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Enhanced Priming of Adaptive Immunity by *Mycobacterium smegmatis* Mutants with High-Level Protein Secretion

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Mycobacteria have features that make them attractive as potential vaccine vectors. The nonpathogenic and rapidly growing *Mycobacterium smegmatis* can express both *Mycobacterium tuberculosis* antigens and heterologous antigens from other pathogens, and it has been used as a viable vector for the development of live vaccines. In order to further improve antigen-specific immunogenicity of *M. smegmatis*, we screened a random transposon mutant library for mutants displaying enhanced efficiency of protein secretion ("high secretors") and isolated 61 mutants showing enhanced endogenic and transgenic protein secretion. Sequence analysis identified a total of 54 genes involved in optimal secretion of insert proteins, as well as multiple independent transposon insertions localized within the same genomic loci and operons. The majority of transposon insertions occurred in genes that have no known protein secretion function. These transposon mutants were shown to prime antigen-specific CD8$^+$ T cell responses better than the parental strain. Specifically, upon introducing the simian immunodeficiency virus (SIV) *gag* gene into these transposon mutant strains, we observed that they primed SIV Gag-specific CD8$^+$ T cell responses significantly better than the control prime immunization in a heterologous prime/boost regimen. Our results reveal a dependence on bacterial secretion of mycobacterial and foreign antigens for the induction of antigen-specific CD8$^+$ T cells *in vivo*. The data also suggest that these *M. smegmatis* transposon mutants could be used as novel live attenuated vaccine strains to express foreign antigens, such as those of human immunodeficiency virus type 1 (HIV-1), and induce strong antigen-specific T cell responses.

Live bacterial vaccines are relatively cheap to produce and easy to apply. Thus, they are suitable to immunize large populations (16, 44). Induction of both cell-mediated immunity and antibody-mediated immunity is obtained by the vaccine strain's ability to colonize and multiply in the host without causing disease. In general, live bacterial vaccines require no additional adjuvant component to evoke immune responses (4). Numerous features, such as a strong safety record, make *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) an attractive delivery vehicle for heterologous antigens (37, 47). A range of strategies have been developed to allow controlled and stable expression of viral, bacterial, and parasite antigens in BCG (4). However, since BCG can cause a clinically significant mycobacterial infection in patients with immune deficiency (60), the nonpathogenic *Mycobacterium smegmatis* has instead been used to engineer stable expression of transgenes to elicit cellular and mucosal immune responses (15). Unlike other mycobacterial strains such as BCG that survive in host cells for months by inhibiting phagosome maturation, *M. smegmatis* is rapidly destroyed by phagolysosomal proteases in the phagosomes of infected cells (10, 27, 34, 51). Nevertheless, *M. smegmatis* induces cytokine production by macrophages better than pathogenic mycobacterial species and can activate and induce the maturation of major histocompatibility complex (MHC) class I and costimulatory molecules (6, 55). *M. smegmatis* also facilitates rapid uptake of expressed antigens and cross-presentation of antigens (12, 35). Moreover, preexisting immunity to BCG may have only a marginal effect on the immunogenicity of recombinant *M. smegmatis* (9). Accordingly, *M. smegmatis* has been used as a valuable vector for the development of live vaccines against pathogens such as human immunodeficiency virus (HIV), hepatitis B virus, and *Helicobacter pylori* (9, 11, 32, 33, 49, 57, 59). Recently, recombinant *M. smegmatis* was used successfully as a potential tuberculosis (TB) vaccine for eliciting protective immunity against *Mycobacterium tuberculosis* (23, 49).

Despite the success of heterologous antigen expression and, in some cases, protection induced by recombinant mycobacteria *in vitro* and *in vivo*, the stability of the recombinant vaccine is of great concern. Expression of heterologous antigens in recombinant mycobacteria necessarily imposes a metabolic burden, the extent of which will determine the degree to which the recombinant mycobacterium is compromised. Thus, the relative level of fitness of the recombinant and any derived mutants will affect the rate at which the inserted element or its expression is lost from the bacterial population (24). Another critical issue for engineering a stable and immunogenic mycobacterium-based vaccine vector is antigen localization. Membrane-anchored and secreted antigens from mycobacteria could result in earlier presentation to the immune system. In fact, it has been demonstrated that anti-HIV-1 T cell responses in splenocytes and antibody responses against HIV-1 Env were optimal in mice immunized with recombinant mycobacteria expressing HIV-1 Env engineered as secreted antigens (57). Additionally, it has been suggested that removal of foreign antigens from the mycobacterial cell by secretion may support higher levels of transgenic protein production (21).

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TABLE 1 Plasmids, phages, and strains used in this study

Plasmid, phage, or strain	Description	Reference or source
Plasmids		
pJSC347	Vector for cloning allelic exchange substrates to be used for specialized transduction	26
pMV361Apra	*aacC41 Phsp60 bla* L5 *attP int* ColE1; single-copy mycobacterial shuttle plasmid	48
pMV261Km	*aph Phsp60 oriM* ColE1; multicopy mycobacterial shuttle plasmid	48
pMV261Apra	*aacC41 Phsp60 oriM* ColE1; multicopy mycobacterial shuttle plasmid	This work
pSL120	*hyg ′phoA bla* L5 *attP int* ColE1; a promoterless truncated *E. coli* PhoA plasmid	This work
pSL300	*hyg* P_{Ag85B}::*′phoA* ColE *hyg* L5 *attP int*; PhoA reporter plasmid, pMV306Hyg-*′phoA*	This work
pSL301	*aph Phsp60*-LpqH-SIINFEKL *oriM* ColE1 pMV261Km-*lpqH*-SIINFEKL	This work
pSL302	*aph Phsp60-fbpB*(Ag85B) *oriM* ColE1 pMV261Km-Ag85B	This work
pSL330	*hyg* Δ*lpqM* ColE1; derivative of pJSC347 designed for allelic exchange of *M. smegmatis lpqM*	This work
pSL331	*aacC41 Phsp60 lpqM* (*M. smegmatis*) *int attP* ColE pMV361Apra-*lpqM*	This work
pSL7	*aacC41 Phsp60*-SIV Gag-HA *oriM* ColE1 pMV261Apra-SIV Gag	This work
Phages		
phAE159	Deletion mutant of mycobacteriophage TM4	26
phAE200	phAE159 (Tn*5372*)	26
I3	Generalized transduction phage	26
Strains		
E. coli		
DH5α	F [ϕ80dΔ*lacZ*M15] Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17 glnV44 thi-1 gyrA96 relA1*	Gibco Life
TransforMax EC100D pir-116	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ^- *rpsL nupG pir-116*(DHFR)	Epicentre
HB101	F *supE44 ara14 galK2* Δ(*gpt-proA*)*62 lacY1 hsdS20 rpsL20 xyl-5 mtl-1 recA13* Δ(*mcrC-mrr*)	Stratagene
M. smegmatis		
mc^2155	*ept-1* mutation conferring high efficiency of plasmid transformation	45
SLMS300	mc^2155 *attB*::pSL300, PhoA reporter strain	This work
SLMS301	mc^2155(pSL301), expresses MHC class I epitope SIINFEKL	This work
SLMS302	mc^2155(pSL302), expresses MHC class II epitope of mycobacterial antigen Ag85B	This work
SLMS330	mc^2155 Δ*lpqM*::*hyg*	This work
SLMS331	SLMS330 *attB*::pSL331 Δ*lpqM* complemented strain	This work
SLMS332	SLMS330(pSL301) Δ*lpqM* expressing LpqH-SIINFEKL	This work
SLMS333	SLMS331(pSL301) Δ*lpqM* complemented strain expressing LpqH-SIINFEKL	This work

It has been reported that mycobacteria secrete a very large number of proteins, and even more proteins are cell surface associated. Considering the importance of secreted proteins in virulence and immunogenicity, protein secretion should be tightly regulated and well synchronized by many mycobacterial gene products (1, 14, 39, 43). Thus, we hypothesize that random mutations in those secretion-associated mycobacterial genes will result in various mutants displaying enhanced efficiency of secretion of homologous proteins as well as other heterologous proteins (here called "high secretor mutants"). The high secretor mutants of *M. smegmatis* will mount a better protective immune response, resulting from the efficient delivery of foreign antigens to the MHC class I and II pathways to generate protective cytotoxic $CD8^+$ T and $CD4^+$ T cells. In order to screen for mycobacterial mutants with enhanced secretion, we used the simple and efficient *phoA* reporter system. This system combines a transposable element (*Himar1* transposon) of broad host range and an alkaline phosphatase gene (*phoA*) derived from *Escherichia coli* that has been modified by fusing the region encoding its mycobacterial *secA1*-dependent export signal. PhoA is an exported enzyme that is activated only after its translocation into the bacterial periplasmic space and is assayed by blue color production on plates containing the chromogenic substrate of PhoA, BCIP (5-bromo-4-chloro-3-indolylphosphate) (7, 26). Because of this feature of PhoA, it can be considered a sensor for protein export and a useful reporter for studies on subcellular location of a protein. Mutagenesis of recombinant *M. smegmatis* expressing *phoA* with the *Himar1* transposon identified mutants with enhanced secretion of alkaline phosphatase (PhoA). In this way, we have assessed the efficacy of the isolated *M. smegmatis* transposon mutants with an improved secretion phenotype and strong T cell responses.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains, bacteriophages, transposons, and plasmids used in this study are listed in Table 1. *M. smegmatis* strains used in this study were grown in 7H9 or 7H10 medium supplemented with 0.5% glycerol and 0.05% tyloxapol. *E. coli* strains were grown in Luria-Bertani (LB) medium. Antibiotics were used at the following concentrations: kanamycin, 40 μg/ml for *E. coli* and 20 μg/ml for *M. smegmatis*; apramycin, 60 μg/ml for *E. coli* and 40 μg/ml for *M. smegmatis*; and hygromycin, 150 μg/ml for *E. coli* and *M. smegmatis*. LB agar containing 60 μg of BCIP (Sigma, St. Louis, MO) per ml was used for screening of PhoA-expressing clones.

Construction of plasmids and strains. The PhoA construct was generated as follows. The antigen 85 promoter and signal peptide were PCR amplified from pJH154 (57) using primers XbaI-F (5′-ATCTAGAATAC GGAAATGAGACGACTTTGC) and NdeI-R (5′-CATATGCAACCGCG GGCGCGTTCGGT). To generate the PhoA reporter plasmid (pSL300), the resulting product was cloned directly upstream of *phoA* of the integrating mycobacterial plasmid pSL120 containing the truncated *E. coli phoA* (8). When integrated into the *M. smegmatis* chromosome, pSL300

confers a light-blue phenotype (PhoA$^+$) on BCIP-containing medium. The resulting recombinant *M. smegmatis* strain is SLMS300. Plasmid DNA was introduced into *M. smegmatis* mc^{2}155 by electroporation as previously described (45). For epitope tagging of the 19-kDa lipoprotein, the 19-kDa lipoprotein gene was amplified from 19OVA (22) using a primer directed at the 5′ end of the coding sequence for the 19-kDa lipoprotein gene (5′-CAGGAGGAACGCAGATATCGTGAAGCGTGGACTGA-3′) in combination with a 3′ primer containing a sequence from OVA encoding amino acids 254 to 269 (5′-AATCGCGGCCTCGAGCAAGACG-3′). The PCR product containing the 19-kDa OVA sequence was ligated into pMV261Km, a replicating mycobacterial vector with an Hsp60 promoter. The resulting plasmid, designated pSL301 (LpqH-SIINFEKL), was used to transform wild-type (WT), Δ*lpqM* mutant (SLMS330), and Δ*lpqM* complemented (SLMS331) strains of *M. smegmatis*, which resulted in SLMS301, SLMS332, and SLMS333, respectively. Strain SLMS330 was generated by a specialized transduction (3) of conditionally replicating shuttle phasmid pSL330. The Δ*lpqM* complemented strain, SLMS331, was PCR amplified using 5′-AAGCTTTGAGCCGACGTCACCTGCTC-3′ and 5′-GCTAGCACTAACTGACCGTGATATCGGAGAACC-3′. The amplicon was cloned into the HindIII and NheI sites of pMV361Apra to generate the complementation vector pSL331. This construct was integrated into *M. smegmatis*::Δ*lpqM* (SLMS330) by electroporation. In order to generate pSL302, the full-length *fbpB* was amplified without the promoter region and cloned into integrating mycobacterial vector pMV361Apra.

To determine if *M. smegmatis* transposon mutants we identified might be effective vectors for priming CD8$^+$ T cell responses to simian immunodeficiency virus (SIV), we transformed the *M. smegmatis* transposon strains with a plasmid construct containing the SIVmac239 *gag* transgene. The SIV Gag protein includes the H-2D^b-restricted immunodominant AL11 epitope, and therefore the immunogenicity of this vaccine construct could be evaluated. The SIV *gag* gene was cloned into a multicopy episomal *E. coli*-mycobacterial shuttle plasmid to generate pSL7, which expresses a fusion protein containing the 19-kDa protein secretion sequence, the full-length SIV Gag protein, and a hemagglutinin (HA) tag (Table 1).

Transposon mutagenesis and generalized transduction in *M. smegmatis*. In this study, Tn*5372* (apramycin resistant) was used for transposon mutagenesis of *M. smegmatis*. Tn*5372* is a *Himar* derivative (42), the construct of which was cloned into a temperature-sensitive mycobacteriophage, phAE159 (26). This transposon phage was used as a vehicle for transposon delivery and for random mutagenesis of *M. smegmatis* expressing pSL300. The isolation of mutant strains and the identification of transposon insertion sites were performed as described previously (26). Generalized transduction with mycobacteriophage I3 (40) was performed as described previously (29). High-titer phage lysates (10^7 PFU/ml) of phage I3 were prepared on the donor strain as a host, and then the phage lysates were used to transfer the marker of interest into the recipient strain, SLM301.

DNA sequence analysis. Genomic DNAs were isolated from transposon mutants by use of the cetyltrimethylammonium bromide (CTAB) protocol as described previously (28). Genomic DNA was digested with BssHII and self-ligated, producing a plasmid containing the transposon and flanking *M. smegmatis* genomic DNA. These plasmids were recovered by transformation into *E. coli* DH5α λ*pir* and selection on LB agar with apramycin (42). Resulting plasmid DNAs were then sequenced to map the insertion sites. For DNA sequence analysis, the following bioinformatic algorithms were used: NCBI Advanced BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), the Comprehensive Microbial Resource (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi), and the SmegmaList database (http://mycobrowser.epfl.ch/smegmalist.html).

Western blot analysis of recombinant *M. smegmatis*. To monitor the expression of the *M. tuberculosis lpqH* transgene, individual recombinant *M. smegmatis* strains were grown in Middlebrook 7H9 broth in the presence of kanamycin and apramycin. The lysate of grown recombinant *M. smegmatis* was fractionated on 4 to 20% SDS-polyacrylamide gels and blotted onto nitrocellulose filters (Invitrogen). Mycobacterial Hsp65 protein bands were detected with monoclonal antibody Hsp65 (Santa Cruz Biotechnology) at 1 μg/ml, followed by incubation with goat anti-mouse (GAM) IgG-alkaline phosphatase (AP) antibody (GenScript). Mycobacterial DnaK protein bands were detected by incubation of the filters with monoclonal antibody IT-40 (BEI Resources) at 1 μg/ml followed by incubation with GAM IgG (Sigma, St. Louis, MO)-AP. SIINFEKL epitope bands were detected with anti-SIINFEKL rabbit serum (1/10,000 dilution) and goat anti-rabbit (GAR) IgG-AP (Sigma). The LpqH/Hsp65 expression ratio in scanned gels was analyzed with ImageJ.

Tetramer staining and flow cytometric analysis. Female C57BL/6 mice (Jackson Laboratory), 6 to 8 weeks of age, were used for immunogenicity studies. All animal studies were approved by the institutional animal care and use committees of Duke University. At 7 days after immunization, mice were anesthetized with isoflurane and blood was collected in RPMI 1640 containing 40 U of heparin (American Pharmaceutical Partners, Schaumburg, IL) per ml. Peripheral blood mononuclear cells (PBMCs) were isolated using Lympholyte-M (Cedarlane, Burlington, NC) and stained with SIINFEKL-specific class I MHC tetramers (catalog no. T03000; Beckman Coulter, FL) conjugated with phycoerythrin (PE) and anti-CD8$^+$ monoclonal antibody (Ly-2; BD Bioscience, San Jose, CA) conjugated with allophycocyanin (APC) to detect SIINFEKL-specific CD8$^+$ T cells. The cells were washed in phosphate-buffered saline (PBS) containing 2% fetal bovine serum and fixed with PBS containing 2% ultrapure methanol-free formaldehyde (58). CD8$^+$ T cells were analyzed for tetramer staining with FlowJo software (Tree Star, OR).

IFN-γ ELISPOT assays. Enzyme-linked immunospot (ELISPOT) assays were performed as described previously (56). Flat-bottom 96-well plates (Millipore, MA) were coated with anti-mouse gamma interferon (IFN-γ) antibody (BD Biosciences, CA) before being washed with PBS and blocked with medium. Freshly isolated spleen cells were mixed with an MHC class II antigen 85B (Ag85B) peptide (p25, FQDAYNAAGGHNAVF) (53) or an MHC class I AL11 epitope (AAVKNWMTQTL) and then incubated for 24 h at 37°C in 5% CO_2. Following incubation, the plates were washed, biotinylated anti-IFN-γ antibody (BD Biosciences, CA) was added, and the plates were incubated overnight at 4°C. After the plates were washed with PBS, streptavidin-horseradish peroxidase (BD Biosciences, CA) was added to each well and developed using 3-amino-9-ethylcarbazole (AEC) substrate. To count spot-forming cells (SFCs), spots in the ELISPOT plates were scanned using an ImmunoSpot series I analyzer and quantified by using ImmunoSpot 2.1 software (CTL Analyzers, Cleveland, OH). Control wells included cells cultured in medium in the absence of peptide stimulation. The frequency of IFN-γ SFCs in control wells was subtracted from the frequency of IFN-γ SFCs detected in the peptide-stimulated cells to calculate antigen-specific IFN-γ responses.

Statistical analysis. Statistical significance was assessed by comparison with data from the control groups using analysis of variance (ANOVA) with Tukey's posttest. Data are expressed as the means ± standard deviations of the means. A P value of ≤0.05 was deemed to be significant.

RESULTS

***M. smegmatis* transposon mutants screened for elevated levels of transgene secretion.** In an effort to study mutations conferring protein export enhancement in mycobacteria, we constructed a mariner-based *Himar1* transposon (Tn*5372*) library in a PhoA reporter strain of *M. smegmatis*. The schematic plan for the screening is outline in Fig. 1A. The reporter plasmid, pSL300, was designed to express *E. coli* PhoA with the *M. tuberculosis* antigen 85 (Ag85) promoter and export a recombinant fusion of the PhoA protein with the Ag85 signal peptide (*secA1*-dependent signal peptide) (Fig. 1B). The *M. smegmatis* reporter strain, SLMS300, expresses the truncated *E. coli* '*phoA* (pSL300). The secreted fusion

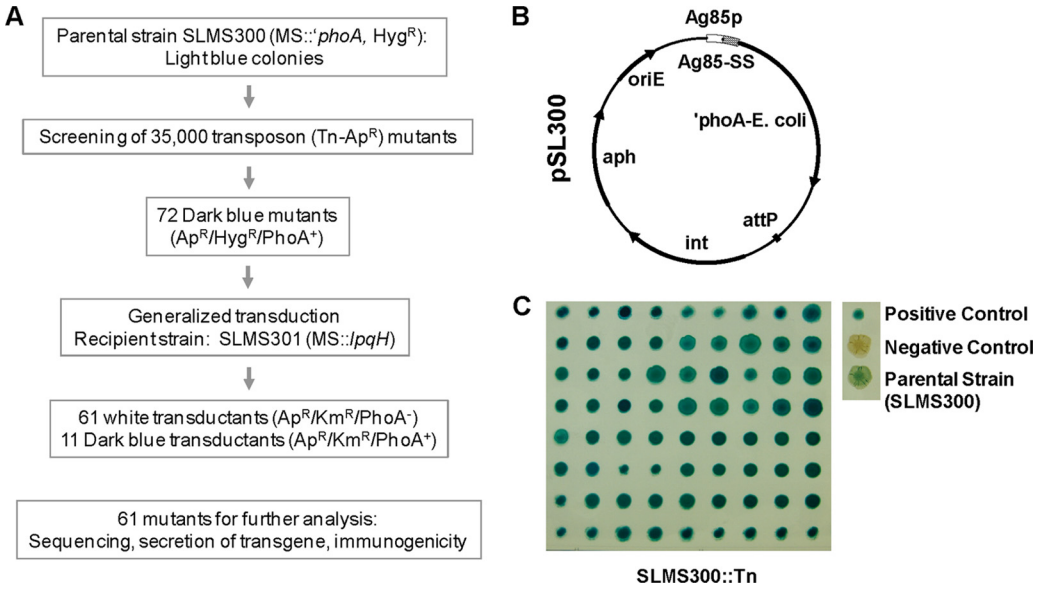

FIG 1 Map of the reporter plasmid and transposon mutagenesis strategy. (A) An efficient screening strategy was developed to isolate *M. smegmatis* transposon mutants with enhanced expression and secretion of transgene products, as described in the text. (B) A reporter plasmid, pSL300 contains the *M. tuberculosis* antigen 85 promoter (Ag85p) and the SecA1-dependent signal sequence from the same protein (Ag85ss) cloned upstream of the truncated *E. coli* PhoA ('phoA) in the mycobacterial integrating vector. (C) The parental strain (SLMS300), *M. smegmatis* expressing pSL300, was randomly mutated using a mariner-based *Himar1* transposon (Tn*5332*). Among 35,000 transposon mutants, 72 dark-blue colonies on BCIP medium were selected. To demonstrate the difference in blue phenotype, wild-type mc^{2}155 expressing empty vector and the intense-blue mutants resulting from transposon insertion into the *pstS* gene (*pstS*::Tn) were used as negative and positive controls, respectively. All other dark-blue colonies represent the individual 72 transposon mutants.

protein was detected by its extracellular alkaline phosphatase activity, shown as pale-blue colonies on BCIP-containing LB medium (Fig. 1C). Apramycin-resistant transposon mutants with an increased protein export would be expected to form colonies with an intense blue color compared to that of the parental strain (SLMS300). From approximately 35,000 transposon mutants, 72 intense-blue mutants were isolated (Fig. 1C). These intense-blue colonies may result from either direct modulation of protein secretion or enhanced expression of the PhoA protein, either of which is followed by higher secretion of PhoA. Another possibility is that some intense-blue colonies resulted from transposon insertion into one of the phosphate transporters or regulators of the endogenous *M. smegmatis phoA* gene, leading to constitutive expression of *M. smegmatis* PhoA protein. In order to exclude these constitutive expressers of *M. smegmatis* PhoA and confirm the phenotype of enhanced secretion of *E. coli* PhoA, we transferred each of 72 transposon insertions from the intense-blue mutants to *M. smegmatis* strain SLMS301 (*M. smegmatis*::*lpqH*) by use of a generalized transduction mycobacteriophage, I3. Strain SLMS301 contains plasmid pSL301, encoding the *M. tuberculosis* LpqH tagged with an SIINFEKL epitope. LpqH is a 19-kDa lipoprotein antigen precursor that is transported to membrane, cell wall, and culture supernatant in a *secA1*-dependent manner (5, 13, 19, 54). Since the recipient strain, SLMS301, does not express *E. coli* PhoA, no transductants should be blue on LB plates containing BCIP unless the transposon insertion is located within either the *M. smegmatis* PhoA regulators (*senX3-regX3*) or the *pst* operon, which encodes high-affinity phosphate transporter components. Transductants were selected as apramycin-resistant (Ap^r) and kanamycin-resistant (Km^r) colonies, as Tn*5372* carries the apramycin resistance gene and the recipient strain SLMS301 has the kanamycin resistance gene in the vector. Seventy-two Ap^r Km^r transductants were evaluated for alkaline phosphatase (PhoA$^+$) activity by plating on BCIP medium. Eleven Ap^r Km^r transductants still exhibited the elevated PhoA$^+$ phenotype (intense-blue colonies) in the recipient SLM301 strain, which lacks *E. coli* PhoA; however, 61 transductants appeared as white colonies on BCIP medium (Ap^r Km^r PhoA$^-$). Subsequent sequence analysis confirmed that the transposons of 11 Ap^r Km^r PhoA$^+$ transductants were inserted into the *M. smegmatis pst* operon (data not shown). Thus, our screening yielded a total of 61 transposon mutants that are able to enhance secretion of the PhoA protein in SecA1-dependent manner.

Mutations in several independent functional groups are responsible for the enhanced secretion. Because Tn*5332* carries the oriR6K origin of replication and the apramycin resistance gene, the transposon insertion site of each mutant was rescued as a plasmid recovered from genomic DNA by restriction endonuclease digestion, self-ligation, and transformation into *E. coli*. DNA sequence analysis of the transposon insertion sites in these mutants revealed that the mutants can be categorized into eight functional groups: (i) cell envelope (11 transposon mutants), (ii) cellular process (3 transposon mutants), (iii) energy metabolism (6 transposon mutants), (iv) fatty acid metabolism and phospholipid (2 transposon mutants), (v) protein folding and stabilization (9 transposon mutants), (vi) regulation (12 transposon mutants), (vii) transport and binding (9 transposon mutants), and (viii) hypothetical protein (9 transposon mutants). A complete list of the transposon insertion sites is in Table S1 in the supplemental material. As expected, several mutants showed transposon insertion within the same gene (Table 2). Six independent transposon mutations were mapped at different locations of the *lpqM* gene, encoding lipoprotein peptidase. Two transposon insertions were located in the MSMEG_6136 gene, which encodes TerC family

TABLE 2 Summary of sequence analysis of high secretor mutants having multiple transposon insertions in the same gene

Transposon mutations[a]	*M. smegmatis* gene	Protein function
6, 15, H92, TH46, TH63, TH65	MSMEG_ 4913	Lipoprotein peptidase, LpqM
H17, H24	MSMEG_6136	TerC family protein, membrane protein
TH66, TH24	MSMEG_1254	DEAD/DEAH box helicase

[a] Bold indicates mutants used for immunogenicity studies (Fig. 2).

proteins containing a number of integral membranes. Similarly, two independent transposons were found in MSMEG_1254, which encodes DEAD/DEAH box helicase. In addition, there are several mutants with multiple transposon insertions in the same operon (Table 3). In sum, 54 *M. smegmatis* genes likely have important roles in *M. smegmatis* protein expression and secretion. At least one transposon mutant of a gene or operon with multiple insertions was tested for its immunogenicity and compared to wild-type *M. smegmatis*. The bolded transposon identification in Tables 2 and 3 represents the mutants used for immunogenicity studies (Fig. 3).

Immunoblot analysis reveals that cell culture supernatants of the transposon mutant candidates contain larger amounts of transgene products. In order to further characterize the mutations responsible for the enhanced secretion of the transgene products, the cell-associated pellet (PPT) fraction and the short-term culture filtrate (CF) fraction of transposon mutant growth cultures were separated by SDS-PAGE and analyzed by Western blotting. Transposon mutants expressing *M. tuberculosis* antigen LpqH tagged with the immunodominant H2-K^b-presented MHC class I peptide SIINFEKL (pSL301) (20) were used to determine the level of transgene product secretion in the transposon mutant backgrounds. Western blot analysis was performed using antibodies against SIINFEKL to detect LpqH and against Hsp65 as a loading control. Since each mutant is potentially unique in its respective secretion of transgene product, as well as endogenous protein, no loading control for culture filtrates was used. Instead we used the LpqH/Hsp65 ratio in cell lysates as an indicator of similar loading of the proteins in the culture filtrates, since the same volume and growth stage of the cultures were processed equally for the Western blot. The expression of LpqH in PPT fractions of the transposon mutants was comparable to that in PPT fractions of the parental strain (Fig. 2). As expected, the mutant strains demonstrated significantly better secretion of *M. tuberculosis* LpqH than the parental strain (Fig. 2). Based on the similar amounts of Hsp65 and LpqH proteins in cell pellets, we conclude that the mutants expressed comparable quantities of endogenic and transgenic proteins. To rule out autolysis as a source of the extracellular LpqH, Hsp65 (a commonly used marker for autolysis) (31, 46) was also measured in these short-term culture filtrates. The presence of LpqH in culture filtrates was not due to leakage or shedding of the protein, since no detectable Hsp65 (a cytoplasmic protein) was found in these short-term CFs (data not shown). In order to determine if the mutations affect endogenous protein secretion, protein extracts representing whole-cell lysates and culture supernatant fractions were analyzed using DnaK antibody. DnaK is a heat shock protein that is known to be associated with the bacterial surface (20) and secreted to the culture supernatant (38). Although similar amounts of DnaK protein were detected in the PPTs of the mutant strains and the wild-type *M. smegmatis*, the amount of DnaK protein in the culture supernatants of the mutants was generally higher than that in the wild-type culture. Some mutants (bold numbers in Fig. 2) used for the Western analysis were further tested for their immunogenicity (Fig. 3).

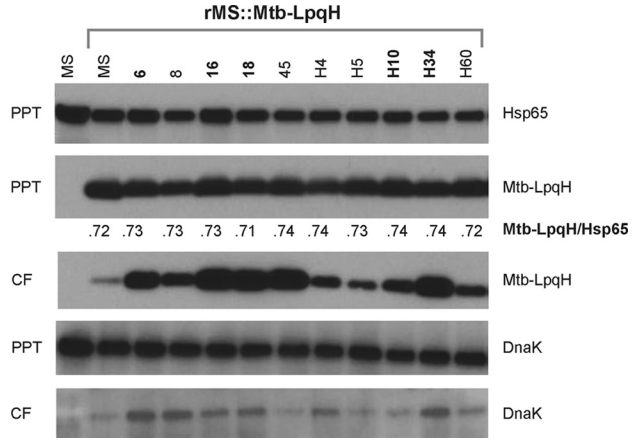

FIG 2 Significantly higher levels of protein secretion are detected in *M. smegmatis* transposon mutants. The parental strain and transposon mutants expressing *M. tuberculosis* LpqH protein tagged with the SIINFEKL epitope were grown to log phase in 7H9 medium without oleic acid-albumin-dextrose-catalase (OADC). Proteins were fractionated into a cell-associated pellet (PPT) fraction and a short-term culture filtrate (CF) fraction and analyzed by Western blotting for the indicated proteins. SIINFEKL polyclonal antibody for LpqH, Hsp65-specific monoclonal antibody for a protein loading control, and DnaK antibody for *M. smegmatis* secreted protein were used. Bolded transposon mutants are those evaluated in immunogenicity studies. The protein expression levels of the transposon mutants and the parental strain were compared by calculating the LpqH/Hsp65 ratio in PPTs.

TABLE 3 Summary of sequence analysis of high secretor mutants having the transposon insertion in the same operon

Transposon mutation[a]	*M. smegmatis* gene	Protein function
TH27	MSMEG_5166	Na^+/solute symporter
30	MSMEG_5167	Major facilitator superfamily protein MFS_1
H10	MSMEG_1253	Hypothetical protein
TH66	MSMEG_1254	DEAD/DEAH box helicase
H34	MSMEG_0136	Virulence factor Mce family protein, putative
H84	MSMEG_0137	Probable conserved Mce-associated membrane protein
TH43	MSMEG_6756	Glycerol kinase, GlpK
20	MSMEG_6758	Glycerol kinase, GlpK
22	MSMEG_6750	Hypothetical protein
16	MSMEG_6752	Endoglucanase A

[a] Bold indicates mutants used for immunogenicity studies (Fig. 3).

Improved T cell response in high secretor mutants. To facilitate analysis of T cell responses, we took advantage of recombinant transposon mutants expressing pSL301 (LpqH-SIINFEKL)

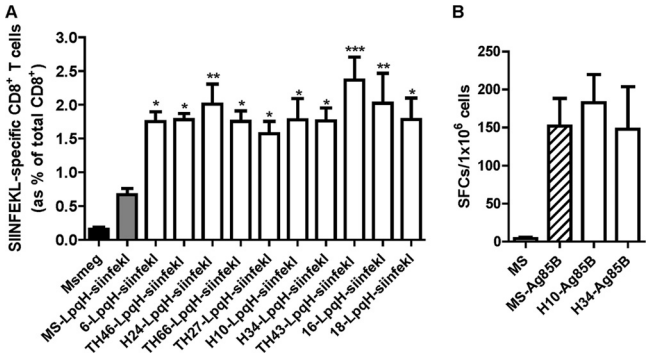

FIG 3 Enhanced immunogenicity of transposon mutants with higher secretion of the transgene product. C57BL/6 mice were immunized intravenously with approximately 1×10^7 CFU of either parental or transposon mutant strains expressing LpqH-SIINFEKL or Ag85B. (A) The mean (±standard error of the mean) percentages of SIINFEKL-specific tetramer positive $CD8^+$ T cells from PBMCs collected at week 1 are shown ($n = 5$ per group). (B) MHC II-specific T cell responses in spleens were assessed by using IFN-γ ELISPOT assays to measure the recall response of CD4 T cells specific for p25 in spleen suspensions; mean (±standard error of the mean) results are shown. The experiments were repeated two times, and results of a representative experiment are shown. Statistical significance was assessed by comparing immunogenicity results for the parental strain group (MS-LpqH-SIINFEKL or MS-Ag85B) to those for recombinant transposon mutants expressing the transgenes ($n = 5$ per group) using ANOVA one-way analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to results for MS-SIINFEKL).

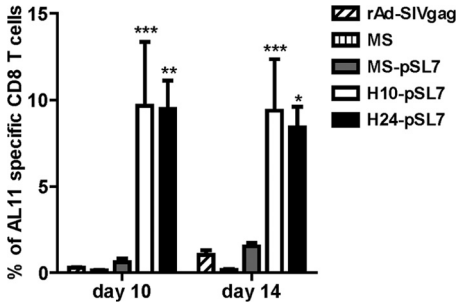

FIG 4 Improved SIV Gag-specific $CD8^+$ T cell responses of *M. smegmatis* transposon mutants. Mice were immunized via the intravenous route with 1×10^7 CFU of actively growing log-phase *M. smegmatis* transposon mutants, H10 and H24, expressing pSL7 (SIV Gag). Heterologous boost was accomplished by injecting 10^6 PFU rAd5-SIV Gag via the intramuscular route. Recombinant transposon mutants, H10-pSL7 and H24-pSL7, were primed for higher Gag-specific $CD8^+$ T cell responses following suboptimal rAd5-Gag (1×10^6 PFU) boosting than the parental *M. smegmatis* strain expressing SIV Gag. The *P* values were determined by ANOVA. * and **, $P < 0.05$ and $P < 0.01$, respectively.

for $CD8^+$ T cell responses or expressing pSL302 (Ag85B) for $CD4^+$ T cell responses. The recombinant strains were generated by generalized transduction as described in Fig. 1. Mice were immunized with 1×10^7 CFU of wild-type *M. smegmatis*, and transposon mutants expressing pSL301 and immunogenicity were assessed by SIINFEKL-specific tetramer responses at 1 week postinfection. All of the tested transposon mutants showed significantly improved $CD8^+$ T cell responses compared to those of the parental strain expressing SIINFEKL ($P < 0.05$). On average, 2.8-times-higher SIINFEKL-specific tetramer positive responses were detected in animals immunized with recombinant transposon mutants (Fig. 3A). The magnitude of induced $CD4^+$ T cell responses was also determined by the IFN-γ ELISPOT assay against immunodominant peptide 25 of Ag85B (p25, FQDAYNAAGGH NAVF) at 1 week after immunization. Uniformly strong $CD4^+$ T cell responses were observed in animals that received either the wild type or transposon mutants expressing pSL302 (Fig. 3B). The immunogenicity data demonstrated that enhanced protein secretion improved antigen-specific $CD8^+$ T cell responses.

To assess the ability of these strains to prime for SIV Gag-specific $CD8^+$ T cell responses, C57BL/6 mice were administered either wild-type *M. smegmatis* or two transposon mutants (H10 and H24) expressing plasmid pSL7. Since responses to the SIV Gag AL11 epitope presented on H-$2D^b$ are not dominant in mice, we did not observe significant SIV Gag AL11-specific primary $CD8^+$ T cell responses following a prime immunization (data not shown). However, since recombinant strains expressing transgenes can efficiently prime for robust secondary responses following a heterologous boost even in the absence of a detectable primary transgene-specific response, these mice were boosted with a suboptimal dose of recombinant adenovirus type 5 (rAd5)-SIV Gag (1×10^6 PFU) at 4 weeks after vaccination with recombinant *M. smegmatis* and controls. At 10 and 14 days after boosting with rAd5-Gag, we observed significantly enhanced AL11-specific $CD8^+$ T cell responses in mice primed with H10 and H24 expressing SIV Gag compared to those in mice primed with parental *M. smegmatis* expressing SIV Gag (Fig. 4). Vaccination with H10-pSL7 and H24-pSL7 primed for an AL11-specific $CD8^+$ T cell response (mean tetramer responses, 9.6% and 9.3% of total peripheral blood $CD8^+$ T cells, respectively) that was approximately 6-fold higher than the response primed by vaccination with the recombinant *M. smegmatis* strain (mean tetramer response, 1.5%). Based on this study, a patent application (S. Lee, B. F. Haynes, W. R. Jacobs, and N. L. Letvin, U.S. patent application 61/677,913 [31 July 2012]) has been filed for a method of preventing HIV-1 transmission that includes high secretor mutant screening and expression vector construction.

Mutation in *lpqM* confers enhanced secretion of transgene products in *M. smegmatis*. We successfully generated a deletion mutant (SLMS330, Δ*lpqM*) by specialized transduction for the purpose of further studying LpqM protein function with respect to the secretion and immunogenicity of transgene products. *M. smegmatis* Δ*lpqM* contains a disrupted *lpqM* gene; a 1,093-bp DNA fragment in the gene's coding region was deleted and was replaced with a hygromycin cassette (Fig. 5A). The mutation was confirmed by PCR using three sets of primers, LL/RR, LL/Hyg1, and RR/Hyg 2 (Fig. 5B). The complemented Δ*lpqM* strain (SLMS331) was generated by expressing the deleted gene in the integrating mycobacterial vector (pMV361Apra). The recombinant *lpqM* deletion mutant (SLMS332, Δ*lpqM*-SIINFEKL) secreted the transgenic *M. tuberculosis* LpqH-SIINFEKL protein better than the parental strain (SLMS301) and the complemented strain (SLMS333) (Fig. 6A). The complemented strain showed a low level of protein secretion to culture filtrates, similar to the wild-type level. We observed a slight decrease in the LpqH protein secretion by the complemented strain compared to the wild-type strain, probably due to its constitutive *lpqM* expression by the Hsp60 promoter. Reflecting the enhanced protein secretion, the SIINFEKL-specific tetramer responses were significantly higher in the mice immunized with the new recombinant Δ*lpqM* mutant expressing the *M. tuberculosis* LpqH-SIINFEKL protein (SLMS332, Δ*lpqM*-SIINFEKL) than in those

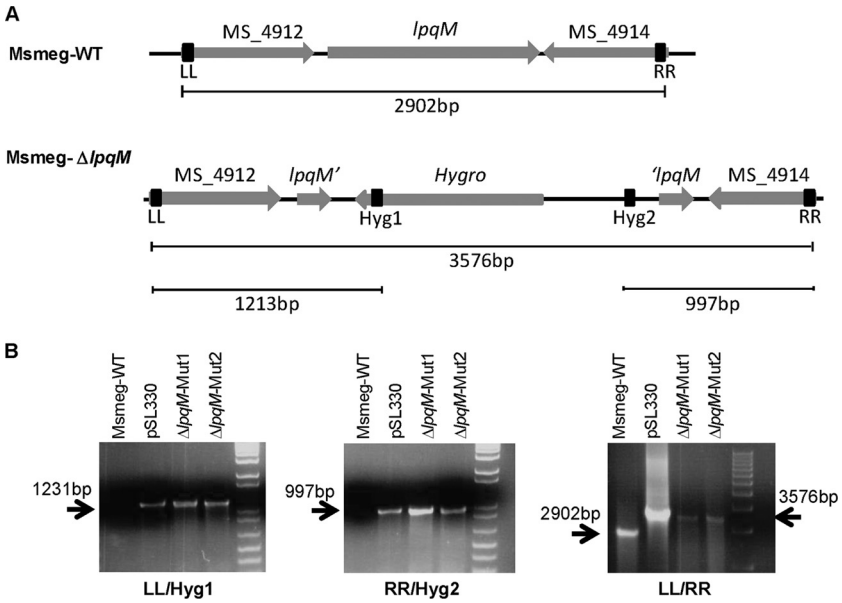

FIG 5 Construction of the *lpqM* mutant. (A) Maps of the *lpqM* genomic regions of wild-type *M. smegmatis* (Msmeg) and a representative *lpqM* mutant. The primer sites (LL, RR, Hyg1, and Hyg2) used for PCR confirmation of mutant clones are indicated as black boxes. (B) PCR products of genomic DNAs from wild-type *M. smegmatis* (lane 1), an allelic exchange plasmid construct (pSL330) for *lpqM* mutagenesis (lane 2), and two independent *lpqM* mutant clones from *M. smegmatis* (lanes 3 and 4). Primers LL/Hyg1, RR/Hyg2, and LL/RR were used, and the expected sizes of the PCR products are indicated. A molecular size marker is shown on the right.

immunized with both the recombinant complemented (SLMS333, Δ*lpqM*-C-SIINFEKL) and wild-type (SLMS301, MS-SIINFEKL) strains (Fig. 6B). However, no statistical difference was measured for the CD4 T cell response, as determined by the IFN-γ ELISPOT assay (Fig. 6C).

DISCUSSION

Attenuated strains of bacteria have been developed as potential live vectors to express homologous or heterologous antigens of many pathogens to induce protective immune responses. Nonpathogenic *M. smegmatis* can effectively express pathogenic antigens and has been used as an important vector for the development of live vaccines (9, 11, 32, 33, 49, 57, 59). In this study, genome-wide screens were employed to identify *M. smegmatis* genes affecting transgene product expression, secretion, and immunogenicity. We have isolated 61 mutations in 54 *M. smegmatis* genes that can enhance stable secretion/expression of foreign antigens (Fig. 1) and further enhance cellular immune responses (Fig. 3). We mapped mutations to different categories: cell envelope, cellular processes, energy metabolism, fatty acid and phospholipid metabolism, protein fate, regulatory functions, and transport and binding proteins (see Table S1 in the supplemental material). These gene loci may not represent the entire list of genes involved in *M. smegmatis* protein expression and secretion due to

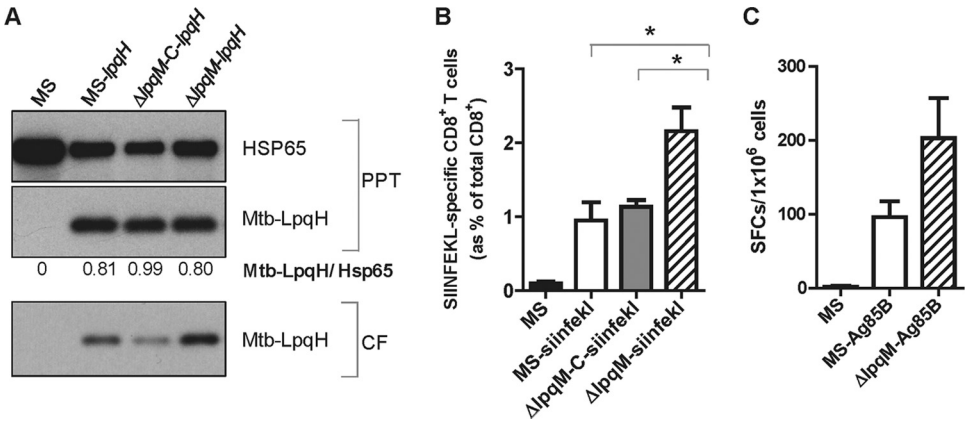

FIG 6 Improved transgene secretion and CD8$^+$ T cell responses by the *M. smegmatis* Δ*lpqM* mutant. (A) The cell-associated pellet (PPT) fraction and the short-term culture filtrate (CF) fraction of the gene deletion mutant (Δ*lpqM-lpqH*) and the complemented strain (Δ*lpqM-C-lpqH*) expressing LpqH-SIINFEKL were analyzed by Western blotting for the indicated proteins. As a control, wild-type *M. smegmatis* (MS) and *M. smegmatis* expressing LpqH-SIINFEKL (MS-*lpqH*) were included. (B and C) SIINFEKL-specific tetramer-positive CD8$^+$ T cells from PBMCs (B) and the Ag85B-specific CD4$^+$ T cell response determined by IFN-γ ELISPOT assay in splenocytes (C) of mice immunized with the indicated recombinant strains (1×10^7 CFU). *, $P < 0.05$.

the limitation of the screening method. For example, our screening used a Sec-dependent signal peptide fused with PhoA as a reporter, but it is likely that transposon mutagenesis using reporters fused with different secretion signals will identify other novel secretion mutants.

Three genomic loci and five additional operons were found more than once during our screening, which strongly suggest that these genes are important to enhance the expression and secretion of the transgene products (Tables 2 and 3). However, how these genomic loci contribute to enhanced secretion of the transgene products remains to be determined. Six independent transposon insertions in *lpqM* were responsible for higher levels of secretion of transgene products (PhoA and LpqH). LpqM, a putative lipoprotein-metalloproteinase, has been shown to be essential for efficient DNA transfer by conjugation (36). LpqM is a membrane-associated protein and requires an intact signal and lipobox for membrane localization. It has been proposed that LpqM probably does not contribute directly to DNA transfer but instead may generate extracellular signal molecules through the proteolysis of proteins released by the Esx-1 or Tad system (2). Similarly, a possible role for LpqM in protein secretion would be to process extracellular proteins into a form able to inactivate secretion. The enhanced secretion of the transgene product shown in the *lpqM* deletion mutant and the reversion of the phenotype to wild type indicate that LpqM is involved in the protein secretion pathway of *M. smegmatis* (Fig. 6). Further investigation is needed to assess potential functions of the LpqM protein in protein secretion. Possible roles of the TerC family protein or DEAD/DEAH box helicase in enhanced protein secretion phenotype are less clear. Both genomic loci have not been shown to be important players in protein secretion and regulation. Since the TerC family protein is hypothesized to catalyze efflux of tellurium ions (25), it may also have an extra activity of secreting the target proteins directly or indirectly. We also isolated two transposon mutants (TH66 and TH24) with mutations in MSMEG_1254, encoding DEAD/DEAH box helicases, and another (H10) with a mutation upstream of MSMEG_1254. The DEAD/DEAH box helicases are a family of proteins whose purpose is to unwind nucleic acids (30, 41). RNA helicases of the DEAD box, and related DExD/H proteins, constitute a very large superfamily of proteins conserved from bacteria and viruses to humans. They are associated with all processes involving RNA molecules, including transcription, editing, splicing, ribosome biogenesis, RNA export, translation, RNA turnover, and organelle gene expression (17, 30, 50). In the context of protein secretion, disruption of DEAD/DEAH box helicase protein may affect transcription of a rate-limiting factor(s) of the secretion machinery and thus cause decreased regulation in protein secretion. Interestingly, there are 6 additional annotated DEAD/DEAH box helicases in the *M. smegmatis* genome, but the precise molecular role of these proteins remains obscure.

In a similar manner, the majority of the disrupted genes in the same operons (Table 3) encode either unknown functions or known functions that are, at this time, difficult to associate with protein secretion. For example, a gene we identified, an endoglucanase A gene homolog, encodes a functional cellulase that is also encoded in the genome of *M. tuberculosis* and in other mycobacterial genomes. Considering that the function of this protein is to utilize plant cell wall material as a nutrient, it may reflect the ancestral soil origin of *Mycobacterium* or hint at a novel environmental niche (52). It is unclear how this protein may play a role in protein secretion. Thus, further studies, including complementation analysis, are needed to dissect the functions of this protein, as well as the other protein candidates. For some categories of genes involved in protein fate, transport and binding, and regulatory functions, their respective roles in protein expression and secretion can be speculated on easily. For others, it is not so straightforward. We speculate with caution that cell envelope, fatty acid biosynthesis, and phospholipid metabolism may influence cell membrane integrity and thus assist protein transport. Mutations in cellular processes and energy metabolism may affect the growth of the bacteria and protein expression. For the purpose of prioritizing further studies to determine if the enhanced secretion by these mutants affects immunogenicity, we chose high secretor mutants displaying multiple insertions in the same gene or operon, since they likely have a more significant role in protein secretion than the mutants with a single transposon insertion.

It has been shown that higher transgene product expression and secretion are directly related to improved immunogenicity (18, 56, 58). As we expected, significantly higher immunogenicity was observed in mice infected with the transposon mutant candidates associated with higher secretion of the transgene products. Analysis by use of the SIINFEKL-specific $CD8^+$ tetramer response and the IFN-γ ELISPOT assay of responses against p25 (Fig. 3) revealed uniformly strong antigen-specific $CD8^+$ responses in animals that received transposon mutants expressing the transgenes (Fig. 3A). However, no significant differences in $CD4^+$ T cell responses were detected among the immunized mice (Fig. 3B). We speculate that the lack of enhanced $CD4^+$ T cell responses in the secreted mutants may be due to a stronger innate capacity of MHC class II-restricted antigen presentation than of MHC class I-restricted antigen presentation in mycobacteria. Thus, even though there is a slight increase in antigen 85 presentation with the mutant strains, the difference may not be statistically significant. Recently it has been shown that hyperexpression of Ag85B in wild-type *M. smegmatis* led to autophagy that increased its MHC class II-dependent presentation to T cells, which again may lessen the possible $CD4^+$ T cell response difference that can be observed from the immunization with the high secretor mutants. Alternatively, highly secreted antigens from the phagosomes of transposon mutants would have easy access to the MHC class I antigen-processing pathway to activate $CD8^+$ T cells. Since a major goal of this work is to develop novel vectors for use as priming immunogens, we also examined whether the transposon mutants might be effective vectors for priming $CD8^+$ T cell responses to a potential vaccine antigen, SIV Gag. Two mutants, H10 and H24, expressing SIV Gag showed enhanced immunogenicity compared to the parental strain in prime/boost vaccination (Fig. 4). These results support the view that mycobacteria possess mechanisms to regulate protein secretion, which in turn affect stimulation of $CD8^+$ T cell responses. The manipulation of such mechanisms can contribute significantly to protective immunity against the pathogen.

In sum, we were able to isolate over 60 transposon mutants that enhance both secretion of transgene products and transgene-specific immunogenicity. The ability of the targeted genes to alter the immunogenicity of the vaccine construct was verified through several approaches. First, we identified more than one strain with independent transposon disruptions giving rise to the same immunogenicity phenotype. Second, we employed site-directed mutagenesis to delete targeted genes in the wild-type *M. smegmatis* construct; the resulting mutant strains displayed the phenotype of

the transposon-disruption mutant strains. Third, we complemented one of the transposon-disrupted genes with a wild-type copy of the gene and observed a reversion to the wild-type phenotype. Thus, we formally confirmed the contributions of the genes identified in the transposon mutant library screen of *M. smegmatis* strains.

A problem facing those attempting to generate live vaccine vectors has been the instability of the inserts due to either high or insufficient expression/secretion of transgene products. Our study shows that genetic modification of a live vector can overcome those issues without sacrificing high-level transgene product expression/secretion or immunogenicity. This novel and effective approach, of enhancing $CD8^+$ T cell responses by deregulating secretion pathways of key antigens, may also be applied to and utilized with vaccine development strategies for numerous other significant diseases.

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