mice were vaccinated either by intradermal injection (i.d.), as above described, or by intranasal inoculation (i.n.) of 10^3 LVS, 10^3 LVS-R, or 10^7 HK-LVS. Mice treated i.n. with phosphate-buffered saline (PBS) were used as negative controls. Four to 6 weeks after vaccination, mice were challenged with a lethal dose of LVS i.p. (10^6). Table 1 shows the survival of differentially vaccinated, LVS-challenged mice. All mice vaccinated either i.d. or i.n. with LVS survived i.p. challenge with LVS. In contrast, nonvaccinated mice did not survive LVS challenge, as expected. Mice vaccinated i.d. with LVS-R or HK-LVS exhibited, respectively, 50% or 5% survival of i.p. LVS challenge. Intranasal inoculation of the two suboptimal vaccines resulted in 80% or 4% of the survival of mice challenged i.p. with LVS, which for the LVS-R-vaccinated group was substantially better than the survival provided by i.d. vaccination.

In parallel with survival studies, spleen, liver, and lung lymphocytes derived from both i.d. and i.n. vaccinated mice (and naive mice) were analyzed for *in vitro* activities. Immune lymphocytes derived from either i.d. or i.n. vaccinated mice were cocultured with LVS-infected macrophages, as described above. After 2 days, cells and supernatants were recovered, macrophages were lysed, and lysates were plated for bacterial quantification. Spleen cells derived from either i.d. or i.n. vaccinated mice exhibited similar abilities to control *in vitro* bacterial growth (Fig. 4). However, liver and lung cells derived from mice vaccinated i.n. with LVS, LVS-R, and HK-LVS showed less control of intramacrophage LVS replication than those derived from i.d. vaccinated mice (data not shown).

To further evaluate *in vitro* T cell activities, production of IFN-γ was measured in supernatants collected after coculture of immune spleen (Fig. 5), liver, or lung lymphocytes (data not shown) from either i.d. or i.n. vaccinated mice with LVS-infected macrophages. As with i.d. vaccination, lymphocytes derived from all three organs of i.n. vaccinated mice produced IFN-γ in response to stimuli by *in vitro* coculture with LVS-infected macrophages. The substantial production of IFN-γ by lymphocytes from all organs and after both vaccination strategies indicates successful priming and activation of T cells during coculture, since only T cells produce IFN-γ in this tissue culture model (18). However, as noted previously (18), the relative amounts of IFN-γ produced in cocultures were not directly related to the extent of control of intramacrophage LVS growth.

To evaluate whether i.n. vaccination influences relative gene expression of potential correlates, splenocytes derived from mice vaccinated with the panel of different vaccines by either the i.d. or the i.n. route were recovered from coculture experiments and analyzed for relative gene expression of 12 potential correlates of protection (Table 3). Compared to splenocytes from i.d.-vaccinated mice, splenocytes from i.n.-vaccinated mice exhibited differential patterns of gene expression only for IL-27 and T-bet, and to a lesser extent GM-CSF. These mediators were expressed at higher levels in LVS-derived splenocytes than in splenocytes from LVS-R- and HK-LVS-vaccinated mice. In addition, a few more genes (e.g., the TNF-α gene) were more upregulated in the LVS and LVS-R groups than in the HK-LVS group; other genes (e.g., the IFN-γ and IL-18bp genes) were more upregulated in the LVS group, but there were no differences between levels of expression

TABLE 3 Relative gene expression of potential correlates of protection in coculture-derived splenocytes from i.d. and i.n. vaccinated mice

Gene	Fold change in expression after administration of the indicated vaccine by the following route[a]:					
	i.d.			i.n.		
	LVS	LVS-R	HK-LVS	LVS	LVS-R	HK-LVS
IFN-γ	52.3	34.4	1.7	11.5	4.1	3.9
TNF-α	1.9	1.6	1.0	2.6	2.2	1.5
IL-6	5.6	3.3	1.1	1.2	1.3	0.9
IL-12rβ2	4.2	3.1	1.2	2.5	5.0	4.1
IL-18bp	1.6	2.1	0.9	1.8	0.7	1.0
IL-27	2.3	2.7	1.2	3.3	1.9	0.8
GM-CSF	5.1	6.3	1.4	2.2	1.8	1.5
T-bet	4.2	1.8	1.2	4.3	3.8	1.7
IL-22	3.5	4.9	1.5	1.1	1.6	2.5
CCL7	0.8	0.6	0.5	0.7	0.3	0.6
Irf-1	1.7	1.4	1.3	1.7	0.9	1.4
SOCS-1	1.4	1.9	2.0	3.6	3.5	3.1

[a] Mice were vaccinated i.d. or i.n. with each of the indicated vaccines. Six weeks later, lymphocytes from spleens were recovered from vaccinated mice and cocultured with LVS-infected macrophages. Nonadherent cells were recovered after 2 days of coculture, RNA was prepared, and gene expression was analyzed as described in Materials and Methods. The median fold change in the expression of each gene compared to its expression in naive cells (calculated from 3 to 5 independent experiments) is shown.

in the LVS-R and HK-LVS groups. However, for the remainder of the genes, few differences were observed between the three groups. The more subtle difference between splenocytes from i.n. vaccinated mice is illustrated by IFN-γ gene expression, whose median level in splenocytes derived from mice vaccinated i.n. with LVS was only 11.5-fold higher than its level in splenocytes of nonvaccinated mice. This is much less than the 52-fold upregulation observed in cells from mice vaccinated with LVS i.d. Taken together, these data indicate that spleen, liver, and lung lymphocytes obtained following i.n. and i.d. vaccination possess similar abilities to control intramacrophage bacterial growth. However, cytokine production and relative gene expression did not entirely correlate between splenocytes of i.d. and i.n. vaccinated mice, likely reflecting different biological mechanisms.

Multivariate analyses and modeling. Quantification of correlates of protection is critical to establishing vaccination status. However, biological or technical variability in any single parameter may limit robust correlations between vaccine efficacy and *in vitro* data. To overcome this problem, we examined the mathematical models of multivariate analyses to distinguish nonvaccinated and differentially vaccinated groups of mice. Using results from splenocytes of naive and i.d. vaccinated mice, a large number of logistic regression models were fitted to normalized data from one, two, or three variables. Variables used were control of intramacrophage bacterial replication (as reflected by CFU data) or normalized relative gene expression of selected mediators. Five types of models were considered, and associated parameters were calculated (see Materials and Methods). Analyses of relative gene expression of the panel of 12 genes confirmed our previous findings (15). Not surprisingly, the gene whose expression best distinguished between naive mice and mice from any vaccine group, as well as between vaccine groups, was IFN-γ, but relative expression of IFN-γ alone did not always distinguish well between cells from naive mice and cells from mice vaccinated with HK-LVS or mice vaccinated with LVS-R versus with LVS (Fig. 6A). We observed a different, but still imperfect, pattern of separation between vaccine groups when CFU data were subjected to the same type of analyses (Fig. 6B); in this case, the resulting model successfully discriminated between the LVS group and the LVS-R group. Notably, when Spearman correlation analyses were performed between any two variables, numbers of CFU were not strongly related to the relative gene expression of any individual mediator, except IFN-γ, for which there was a significant inverse correlation with numbers of CFU (see Table S1 in the supplemental material). The latter results imply that control of intramacrophage CFU levels reflect a mechanism(s) that is largely independent of the expression of any single cytokine gene, except IFN-γ. Therefore, we next integrated the CFU data with the gene expression data, either individually or in multiple combinations; groups of two or three cytokine genes without CFU data were also analyzed. A model combining IFN-γ and CFU data separated almost all groups but did not differentiate between responses from the naive mice and HK-LVS-vaccinated mice (Fig. 6C). Notably, combinations of two mediators, such as IFN-γ and T-bet, or several combinations of three genes distinguished well between most vaccination groups (Table S2). However, the strongest separation with the best *P* values, R^2 values, and cross-validation (CV) values resulted from combining CFU data with expression data from two cytokine genes, such as T-bet and GM-CSF (Fig. 6D). Of note, several combinations, including numbers of CFU and data from two cytokine genes, resulted in similar findings (Tables 4 and S2). Collectively, these results indicate that including more complex models with three variables, particularly mechanistically independent variables, provides a powerful and robust means of predicting the extent of protection.

DISCUSSION

New protective vaccines against intracellular bacteria such as *Francisella* and *Mycobacterium tuberculosis* are needed, as are methods to monitor immune responses of vaccinated subjects that reflect protection. For infections such as these for which field trials are difficult, the identification of correlates of protection may represent the most feasible means to extrapolate efficacy to humans. However, at present there are no validated methods for predicting vaccine-induced protection for *Francisella* or any other intracellular pathogen. Different approaches have been investigated, particularly in studies of *M. tuberculosis*, but have not yet been successful. Although Th1 immune responses are readily in-

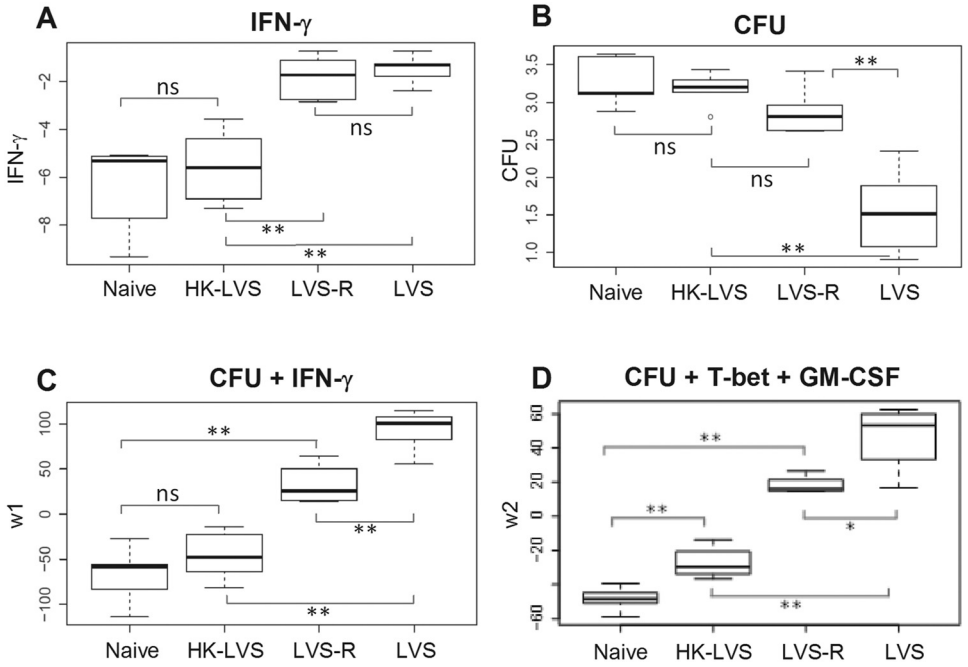

FIG 6 Gene expression and CFUs separate cells from different vaccine groups. Data utilized in these analyses were obtained from naive and immune splenocytes evaluated in five independent experiments in which all four vaccines were given i.d. Models were generated as described in Materials and Methods, using normalized data from splenocytes' control of intramacrophage bacterial replication, as reflected by recovery of CFU (Fig. 2) and relative gene expression (Table 2). Results are shown for IFN-γ, CFU, and two selected models. Here, ±, *, and ** denote nonsignificant (ns) positive/negative differences (P value ≥ 0.05), significant differences ($0.05 > P$ value ≥ 0.01), and highly significant differences (P value < 0.01), respectively. Box plots with the dark line depicting the median value, the box representing the range between the 1st and 3rd quartiles, and the whiskers representing the minimum and maximum values are shown for normalized IFN-γ values (A), normalized numbers of CFU (B), a linear combination of normalized IFN-γ and CFU values, where $w1 = 180 - 38.7$ CFU $+ 18.5$ IFN-γ (C), and a linear combination of normalized CFU, T-bet, and GM-CSF values, where $w2 = 260 - 15.3$ CFU $+ 10.6$ T-bet $+ 18.5$ g $-$ Csf2 (D).

duced by *M. tuberculosis* infection or *Mycobacterium bovis* BCG vaccination, neither IFN-γ production nor TNF-α production has correlated with protection against tuberculosis in either animal studies or clinical trials (19–21). More recently, multifunctional cytokine-producing T cells (MFCs) have been proposed to correlate with vaccine efficacy (22, 23). Although BCG vaccination appears to provide better protection against tuberculosis in children than in adults (24, 25), both children and adults immunized with BCG exhibited similar levels of *ex vivo* cytokine production and MFC frequencies (26), and in BCG-vaccinated babies neither parameter correlated with protection against disease (27).

As in *M. tuberculosis* infections, T cell-mediated Th1 immune responses are considered essential to provide protective immune responses against infections with *Francisella*, but correlates are unknown. Eneslätt and colleagues (28) discriminated between *Francisella*-immune people and nonimmune people by developing T cell signatures, based on assessing the ability of *in vitro*-stimulated peripheral blood mononuclear cells (PBMCs) to proliferate and to produce Th1-related cytokines such as IFN-γ and MIP-1β. Similarly, using a mouse model, the systemic production of IFN-γ, TNF-α, and MCP-1 within a few days after vaccination with attenuated type A *Francisella* mutants of different efficacies partially predicted the degree of protection against respiratory type A challenge, but measurement of serum antibodies did not (10). However, some evidence indicates that antibody responses may contribute to protection against *Francisella*. The relative importance of cell-mediated and humoral immune responses appears to vary according to the use of different animal models and types of vaccine (29, 30). Nonetheless, anti-*Francisella* antibody levels alone have generally not been useful in predicting protection following either tularemia infection or vaccination in people (14).

Using a mouse model, we previously discriminated between vaccines of different efficacies against systemic (i.p.) LVS challenge by analyzing the ability of *Francisella*-immune splenocytes to control intramacrophage LVS replication *in vitro*, which further correlated with upregulation of certain genes during coculture (15). Future studies will be needed to examine the relationship between control of intramacrophage bacterial growth, gene expression, and respiratory (aerosol or intranasal) challenge. Of note, while control of intramacrophage replication of fully virulent strains of *Francisella* is obviously of interest, the much faster replication rate *in vitro* of strains such as type A *F. tularensis* SchuS4 limits the sensitivity of this coculture system, and thus we have used LVS as a surrogate infecting agent (18). This is further supported by our previous observation that mice vaccinated with LVS, LVS-G, LVS-R, or heat-killed LVS exhibit the same hierarchy of protection against challenge with either LVS i.p. or SchuS4 i.d. (15). In the present report, we show that the properties originally identified using splenocytes are representative when analyzing lymphocytes from other nonprofessional immune organs that are colonized during anti-*Francisella* vaccination, namely liver and lung. The *in vitro* coculture approach is intended to model *in vivo* interactions in tissues between infecting bacteria, their target host

TABLE 4 Discrimination of differentially vaccinated groups of mice by multivariate analyses using CFU, gene expression, or combinations of CFU with one or two genes[a]

Model	Result from a comparison of the indicated groups				R^2	CV
	HK-LVS vs naive	LVS-R vs HK-LVS	LVS vs HK-LVS	LVS vs LVS-R		
CFU	+	+	**	**	0.79	80
IFN-γ	+	**	**	+	0.77	95
IL-6	+	+	+	–	0.21	55
IL-12rβ2	+	*	*	+	0.51	80
T-bet	+	+	+	+	0.23	60
GM-CSF	+	*	*	–	0.46	80
TNF-α	+	+	+	+	0.29	60
IL-27	+	+	+	+	0.34	75
IL-22	+	*	+	–	0.54	70
CFU, IFN-γ	+	**	**	**	0.88	100
CFU, IL-6	+	+	**	**	0.75	70
CFU, IL-12rβ2	*	**	**	*	0.78	85
CFU, T-bet	+	+	**	**	0.78	75
CFU, GM-CSF	+	*	**	*	0.82	85
CFU, TNF-α	+	**	**	**	0.74	90
CFU, IL-27	+	*	**	**	0.71	90
CFU, IL-22	+	**	**	*	0.80	90
IFN-γ, T-bet	+	**	**	*	0.84	90
CFU, IFN-γ, IL-27	*	**	**	**	0.88	90
CFU, TNF-α,IL-22	*	**	**	*	0.80	100
CFU, T-bet, GM-CSF	**	**	**	*	0.92	100
CFU, IL-6, IL-12rβ2	*	**	**	*	0.80	80

[a] Data utilized in these analyses were obtained from splenocytes evaluated in five independent experiments in which all four vaccines were given i.d. Models were generated as described in Materials and Methods, using normalized data from splenocytes' control of intramacrophage bacterial replication, as reflected by the recovery of CFU (Fig. 2) and relative gene expression (Table 2). Results are shown for a set of selected models without interaction terms; see Fig. 6 for related graphic depictions of results using IFN-γ alone, CFU alone, CFU plus IFN-γ, and CFU plus T-bet plus GM-CSF. Summary results for a larger set of selected models are shown here. Each model produces a variable, *w*, that is a linear combination of a set of explanatory variables. The differences between the vaccine groups for variable *w* are indicated as positive and nonsignificant (+, i.e., a positive difference between the group sample means but the *P* for which is >0.05) or negative and nonsignificant (−, i.e., a negative difference between the group sample means but for which the *P* is >0.05), positive and significant (*, $P \leq 0.05$), or positive and highly significant (**, $P \leq 0.01$). R^2 is the model's coefficient of determination, and CV (leave-one-out cross-validation) shows the model's estimated ability to predict an individual as either belonging to the naive/HK-LVS group or the LVS-R/LVS group (a high value corresponds to a high ability to predict). The results correspond to the fitted variable, *w*, and coefficients in the models can be negative; e.g., the slope coefficient in the pure CFU model (1st row) was negative (this explains the positive differences).

cells (in this case, macrophages), and responding lymphocytes, in which the presumed goal of the interactions is to limit bacterial replication and spread (18). In these cocultures, only T cells are active, although *in vivo* other lymphocytes likely regulate interactions and thus have been routinely included in assays (18). The proportion and therefore number of T cells (both T cell receptor β-positive [TCRβ$^+$] CD4$^+$ and TCRβ$^+$ CD8$^+$ cells) added to cocultures were higher among *Francisella*-immune splenocytes than among liver and lung lymphocytes (Fig. 1A and B). Thus, splenocytes might be expected to effect stronger control and differential gene expression, but this was not always the case (Table 2), implying other informative biological differences.

We therefore considered it important to perform detailed comparisons of splenocytes to lymphocytes from other involved tissues, where vaccine-induced control of subsequent infections would be required. Liver is obviously not considered an immune organ, but it is well equipped to protect itself from pathogens, particularly bacterial endotoxin, while tolerating foreign antigens (31). These needs are met by both resident immune cell populations with relatively more innate immune cells, particularly NK cells, dendritic cells, and macrophage-like Kupffer cells (31), as well as recruited effector lymphocytes regulated by liver-specific combinations of adhesion molecules (32). During infections of mice with LVS, lesions in the liver are dominated by Gr-1$^+$ Mac-1$^+$ immature myeloid cells and major histocompatibility complex class II-positive (MHC-II$^+$) Mac-1$^+$ macrophages (33), but the functions of liver T lymphocytes have only rarely been studied (34). The present data suggest that despite the somewhat different compositions of cells in the different preparations (Fig. 1A), the viability of LVS-immune liver lymphocytes in cocultures, the recovery of viable T cells, the quality of mRNA obtained, cytokine production, and, ultimately, functions in terms of intramacrophage growth control are also comparable to those of splenic lymphocytes (Fig. 2 and 3). Similarly, relative gene expression patterns in *Francisella*-immune liver cells were similar to those of *Francisella*-immune splenocytes (Table 2) and reflected the *in vivo* hierarchy of vaccine protection. A few genes, including TNF-α, GM-CSF, and IL-22, were more strongly upregulated in liver lymphocytes than in splenic lymphocytes. We are currently investigating whether these gene expression differences are due to different distributions of T cell subpopulations in liver and spleen and/or reflect different biological activities of liver lymphocytes.

The lung is obviously a critical site for interactions between respiratory pathogens and the immune system, with even fewer resident lymphocytes than other organs but abundant alveolar macrophages. Upon aerosol exposure to *M. tuberculosis*, cytokines produced by macrophages and dendritic cells play critical roles in controlling the initial infection and promoting adaptive T cell responses (35). The functions of lung lymphocytes during infections, however, have received relatively less attention (36–38).

These cells are difficult to study *ex vivo*, particularly when longer time frames in tissue culture are of interest. Here, the numbers and health of lung lymphocytes recovered after 2 to 3 days of *in vitro* coculture were suboptimal; only 10 to 20% were viable, and the quality of RNA purified from lung cells was also reduced, as reflected by a consistently lower RNA integrity number (calculated by Agilent Bioanalyzer software [data not shown]). Despite these suboptimal qualities, a hierarchy of control consistent with *in vivo* protection was still evident: LVS-immune lung cells controlled intramacrophage bacterial growth more efficiently than those cells derived from HK-LVS- and LVS-R vaccinated mice (Fig. 2), which was reflected by similar patterns of IFN-γ production (Fig. 3). The extent of that control, however, was typically less for lung lymphocytes from each vaccine group than for the respective spleen and liver samples. This observation is consistent with previous findings, which demonstrated that lower numbers of lymphocytes in the cocultures still controlled bacterial replication well (15), and illustrates the sensitivity of the methodology. However, differences in relative levels of gene expression in lung lymphocytes obtained after different vaccinations were less evident in lung lymphocytes (Table 1). This likely reflects the lower proportion of T cells in lung lymphocytes (Fig. 1A) and the lower quality of mRNA obtained (see above; data not shown), combined with potential biological differences between organs. The technical issues likely limit full evaluation of lung T cell activities and mRNA. However, as illustrated by the substantial upregulation of IFN-γ gene expression (Table 2) and corresponding production of IFN-γ protein (Fig. 3) in cocultures with lung lymphocytes, protein quantification of selected cytokines by ELISA may be a valid alternative.

Different routes of vaccination induce qualitatively different immune responses under many circumstances, including different responses by cells in involved organs. For example, mice vaccinated with BCG and then boosted i.d. with a recombinant adenovirus expressing *M. tuberculosis* Ag85A exhibited strong splenic Th1 T cell responses in spleens compared to those not boosted, but exhibited no improvement in controlling an aerosol challenge; in contrast, those boosted i.n. had improved control of aerosol *M. tuberculosis* challenge but weak splenic responses (39). Here, LVS-R provided better protection against i.p. challenge *in vivo* when administered i.n. than when administered by i.d. vaccination (Table 1). Improved protection by LVS-R administered i.n. might involve factors not reflected by *in vitro* cocultures, such as mucosal barriers and mucosal antibody responses that may alter LVS-R dissemination and systemic immune responses. Interestingly, *in vitro* control of intramacrophage bacterial growth and IFN-γ production was similar between all types of cells obtained after i.d. vaccination and i.n. vaccination, but differences in relative gene expression between vaccine groups were more subtle (Table 3) when splenocytes from i.n. vaccinated mice were studied (Table 1 and reference 15). Of the 12 candidate correlate genes tested, only IL-27 and T-bet exhibited clear patterns of differential expression in splenocytes from i.n. vaccinated mice. Further studies will be necessary to evaluate whether qualitatively different lymphocyte functions develop after different vaccination routes and/or whether the frequency of LVS-specific T cells is lower in spleens from i.n. vaccinated mice than in those from i.d. vaccinated mice. Such results may suggest that cells from organs other than spleen may better reflect respiratory vaccination.

Finally, we propose an approach to quantitatively model correlates of protection, by applying multivariate analyses to integrate results generated from the measurements of intramacrophage bacterial replication *in vitro* and from the quantification of gene expression by *F. tularensis*-specific T cells. Separately, these two methods have several advantages: the *in vitro* coculture methodology is highly sensitive but may occur via a number of mechanisms, while relative gene expression by lymphocytes provides information regarding individual mediators. By combining data from these two complementary methodologies, we identified several models that strongly differentiate degrees of vaccine efficacy. In ongoing studies, we are exploring the value of adding measurements of serum anti-LVS antibodies, which would provide another complementary method that is distinct from cell-mediated immunity, to further improve prediction of protection. Of note, a major, and unique, goal of this approach is not only to distinguish between "successful" and "failed" vaccinations but also to estimate the relative proportion of protected people within a population. Further, these models may aid in refining the list of candidate correlate mediators so that the biological relevance of each individual mediator during protection against lethal challenge with LVS, and with fully virulent tularemia, can be investigated separately (16, 17). Further, the models may facilitate the identification of useful markers of vaccination, even if the mediators in question are not essential during protective responses. Most importantly, the models may represent a practical approach to evaluate and monitor correlative anti-*F. tularensis* immune responses after vaccination.

Because relative gene expression is influenced by the sources of cells under study and by vaccination route, additional potential markers and mechanistically important mediators may be identified by further study of different tissue sites. We propose that the *in vitro* coculture strategy is useful not only for exploring such mechanisms but also for functional clinical assays. In tuberculosis studies, similar approaches often referred to as "mycobacterial growth inhibition assays" have shown promise in evaluating mouse (40) and human (19) immune responses to vaccination or infection and potentially in predicting protection. These include formats such as use of human peripheral blood cells and whole blood, which can lead to transcriptional signatures and be informative in clinical settings (41, 42). To that end, we are currently evaluating the functions of mouse peripheral blood lymphocytes (PBLs). Indeed, PBLs obtained from mice vaccinated with LVS control the intramacrophage replication of LVS as effectively as LVS-immune splenocytes, and exhibit upregulated expression of a panel of genes similar to those detected in LVS-immune splenocytes (R. De Pascalis, L. Mittereder, N. J. Kennett, K. L. Elkins, unpublished results). The success demonstrated here therefore justifies further development of this approach, with the goal of advancing to human studies.

MATERIALS AND METHODS

Experimental animals and design. Six- to 12-week-old wild-type male C57BL/6J mice were purchased from Jackson Laboratories, Bar Harbor, ME. All mice were housed in sterile microisolator cages in a barrier environment, fed autoclaved food and water *ad libitum*, and routinely tested for common murine pathogens by the diagnostic service of the Division of Veterinary Service, CBER. The animals were matched so that their variability with respect to age and time of challenge was minimized within the experiments. For model development, five independent experiments were performed as described below. Within each experiment, all four vaccine

groups (LVS, LVS-R, HK-LVS, and naive) were represented. Other results reflect outcomes from 3 to 7 experiments.

Bacteria and growth conditions. Frozen aliquots of mid-log-phase growth of *F. tularensis* LVS (American Type Culture Collection catalog no. 29684) and *F. tularensis* LVS-R, originally obtained from Francis Nano (University of Victoria, Victoria, British Columbia, Canada), were used for vaccination and lethal challenges. Intraperitoneal, i.n., and i.d. 50% lethal doses (LD_{50}s) and times to death of BALB/cByJ male mice were previously assessed for each bacterial stock at the time of preparation (18). In addition, to obtain heat-killed LVS (HK-LVS), aliquots of LVS were treated at 60°C for 40 min, and bacterial killing was confirmed by plating.

Bacterial immunization and challenge. Parenteral i.d. immunizations were performed as previously described (15). Briefly, 1×10^4 CFU LVS, 1×10^4 CFU LVS-R, or the amount equivalent to 1×10^8 HK-LVS diluted in 100 μl phosphate-buffered saline (PBS) (BioWhittaker, Walkersville, MD) were used. For i.n. immunization, groups of mice were initially anesthetized and then administered 1×10^3 CFU LVS, 1×10^3 CFU LVS-R, or the amount equivalent to 1×10^7 HK-LVS diluted in 20 μl PBS i.n. in one nostril. Four to 6 weeks after vaccination, mice were challenged with 10^6 LVS i.p. and monitored for survival. At the time of vaccination, the number of CFU of actual doses inoculated i.d. or i.p. or administered i.n. were confirmed by plate counting.

Preparation of lymphocytes and flow cytometry. Single-cell suspensions of spleen, liver, and lung lymphocytes were generated for *in vitro* culture and flow cytometry by standard techniques, as previously described (15). No bacterial CFU were detected in organ homogenates at the time of harvest. Cell viability was assessed by exclusion of trypan blue and flow cytometry using a commercially available kit (Live/Dead Staining Kit; Invitrogen). Cell surface phenotype was determined using a panel of murine cell surface markers, and flow cytometry was performed as previously described (15). Multiparameter analyses were performed using a Becton, Dickinson LSR II flow cytometer (San Jose, CA) and analyzed using FlowJo software (TreeStar, Inc.).

Coculture of lymphocytes with LVS-infected bone marrow-derived macrophages. Cocultures were performed in 24-well plates as previously described, using confluent monolayers of ~10^7 bone marrow-derived macrophages and 5×10^6 lymphocytes (15, 18). Single-cell suspensions of splenic, liver, and lung lymphocytes derived from vaccinated and nonvaccinated control groups were harvested after 48 h of coculture. Cells were assessed for viability and for cell surface phenotype by flow cytometry. Cells to be assessed for gene expression by reverse transcription-quantitative PCR (qRT-PCR) were pelleted, immersed in RNAlater (Ambion, Austin, TX), and stored at −70°C until further characterization. Similarly, supernatants from harvested cells were stored at −70°C for further analyses. Adherent macrophages were lysed and loads of intracellular bacteria determined, as previously described (15, 18).

Assessment of supernatants and lymphocytes. Supernatants recovered from *in vitro* cocultures were assayed using standard sandwich ELISAs, according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). Quantification of IFN-γ, TNF-α, IL-6, and IL-12 was assessed by comparison to recombinant proteins as standards (BD Pharmingen). For evaluation of gene expression, total RNA extraction from samples, assessment with a Bioanalyzer (Agilent Technologies, Santa Clara, CA), and cDNA synthesis were performed using commercially available kits and by following the manufacturers' instructions, as previously described (15). Semiquantitative real-time PCR was completed with a ViiA 7 sequence detection system (Applied Biosystems, Carlsbad, CA). Fiftyfold-diluted cDNA synthesized from 1 μg of total RNA was further diluted in a 20-μl volume of PCR mix (Applied Biosystems) containing primers and probes. Twenty-two genes, including 12 that were previously identified as high-priority potential correlates of protection against *F. tularensis* (15), were analyzed. GAPDH and GUSB housekeeping genes were used to normalize the level of mRNA of each gene. For all cytokines, the change in threshold cycle (ΔC_T) value was calculated by taking the difference between the C_T value of the housekeeping gene and the C_T value of the cytokine.

Multivariate data analyses and modeling. For these analyses, data from five replicate experiments of identical designs were used. To reduce the experimental variation, theΔC_T values were further normalized. Specifically, $\Delta C_{T\,ijk}$ was used to denote the ΔC_T value for gene *i*, treatment *j*, and experiment *k*. Each experiment contained four treatment groups (i.e., LVS, LVS-R, HK-LVS, and naive [$n_{\text{treatment}} = 4$]), and data from the expression of 22 genes were used ($n_{\text{gene}} = 22$), in addition to data from bacterial recovery as reflected by numbers of CFU. The average ΔC_T value for experiment *k* was denoted $\Delta C_{T\,k}$ and was obtained as

$$\Delta C_{Tk} = \frac{\sum_{i=1}^{n_{\text{gene}}} \sum_{j=1}^{n_{\text{treatment}}} \Delta C_{Tijk}}{n_{\text{gene}} n_{\text{treatment}}}$$

The average ΔC_T value for experiments using GAPDH as the housekeeping gene was taken as the baseline and was denoted $\Delta C_{T\,\text{GAPDH}}$. The normalized ΔC_T values, denoted $N\Delta C_{T\,ijk}$, were then obtained as follows: $N\Delta C_{T\,ijk} = \Delta C_{T\,ijk} + (\Delta C_{T\,\text{GAPDH}} - \Delta C_{T\,k})$. The CFU values were also similarly normalized. Specifically, CFU_{jk} denoted the CFU value for treatment *j* in experiment *k*. The normalized CFU values, denoted N-CFU_{jk}, were obtained with the formula $\text{CFU}_{jk} + (\text{CFU}_{\text{GAPDH}} - \text{CFU}_k)$, where

$$\text{CFU}_k = \frac{\sum_{j=1}^{n_{\text{treatment}}} \text{CFU}_{jk}}{n_{\text{treatment}}}$$

and where $\text{CFU}_{\text{GAPDH}}$ is the average CFU_k value for experiments using GAPDH.

After normalization, we first applied logistic regression to distinguish the naive and HK-LVS samples from the LVS-R and LVS samples. A large number of logistic regression models (in total, 741 models) with one, two, or three explanatory variables (CFU and various cytokines) were fitted to the data using five types of models:

$$w = \alpha + \beta_1 x_1, \quad \text{(a)}$$

$$w = \alpha + \beta_1 x_1 + \beta_2 x_2, \quad \text{(b)}$$

$$w = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1 x_2, \quad \text{(c)}$$

$$w = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3, \quad \text{(d)}$$

$$w = \alpha + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1 x_2 + \beta 5 x_1 x_3 + \beta_6 x_2 x_3, \quad \text{(e)}$$

where x_1, x_2, and x_3 denote values for different cytokines or the number of CFU. The models were used to determine the probability that a sample belongs to the LVS-R/LVS group, where the relation between *P* and the fitted value *w* is given by $P = 1/(1 + e^{-w})$. For each model, the value of the linear combination *w* was calculated for all samples. Each model was then further evaluated by considering (i) the model's performance as a predictive model (i.e., its ability to distinguish between the two major groups), (ii) the ability of variable *w* to distinguish between pairs of different vaccines (i.e., between HK-LVS and naive, between LVS-R and HK-LVS, between LVS and HK-LVS, and between LVS and LVS-R), and (iii) the value of the coefficient of determination (R^2). The model's performance as a predictive model was estimated using leave-one-out cross-validation (CV) (Table S2). The nonparametric Wilcoxon rank sum test with a one-sided alternative hypothesis (>) was used for the pairwise comparisons. The coefficient of determination (R^2) for variable *w* was obtained using standard techniques. We considered a "good" model to have a high CV value, a high R^2 value, and significant separation between all four vaccine groups.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00936-13/-/DCSupplemental.

Table S1, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.1 MB.

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