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Activities of Murine Peripheral Blood Lymphocytes Provide Immune Correlates That Predict *Francisella tularensis* Vaccine Efficacy

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We previously identified potential correlates of vaccine-induced protection against *Francisella tularensis* using murine splenocytes and further demonstrated that the relative levels of gene expression varied significantly between tissues. In contrast to splenocytes, peripheral blood leukocytes (PBLs) represent a means to bridge vaccine efficacy in animal models to that in humans. Here we take advantage of this easily accessible source of immune cells to investigate cell-mediated immune responses against tularemia, whose sporadic incidence makes clinical trials of vaccines difficult. Using PBLs from mice vaccinated with *F. tularensis* Live Vaccine Strain (LVS) and related attenuated strains, we combined the control of *in vitro Francisella* replication within macrophages with gene expression analyses. The *in vitro* functions of PBLs, particularly the control of intramacrophage LVS replication, reflected the hierarchy of *in vivo* protection conferred by LVS-derived vaccines. Moreover, several genes previously identified by the evaluation of splenocytes were also found to be differentially expressed in immune PBLs. In addition, more extensive screening identified additional potential correlates of protection. Finally, expression of selected genes in mouse PBLs obtained shortly after vaccination, without *ex vivo* restimulation, was different among vaccine groups, suggesting a potential tool to monitor efficacious vaccine-induced immune responses against *F. tularensis*. Our studies demonstrate that murine PBLs can be used productively to identify potential correlates of protection against *F. tularensis* and to expand and refine a comprehensive set of protective correlates.

Concerns about potential biowarfare pathogens have prompted the research community to develop vaccines against category A agents of bioterrorism, such as *Francisella tularensis* (1–3). However, the sporadic incidence of tularemia (4, 5), the disease caused by *F. tularensis*, represents a limitation for the testing of new vaccine candidates in humans. Therefore, the identification and the use of correlates of protection may provide a means to monitor immune responses against *F. tularensis* in animal models and to bridge the findings of preclinical studies with animals to the response in humans (2). *F. tularensis* induces antibody responses (6–9), and in mice, B-cell functions contribute to survival against lethal challenge with *F. tularensis* Live Vaccine Strain (LVS) (10–12). However, *F. tularensis* is an intracellular bacterium, and T-cell immune responses play crucial roles for long-term protection (13–15). We have previously focused on the development of methods to identify T-cell-mediated correlates of protection (16). In particular, we have developed a method using *in vitro* stimulation of splenocytes from vaccinated mice with LVS-infected macrophages. This allowed the identification of factors that correlated with different levels of vaccine efficacy (16). The mediators identified included cytokines and other mediators known to play mechanistic roles, such as gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) (13, 14), and those that were subsequently found to play critical roles, such as interleukin-6 (IL-6) (17) and IL-12 receptor β2 (IL-12rβ2) (18), thus validating the approach. In addition, when the quantification of multiple selected markers was combined in multivariate analyses, the resulting models were significantly correlated with survival (19). However, our studies also found that the expression of potential correlates of protection varied slightly among mouse strains (12), routes of injection (19), and, importantly, the tissue sources of immune cells (19).

Peripheral blood leukocytes (PBLs) represent the only easily accessible source of immune cells from human subjects and therefore the ideal source for investigation of the cell-mediated immune responses of infected or vaccinated subjects (20–23). In addition, cell-mediated immunity can readily be detected using PBLs several years after natural *F. tularensis* infection or after vaccination with LVS (24).

In the study described in this report, we extended our work to the identification of potential correlates of protection against *F. tularensis* using PBLs from mice immunized with LVS-derived vaccines, with the purpose of validating this approach with this readily accessible source of immune tissue. Following the initial success using PBLs, we expanded the screening to additional immune-related factors and refined the list of potential correlates of protection. Several of the newly identified mediators were upregulated in PBLs very shortly after vaccination and detected without

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further *ex vivo* restimulation, suggesting their value in monitoring successful responses to vaccination.

MATERIALS AND METHODS

Experimental animals. Six- to- 12-week-old wild-type male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were age matched within each experiment. All mice were housed in sterile microisolator cages in a barrier environment and fed autoclaved food and water *ad libitum*. All experiments were carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Research Council (25) and conducted under protocols approved by the CBER Animal Care and Use Committee.

Bacteria and growth conditions. *F. tularensis* LVS (ATCC 29684), LVS-G, and LVS-R (LVS-G and LVS-R were originally obtained from Francis Nano, University of Victoria, Victoria, British Columbia, Canada) were grown to mid-log phase in supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, MI) (26). The numbers of live CFU and the 50% lethal doses administered by the intraperitoneal (i.p.) and intradermal (i.d.) routes of infection were assessed as previously described (16). *F. tularensis* LVS-G is a spontaneous colony opacity variant isolated from LVS (27); *F. tularensis* LVS-R, isolated after exposure of LVS to acridine orange, has the same colony opacity phenotype as LVS-G (28) and is capsule negative. Heat-killed LVS (HK-LVS) was prepared immediately prior to use by treating LVS at 60°C for 40 min; killing was confirmed by plating.

Bacterial immunization and challenge. Two separate sets of immunization were performed. In the first set, aimed to identify correlates of protection *in vitro*, and for each independent experiment, 10 to 12 mice were vaccinated for each vaccine group. Sublethal immunizations were performed by parenteral i.d. administration of 1×10^4 CFU LVS, LVS-R, or LVS-G or the amount equivalent to 1×10^8 HK-LVS diluted in 0.1 ml phosphate-buffered saline (PBS; BioWhittaker, Walkersville, MD); control groups received 0.1 ml PBS i.d. At 6 weeks after vaccination, seven mice from each group were sacrificed; blood was collected by cardiac puncture, and the spleens were isolated. The remaining three to five mice were challenged i.p. with a lethal dose of 10^6 LVS and monitored for survival. In the second set of immunizations, aimed to assess the *in vivo* gene expression of PBLs, three mice per vaccine group and for each time point, immunized according to the procedure described above, were sacrificed at specific time points after the vaccination; blood was collected, and the spleens were isolated. Additional mice were challenged with 10^6 LVS and sacrificed at different time points for assessment of the gene expression of PBLs and splenocytes.

Preparation of lymphocytes and flow cytometry. Single-cell suspensions of splenocytes were generated for *in vitro* culture, flow cytometry, or RNA purification by standard techniques, as previously described (16). In addition, single-cell suspensions of PBLs were prepared according to the following protocol. Blood was collected, pooled in heparinized tubes, and diluted 1:1 with PBS. The solution was further diluted 4:3 with lymphocyte separation medium (LSM; Mediatech Inc., Manassas, VA). Following centrifugation at $400 \times g$ for 25 min at room temperature, the layer containing the lymphocytes was collected, washed, and suspended in serum-free medium. Cell viability was assessed by exclusion of trypan blue and by flow cytometry (Live/Dead staining kit; Invitrogen).

Coculture of LVS-infected BMMϕ. Cocultures of LVS-infected bone marrow-derived macrophages (BMMϕ) with single-cell suspensions of splenocytes or PBLs derived from the vaccinated and nonvaccinated groups were performed according to a previously described protocol (16, 19, 29). Lymphocytes were harvested after 48 h and assessed for viability and for changes in cell surface phenotype by flow cytometry. Cells whose gene expression was to be assessed by quantitative reverse transcription-PCR were pelleted and stored in RNA*later* (Ambion, Austin, TX). Similarly, supernatants from harvested cells were collected and stored at −70°C for further analyses. Adherent macrophages were lysed and intracellular bacteria loads were determined as described previously (16).

Lymphocyte gene expression analyses. Extraction of total RNA from samples (RNeasy minikits; Qiagen, Valencia, CA), assessment of RNA purity by use of the Bioanalyzer platform (Agilent Technologies, Santa Clara, CA), and cDNA synthesis (high-capacity RNA-to-cDNA kit; Applied Biosystems, Carlsbad, CA) were performed following the manufacturers' instructions, as previously described (16). For the *in vivo* assessment of gene expression, RNA purified from PBLs and splenocytes from mice sacrificed at specific time points was used to amplify selected genes. Semiquantitative real-time PCR was completed with a ViiA 7 sequence detection system (Applied Biosystems). RNA purified from cells recovered from coculture was used for gene expression analyses in two separate sets of quantifications. Specifically, a total of 101 genes were analyzed using Applied Biosystems primers and probes either individually or with a Mouse Immune Array. In addition, two arrays containing primers for the amplification of cytokines, chemokines, and receptor genes were used to test a total of 187 genes (Rt^2 Profiler PCR array; Qiagen). The Rt^2 Profiler PCR array uses SYBR green to quantify the amplicons. Forty genes were quantified by both methods; therefore, the total number of unique genes analyzed was 248. The levels of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and Gusb expression were used to normalize the data; the change in the threshold cycle (ΔC_T) value and the ratio of the ΔC_T values of samples from vaccinated and those from control mice were then calculated.

Evaluation of molecules in culture supernatants. Assessment and quantification of the IFN-γ, TNF-α, IL-6, and IL-12 in the supernatants recovered from *in vitro* cocultures were performed using standard sandwich enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (BD Pharmingen, San Diego, CA); cytokines were quantified by comparison to the concentrations of recombinant standard proteins (BD Pharmingen). Estimation of the amount of nitric oxide in the culture supernatants was performed using the Griess reaction (Sigma-Aldrich, St. Louis, MO) (30) and by comparison to the concentrations of serially diluted $NaNO_2$. In addition, supernatants were used to assess 80 proteins using Quantibody Mouse Cytokine Arrays 4 and 5 (RayBiotech, Inc., Norcross, GA). Supernatants were loaded onto the wells of the chips along with the standards provided with the kits. The chips were processed according to the manufacturer's instructions and read with a GenePix microarray scanner (Molecular Devices, Sunnyvale, CA).

Statistical analysis. All statistical analyses were performed using Microsoft Excel software. Data for the numbers of CFU were $\log_{10}$ transformed, and cytokine and nitric oxide concentrations were measured on a log scale; thus, a normal distribution was assumed. Data are represented as the mean ± standard deviation (SD). Significant differences in the numbers of CFU, cytokine levels, and nitric oxide levels for individual pairs of means were evaluated using a two-tailed Student's *t* test, with a *P* value of <0.05 indicating significance. In addition, corrections for multiple comparisons, as seen in Fig. 4 and 5 and in Fig. S1 in the supplemental material, were performed using the Bonferroni method.

RESULTS

***In vivo* determination of gene expression of PBLs in response to LVS-derived vaccines.** To determine whether PBLs express potential correlates of protection, we performed an exploratory assessment of the relative expression of selected genes *in vivo* after LVS vaccination and after challenge of vaccinated mice with a lethal dose of LVS. The genes chosen were those previously identified to be potential predictors of vaccine efficacy on the basis of *in vitro* restimulation of *Francisella*-immune splenocytes (16, 19). As shown in Fig. 1A, the expression of the IFN-γ, IL-12rβ2, T-bet, and granulocyte-macrophage colony-stimulating factor (GM-CSF) genes reached a peak between 2 and 10 days after primary LVS vaccination. Increases in the levels of expression of TNF-α and IL-6 were modest at all time points; by day 60 after vaccination, only the expression of IFN-γ re-

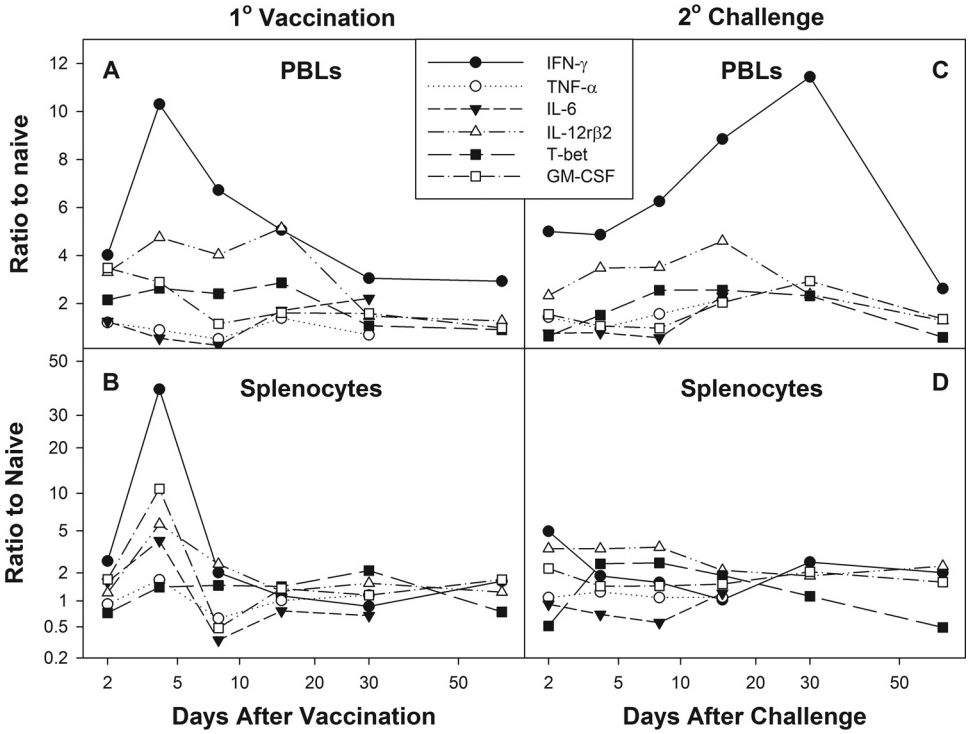

FIG 1 Expression of genes that are potential correlates of protection is upregulated in mouse PBLs after vaccination with LVS. PBLs (A and C) and splenocytes (B and D) obtained from naive mice or from mice immunized with LVS were used to purify total RNA at the indicated time points after vaccination (A and B) or after vaccination and lethal challenge with LVS (C and D). Semiquantitative analyses of gene expression were performed using selected sets of primers/probes, chosen from among those that best reflected the hierarchy of *in vivo* vaccine efficacy (16, 19). The values shown are the median fold changes of expression of the indicated genes in immunized mice compared to their levels of expression in naive mice calculated from three independent experiments.

mained slightly upregulated. In comparison, the expression of IFN-γ, IL-12rβ2, IL-6, and GM-CSF was upregulated in splenocytes 4 days after vaccination; however, the relative values in splenocytes declined faster than those in PBLs (Fig. 1B). After primary vaccination and secondary challenge with LVS, the expression of IFN-γ, IL-12rβ2, and, to a lesser extent, the other genes was upregulated in PBLs between 2 and 30 days after challenge; again, after 60 days, only the expression of IFN-γ remained slightly upregulated (Fig. 1C). In contrast, the expression of all genes, including IFN-γ, was minimal in splenocytes after challenge (Fig. 1D).

Determination of *F. tularensis*-specific PBL activities. To determine whether PBL preparations can be used in the *in vitro* coculture system and whether the activities in those preparations are comparable to those in splenocyte preparations, we first compared the characteristics and functions of PBLs derived from naive mice with those of PBLS derived from LVS-vaccinated C57BL/6J mice 6 weeks after vaccination. As shown in Fig. 2, analyses of the distribution of leukocytes from naive mice revealed that PBLs generally had a higher percentage of NK1.1$^+$ and CD11b$^+$ cells and a lower percentage of CD4$^+$ and CD8$^+$ cells than splenocytes, although the differences were not significant. Figure 3A shows that PBLs obtained from LVS-vaccinated mice were effective in controlling the intramacrophage growth of LVS and that the degree of control reflected the number of input cells used in the coculture. Similarly, analyses of supernatants demonstrated that the relative amounts of IFN-γ (Fig. 3B) and NO (Fig. 3C) produced by the PBLs of LVS-vaccinated mice reflected the number of input cells.

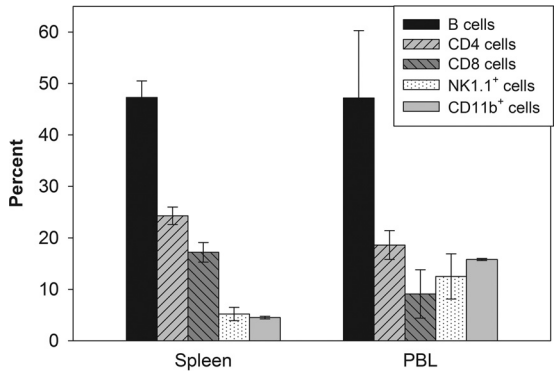

FIG 2 PBL populations differ slightly from splenocyte populations. Single-cell preparations obtained from naive mouse spleens and PBLs were stained with a panel of fluorescent antibodies to cell surface markers and with a fluorescent viability dye. After exclusion of fragments and aggregates by side scatter A (SSC-A) versus forward scatter A (FSC-A) and FSC-W versus FSC-A, cells were initially analyzed for viable leukocytes (live CD45$^+$ leukocytes). Subpopulations were then quantified. B and T cells were identified as B220$^+$ CD19$^+$ T-cell receptor β (TCRβ) negative (TCRβ$^-$) and TCRβ positive (TCRβ$^+$) B220$^-$ CD19$^-$, respectively. 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 cells (NK1.1$^+$ TCRβ$^-$) and NK T cells (NK1.1$^+$ TCRβ$^+$). CD11b$^+$ cells include neutrophils (CD11b$^+$ Gr-1$^+$), dendritic cells (CD11c$^+$ CD11b$^+$ Gr-1$^-$), and macrophages (for spleens) or monocytes (for PBLs) (CD11b$^+$ Gr-1$^-$ CD11c$^-$). Shown are the percentage of leukocytes identified using naive cells from five experiments; error bars indicate standard deviations.

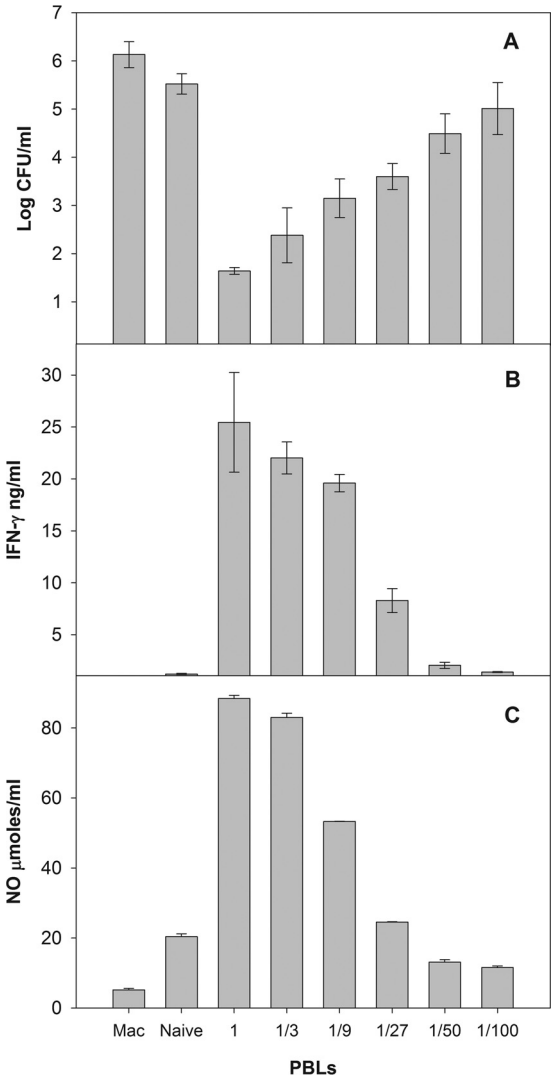

FIG 3 *In vitro* functions correlate with the numbers of PBLs added to cocultures. (A) Decreasing numbers of PBLs derived from LVS-vaccinated mice were added to a constant number of LVS-infected macrophages. After 3 days of coculture, BMMϕ were washed, lysed, and plated to evaluate the recovery of intracellular bacteria. The values shown are the mean numbers of CFU per milliliter ± SD for viable bacteria from triplicate independent experiments. (B and C) Supernatants were collected and separated from cells for analyses of IFN-γ by ELISA (B) and NO by the Griess reaction (C). The concentrations of each were calculated using standard curves as references. The values shown are the mean concentration ± standard deviation from triplicate independent experiments of similar designs and outcomes. Mac, macrophages.

In particular, the relative levels of IFN-γ and NO production exhibited a pattern inverse to that observed in the *in vitro* control of intramacrophage LVS replication: cocultures with larger amount of PBLs from LVS-vaccinated mice produced the largest amounts of IFN-γ and NO. Collectively, these data indicated that despite slight differences in cell composition, PBL preparations retained the same *in vitro* activities previously observed with splenocytes (12, 16) and that those activities were inherently related to the number of PBLs used.

To determine whether the relative degree of *in vitro* coculture activities in PBLs reflected *in vivo* protection, we vaccinated C57BL/6J mice i.d. with LVS, LVS-G, LVS-R, or HK-LVS. This panel of vaccines provides strong (LVS), intermediate (LVS-G and LVS-R), and weak (HK-LVS) protection against secondary challenge with LVS, which is lethal in mice when administered i.p. (16). Single-cell suspensions of PBLs and splenocytes were prepared, and their activities were compared. Flow cytometry analyses of the distribution of input PBLs did not reveal any obvious differences between naive and vaccinated mice (see Table S1A in the supplemental material). However, after 2 days of coculture, the analyses revealed a relative enrichment of $CD4^+$ and $CD8^+$ T cells (see Table S1B in the supplemental material). Figure 4A shows that the hierarchy of *in vitro* activities of PBLs was similar to that of splenocytes (Fig. 4B) (16). In particular, PBLs obtained from LVS-vaccinated mice were most effective in controlling the intramacrophage growth of LVS, followed by those from LVS-G- and LVS-R-vaccinated mice. Cells derived from HK-LVS-vaccinated mice were the least effective, resulting in no bacterial control. In parallel with the *in vitro* studies, additional vaccinated mice were challenged with a lethal dose of LVS i.p. (10^6 CFU) to monitor the differences in the degree of protection afforded by different vaccines. Similar to the findings of previous studies using C56BL/6J mice (12, 16, 19), all mice vaccinated with LVS survived the challenge dose; good protection was obtained with LVS-G vaccination and less protection was obtained with LVS-R and HK-LVS vaccination (data not shown), confirming the hierarchy of relative protection (16).

Analyses of the supernatants demonstrated a few differences between PBLs and splenocytes. Specifically, the relative amounts of IFN-γ produced by cocultures containing PBLs were high in all vaccine groups, including the LVS-R vaccine group but not the HK-LVS vaccine group (see Fig. S1A in the supplemental material). Although the level of IFN-γ production in the supernatants from cocultures containing splenocytes derived from LVS-R-vaccinated mice showed considerable variability, overall it was lower than that in supernatants from cocultures containing splenocytes derived from the LVS- and LVS-G-vaccinated groups (see Fig. S1B in the supplemental material). While the level of NO production was somewhat higher in cultures with LVS-vaccinated splenocytes, NO production otherwise exhibited patterns in cocultures containing PBLs (see Fig. S1C in the supplemental material) or splenocytes (see Fig. S1D in the supplemental material) similar to those associated with the *in vitro* control of intramacrophage LVS replication. In contrast to the results obtained with IFN-γ, the levels of IL-6, IL-12, and TNF-α production did not show any significant differences among the vaccine groups (data not shown).

Relative gene expression of PBLs following *in vitro* coculture. We next focused our studies on the analysis of the gene expression of nonadherent PBLs recovered from the cocultures. Table 1 indicates the genes that were quantified by two different methods using primer-probe pairs or SYBR green. The results obtained by both methods correlated for 35 of the 40 genes overlapping between the two arrays. Table 1 includes the genes whose results were consistent across five replicate experiments; it excludes genes that had more variable outcomes, even though median fold change calculations suggested differential gene expression among the vaccine groups. The genes were divided into three groups on the basis of the relationship between their relative levels of expression and the vaccine efficacy hierarchies (see footnote *a* of Table 1).

Protein expression of coculture supernatants. In parallel with

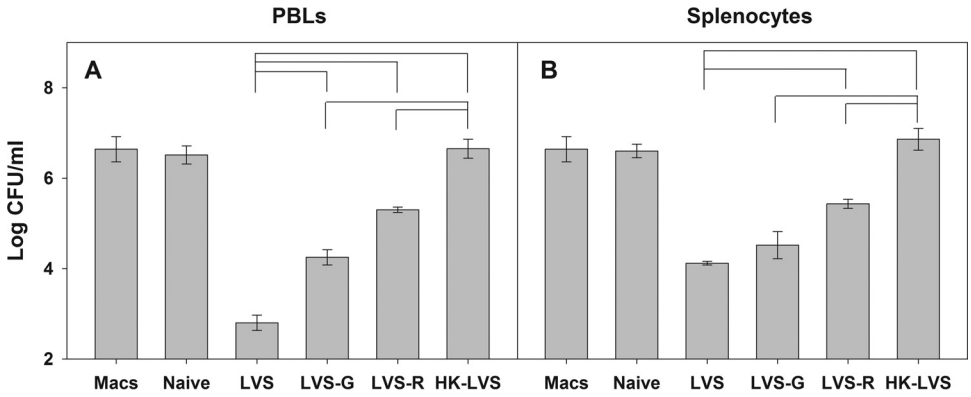

FIG 4 PBLs from mice vaccinated with LVS-related vaccines exhibit a hierarchy of control of intramacrophage LVS growth comparable to that of splenocytes. BMMϕ from C57BL/6J mice were infected with LVS (macrophages alone [Macs]) and cocultured with PBLs (A) or splenocytes (B) obtained from naive or vaccinated C57BL/6J mice, as indicated. After 2 days of coculture, BMMϕ were washed, lysed, and plated to evaluate the recovery of intracellular bacteria. The values shown are the mean numbers of CFU of viable bacteria per milliliter ± SD for triplicate samples. The results shown are from one representative of five independent experiments of similar designs and outcomes. Brackets indicate a significant difference ($P < 0.05$) between the recoveries of the bacteria in cocultures. There were no significant differences in the recovery of bacteria from cocultures between LVS-immune cells and LVS-G-immune cells (B).

the gene expression analyses, supernatants derived from cocultures were assessed for protein expression using protein chip arrays designed to detect immunologically related proteins. For the majority of the 80 proteins tested, either the proteins were not detected or their expression was similar among the four vaccine groups. As illustrated in one representative experiment of five experiments, six proteins, including monokine induced by gamma interferon (MIG), IFN-γ, IL-1ra, and monocyte chemotactic protein 5 (MCP-5) (Fig. 5A), as well as T-cell activation 3 (TCA-3) and VCAM-1 (Fig. 5B), showed moderately differential expression patterns among the vaccine groups. Analyses using the median fold change in expression of these proteins compared to their expression in naive cells, calculated from all five independent experiments, indicated that the relative production of these six pro-

TABLE 1 Relative expression of genes that are potential correlates of protection in cocultures using immune PBLs from differentially vaccinated mice[a]

	Mouse Immune Array				Cytokine, Chemokine, and Receptor Array			
		Median fold change in expression				Median fold change in expression		
Group	Gene	LVS	LVS-R	HK-LVS	Gene	LVS	LVS-R	HK-LVS
Group 1	GZMB	31.02	9.13	0.87	IL-21	167.8	11.71	0.78
	IL-2RA	11.93	4.31	0.99	LTA	11.73	2.46	0.90
	T-bet	6.92	3.27	1.05	TNFSF4	4.48	1.62	0.87
	FasL	6.21	2.82	0.94	CCL28	3.76	1.65	0.89
	C3	7.85	2.56	1.36	CCR5	6.25	3.01	1.31
	CCL5	6.66	2.85	1.55	FasL	3.40	1.71	0.87
	ICOS	4.33	2.71	1.50	CCL5	9.32	4.73	2.01
	Bcl-2	4.19	2.24	1.28	TNFSF8	2.97	1.68	1.00
	CCR2	6.80	2.10	1.23	TNFSF18	2.39	1.29	0.80
	IL-12rβ2	4.53	2.61	1.77				
	NOS2	19.04	3.07	2.16				
Group 2	IFN-γ	79.45	53.89	2.16	IFN-γ	28.74	31.93	1.28
	PRF1	4.70	4.54	0.92	CCR9	2.87	2.54	1.00
	GM-CSF	5.04	6.14	1.47	GM-CSF	4.17	3.58	1.20
	TNFRSF18	3.25	2.24	1.30	CXCL11	3.14	2.55	1.39
	Hmox1	3.48	3.39	1.41				
Group 3	CD80	2.98	1.07	0.96	CMTM3	3.55	1.61	1.17
	Ptgs2	6.86	2.79	1.83	CXCR6	6.46	2.89	2.70
					CX3CR1	5.84	0.71	1.25
					IL-1b	6.40	2.37	2.38
					Nfkb1	4.47	2.06	1.50

[a] The median fold change in the level of expression of the indicated genes compared to that by naive cells was calculated from five independent experiments. Semiquantitative analyses of gene expression were performed using a Mouse Immune Array or Cytokine, Chemokine, and Receptor Arrays, as described in Materials and Methods. Groups 1 to 3 indicate different gene expression hierarchies, as follows: group 1, LVS-vaccinated mice > LVS-R-vaccinated mice > HK-LVS-vaccinated mice; group 2, LVS-vaccinated mice ≈ LVS-R-vaccinated mice > HK-LVS-vaccinated mice; group 3, LVS-vaccinated mice > LVS-R-vaccinated mice ≈ HK-LVS-vaccinated mice.

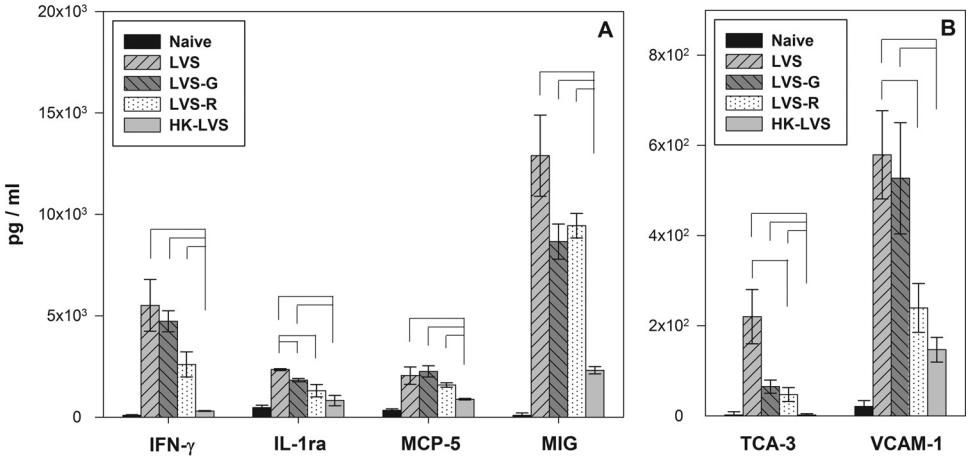

FIG 5 The *in vitro* production of cytokines and chemokines by PBLs from vaccinated mice exhibits differential expression patterns. Supernatants derived from PBLs cocultured for 2 days were collected and separated from cells for analyses of selected cytokines and chemokines by use of a protein array quantification system. The values shown are the mean concentration ± standard deviation for quadruplicate samples. The results shown are from one representative of five independent experiments of similar designs. Proteins with higher or lower concentrations are shown in panels A and B, respectively (note the different scales in each panel). Brackets indicate significant differences ($P < 0.05$) between the amounts of proteins produced in the cocultures.

teins was similar in the LVS and LVS-G groups, somewhat less in the LVS-R group, and notably lower in the HK-LVS group (see Table S2 in the supplemental material).

Relative gene expression of PBLs *in vivo* following vaccination. To determine the utility of the newly identified genes for the monitoring of vaccination, we performed studies similar to those for which the results are shown in Fig. 1 at two time points. PBLs prepared from mice vaccinated with LVS-derived vaccines were analyzed for gene expression 2 and 15 days after vaccination. Figure 6A indicates that CCR5, IL-18 binding protein (IL-18bp), granzyme B, Socs-1, IL-27, and CXCL11 had differential expression patterns among the vaccine groups at day 2 after vaccination. At 15 days after vaccination, in addition to CCR5, granzyme B, IL-27, IL-12rβ2, IFN-γ, and CCL5 were also more upregulated in the PBLs of LVS-vaccinated mice than in the PBLs of LVS-R- and HK-LVS-vaccinated mice (Fig. 6B). These patterns correlated directly with the *in vivo* vaccine efficacies.

DISCUSSION

Although mediators such as IFN-γ are clearly important in controlling intracellular bacteria, such as *Mycobacterium tuberculosis* (20) and *Francisella* (14), to date no correlates of either resistance to infection or vaccine-induced protection have been defined for any intracellular pathogen (21). We and others (12, 16, 19, 31) have therefore used the *in vitro* interactions between murine immune T lymphocytes and bacterium-infected host cells (macrophages) to search for functional and molecular immune correlates. We have identified several candidate mediators, including IFN-γ, IL-6, IL-12rβ2, and T-bet (16). Previous studies relied on splenocytes but also demonstrated that the expression of genes that are potential correlates of protection varies according to the tissue source (19). Ideally, an easily accessible source of lymphocytes, such as PBLs, would provide a means for the convenient and fast assessment of correlative immune responses to vaccination. We therefore evaluated the gene expression of PBLs obtained directly from mice immunized with LVS, which represents the best vaccine available. The results showed that the changes in expression of most previously identified genes in either immune splenocytes or PBLs were modest and limited over time (Fig. 1).

In contrast, *in vitro* restimulation of immune lymphocytes by coculturing of immune cells with macrophages infected with rep-

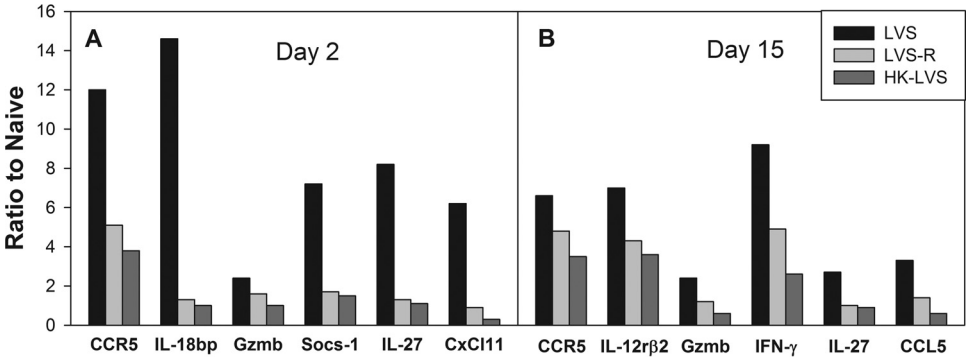

FIG 6 Expression of genes that are potential correlates of protection is differentially upregulated in PBLs after vaccination with LVS-derived vaccines. PBLs obtained from naive mice or from mice immunized with LVS-derived vaccines were used to purify total RNA 2 days (A) and 15 days (B) after vaccination. Semiquantitative analyses of gene expression were performed using selected sets of primers/probes chosen from among those that best reflected the hierarchy of *in vivo* vaccine efficacy in splenocytes (16) or PBLs (Table 1). The values shown are the fold changes in the levels of expression of the indicated genes compared to their levels of expression in PBLs from naive mice calculated from one representative of three independent experiments. Gzmb, granzyme B.

licating LVS has proved effective for the monitoring of T-cell immune functions and the identification of potential correlates of protection. Several differences between PBLs and splenocytes could impact the outcome of the *in vitro* study. For example, the cellular composition of PBLs differs slightly from that of splenocytes (Fig. 2). Also, the method used to isolate PBLs is more complex than the method used to prepare splenocytes. Lastly, the number of PBLs isolated from mice is substantially lower than the number of splenocytes. Nonetheless, here we show that PBLs from LVS-vaccinated mice contain abundant capabilities to control *in vitro* LVS intramacrophage replication, to produce IFN-γ, and to induce NO production (Fig. 3).

Probably due to the small numbers that can be obtained, PBLs from mice have been studied very infrequently and are usually used in descriptive analyses to monitor responses to drug treatments (32) or production of model SCID/human animals (33). We have found in the literature only two examples of the use of mouse PBLs for functional assessments, and both of those studies examined T-cell proliferation *in vitro* (34, 35). Our studies clearly demonstrate that the function of murine PBLs can be measured in complex immunological assessments (Fig. 3 to 6). Thus, mechanistic analyses and comparisons of PBLs with cells from sources such as the spleen and lymph node, as well as comparison with human PBLs, are readily feasible.

An important feature of our model is the ability to correlate *in vitro* functions with *in vivo* efficacies (16). We demonstrated that the ability to control LVS replication *in vitro* was most efficient when PBLs from LVS-vaccinated mice were used, followed by those from LVS-G-, LVS-R-, and HK-LVS-vaccinated mice, in a pattern similar to that obtained with splenocytes. This pattern directly reflects the efficacies of the vaccines in C57BL/6J mice (Fig. 4). Therefore, the *in vitro* coculture conditions obtained using immune PBLs appeared to be suitable to expand and refine the list of potential correlates of protection. For this purpose, RNA purified from PBLs recovered from cocultures was analyzed for relative gene expression, using commercially available arrays that included immunologically related genes. We report the outcomes of the reverse transcription-PCR analysis by dividing the patterns into three groups (Table 1). In particular, the relative expression of the genes in group 1 follows the pattern LVS-vaccinated mice > LVS-R-vaccinated mice > HK-LVS-vaccinated mice. In group 2, the gene expression pattern of the PBLs derived from LVS-vaccinated mice was similar to that of PBLs derived from LVS-R-vaccinated mice but higher than that of PBLs derived from HK-LVS-vaccinated mice. Finally, in group 3 there were minimal gene expression differences between the LVS-R- and HK-LVS-vaccinated groups, but gene expression in both of those groups was less than that in the LVS-vaccinated group. The factors described in group 1 represent the ideal potential correlates, because they follow the same pattern of *in vivo* protection. However, using a multivariate analysis, we previously demonstrated that values from multiple genes in conjunction with *in vitro* LVS replication data are necessary to best differentiate vaccine groups (19). For that purpose, factors included in groups 2 and 3 can still be quite useful.

Two genes (FasL and CCL5) had the same expression patterns by the use of either probes or SYBR green quantification; moreover, the expression of IFN-γ and GM-CSF was somewhat similarly upregulated in the LVS and LVS-R vaccine groups in both quantification methods. These data overall cross validate both quantification methods. In addition to IFN-γ and GM-CSF, two other genes detected in PBLs (T-bet and IL-12rβ2) were previously indicated to be potential correlates of protection by using splenocytes (16, 19) or liver lymphocytes (19). CCR5, which was identified to be a potential correlate candidate using splenocytes from vaccinated BALB/cByJ mice (12), is also differentially expressed in the PBLs of C57BL/6J mice. Interestingly, the findings of concurrent studies, which used the same analytical approach in mice to identify correlates of protection against *M. tuberculosis*, have similarities with our findings (36). In particular, 11 genes, namely, those for IFN-γ, granzyme B, IL-2ra, CXCL11, NOS2, Ptgs2, T-bet, ICOS, GM-CSF, CCL5, and LTA, were among the most upregulated in the PBLs of mice vaccinated with *Mycobacterium bovis* BCG Pasteur. Thus, it may be possible to identify mediators that are common during vaccine-induced protection against intracellular bacteria in general.

Screening of proteins represents an alternative method to identify correlates of protection (24). However, analyses of protein production during cocultures revealed considerable variability between replicate experiments. Only six proteins (Fig. 5; see also Table S2 in the supplemental material) showed differential expression patterns between the live vaccine-immunized groups and the HK-LVS-vaccinated group. The relative expression of the IL-1ra and VCAM-1s genes at a single time point (48 h) was not different among the vaccine groups; this was in contrast to their protein production results, which reflect accumulation over time in culture. The IFN-γ production detected using protein chips confirmed the outcomes observed by standard ELISA (see Fig. S1A in the supplemental material) and was consistent with the gene expression data (Table 1). The relative expression of genes for the remaining proteins has not yet been analyzed.

Transcriptional profiling of PBLs from nonhuman primates and people has been used to characterize the immune responses to a number of vaccines; the resulting signatures often reflect innate immune responses and antigen presentation pathways (37). The only circumstance in which a vaccine-related predictive signature has been defined to date was with the RTS,S malaria vaccine (38). Here, our approach identified new factors that were differentially expressed in PBLs a few days after *Francisella* vaccination, in the absence of *in vitro* restimulation (Fig. 6). In contrast to a modest upregulation during *in vitro* restimulation, IL-18bp, IL-27, and Socs-1 were highly upregulated in murine PBLs obtained only 2 days after LVS vaccination. These results therefore indicate that the approach described here may provide a tool that may be used to quickly monitor whether immune responses are efficacious.

In conclusion, the validity of this approach for the identification of potential correlates of protection using PBLs supports the advancement of studies to different animal species and humans. The ultimate aim is to define a set of functionally relevant markers that best reflect human immune responses against *F. tularensis*, facilitate screening of potential new vaccines, and bridge the results obtained with animal models to people.

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