

Flow Cytometry Sorting of Recombinant Mycobacterial Species Yields Bacterial Clones with Enhanced Insert Expression[∇]

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Recombinant mycobacteria hold promise as vectors for delivery of HIV-1 and other pathogen antigen inserts for inducing systemic and mucosal immune responses. In general, the immunogenicity of the recombinant mycobacterial insert is proportional to the level of insert expression. In this study, a novel flow cytometry-based assay has been developed to sort live recombinant mycobacterial mutants with high expression of foreign inserts and to enrich those sorted bacterial populations. Sorted recombinant mycobacterial clones expressed higher levels of the ovalbumin SIINFEKL epitope, and select sorted clones showed better immunogenicity than unsorted recombinant mycobacteria. Thus, flow cytometry-based sorting can isolate recombinant mycobacteria enriched for higher insert expression.

There are barriers to the development of recombinant mycobacterial vaccine vectors, including low levels of vaccine insert gene expression and vector instability. To develop strategies for obtaining higher levels of insert gene expression in recombinant mycobacteria, we developed a flow cytometry-based assay system for quantitative screening of the surface expression of foreign antigens. We hypothesized that we could isolate mycobacterial mutants that have high surface level expression of foreign gene inserts by a novel flow cytometry-fluorescence-activated cell sorting (FACS) technique. Previous studies have reported the use of flow cytometry analysis of mycobacteria for the identification of different mycobacterial species (20), for the detection of reporter gene expression (13), and for testing for susceptibility to antimicrobials in *Mycobacterium tuberculosis* (11). However, there are no reports of surface staining and sorting of recombinant mycobacteria for the isolation of high insert expressers. The present work determined whether a fluorescence-labeled antibody combined with FACS is a suitable method for isolating high surface expressers of recombinant surface antigens in mycobacteria. We show that the strategy of flow cytometry-based analysis of labeled bacteria coupled with surface insert expression can be used to sort and isolate recombinant mycobacterial clones with enhanced insert expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Mycobacterium smegmatis* mc²155 was used to generate recombinant *M. smegmatis*. Plasmid pMP10 (kindly provided by Steven Porcelli, Albert Einstein College of Medicine, New York, NY) was used to transform *M. smegmatis* mc²155 (*M. smegmatis*/pMP10) for the expression of the *M. tuberculosis* 19-kDa SIINFEKL-E_α(₅₂₋₆₈) fusion protein, which is regulated by the MTB heat shock protein 60 (*hsp60*) expression cassette (15). *Mycobacterium*

bovis BCG-Danish strains were used to develop bacterial surface staining. BCG and *M. smegmatis* mc²155 were grown in Middlebrook 7H9 broth (Difco, Sparks, MD) containing 10% oleic acid-dextrose complex (OADC; Difco), 0.5% glycerol, 0.05% Tween 80 (4).

Generation of transposon mutagenesis library. Tn5371 (*hyg*) is a derivative of the *mariner* family transposons (14) and was used for transposon mutagenesis of *M. smegmatis*/pMP10. Transduction of *Mariner* transposons was performed as described previously (6). Transductants were plated on 7H10 mycobacterial medium supplemented with antibiotics. Briefly, Tn5371 was cloned into a mycobacteriophage TM4-based vector, the temperature-sensitive phage phAE159, resulting in phAE159 (Tn5371) (6). This phage was used as a vehicle for transposon delivery and for random mutagenesis of *M. smegmatis*/pMP10.

Immunizations. Female C57BL/6 mice (Charles River Laboratory, Raleigh, NC) 6 to 8 weeks of age were used for immunogenicity studies of a major histocompatibility complex (MHC) class I molecule Kb binding peptide, SIINFEKL. Sorted recombinant *M. smegmatis* mc²155 strains were resuspended in phosphate-buffered saline (PBS) containing 0.05% Tween 80. Each mouse was immunized intraperitoneally (i.p.) with 1×10^7 CFU of recombinant mycobacteria. Mouse peripheral blood mononuclear cells (PBMCs) were collected 7 days after immunization.

Female rabbits 8 weeks of age were obtained from Charles River Laboratories (Raleigh, NC) and were used for obtaining anti-BCG rabbit serum. BCG-Danish organisms were resuspended in PBS containing 0.05% Tween 80, and each rabbit was immunized i.p. with 1×10^7 CFU of BCG. Animals were housed under AALAC guidelines with animal use protocols approved by the Duke University Animal Use and Care Committee and the Duke University Institutional Biosafety Committee.

Bacterial surface staining with polyclonal antibody. Mycobacteria were prepared as described above. Mycobacteria were washed with 0.3 ml PBS–0.05% Tween 80 ($12,000 \times g$ for 1.5 min at room temperature) and with 0.3 ml PBS. Mice were immunized twice with BCG-Danish (1×10^7 CFU/mouse). Anti-BCG serum was collected 2 weeks after each immunization and assayed by enzyme-linked immunosorbent assay (ELISA) for antibody titers against *M. tuberculosis* whole-cell lysates (Colorado State University, Fort Collins, CO). Two rabbits were immunized with BCG-Danish (1×10^7 CFU/rabbit) three times, serum was collected 2 weeks after each immunization, and ELISA was performed for antibody titers against *M. tuberculosis* whole-cell lysate. To optimize flow cytometry-based assays of antibody binding to recombinant mycobacteria, we assayed BCG cells with mouse anti-BCG serum labeled with goat anti-mouse (GAM) IgG-phycoerythrin (PE). Identification of recombinant mycobacteria that expressed the surface ovalbumin SIINFEKL epitope was performed with anti-SII NFEKL antibody and anti-IgG-Alexa Fluor 488 (AF488) and -AF647 reagents (Invitrogen, CA). Bacteria were resuspended with 100 μ l of anti-SIINFEKL rabbit serum (1:5,000 dilution) (kindly provided by Steven Porcelli, Albert Einstein University Medical Center, New York, NY) and incubated for 1 h at 4°C.

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Stained mycobacteria were washed twice with 0.5 ml PBS and resuspended in 100 μ l PBS, and goat anti-rabbit (GAR) IgG-Alexa Fluor (Invitrogen, Carlsbad, CA) was added. One approach to increase the sensitivity of bacterial surface staining is to reduce fluor-derived background staining. Background staining due to nonspecific binding of fluors to bacterial cells was reduced by labeling bacteria using two different fluors conjugated to goat anti-rabbit reagent and gating on the double-positive bacteria. Therefore, we double labeled bacteria with GAR IgG-AF488 and GAR IgG-AF647 (Invitrogen, CA), and then incubated them for 30 min at 4°C with both reagents and washed them twice with 0.5 ml PBS. Stained bacteria were fixed with PBS with 2% ultrapure methanol-free formaldehyde (Polysciences, Warrington, PA) for flow cytometry analysis.

Flow cytometry-based sorting of mixed populations of *M. smegmatis* and *M. smegmatis*/pMP10. Mixed cultures (1:1 or 1:10) of *M. smegmatis* mc²155 expressing the ovalbumin SIINFEKL epitope (*M. smegmatis*/pMP10; SIINFEKL⁺, Km^r, white colonies) and *M. smegmatis* mc²155, producing red colonies (*M. smegmatis* dsRed; lacking SIINFEKL [SIINFEKL⁻], Km^r, red colonies), were double stained with saturating amounts of anti-SIINFEKL rabbit serum and GAR IgG-AF647 and GAR IgG-AF488. After sorting of the double-positive population (see Fig. 4), the sorted bacterial cells were plated onto 7H10 plates containing 25 μ g/ml kanamycin and 40 μ g/ml hygromycin to determine colony color.

Western blot analysis of sorted bacteria. To monitor the expression of the ovalbumin SIINFEKL epitope, individual colonies of sorted recombinant *M. smegmatis* were grown in Middlebrook 7H9-OADC-Tween broth in the presence of kanamycin and hygromycin. The lysate of grown recombinant *M. smegmatis* was fractionated on 4 to 20% SDS-PAGE gels and blotted onto nitrocellulose filters (Invitrogen, CA). Mycobacterial DnaK protein bands were detected by incubation of the filters with monoclonal antibody HAT-3 (IT41; 71-kDa DnaK antigen) (Colorado State University, Fort Collins, CO) at 1 μ g/ml followed by incubation with GAM IgG (Sigma, St. Louis, MO)-alkaline phosphatase (AP). SIINFEKL epitope bands were detected by anti-SIINFEKL rabbit serum (1/10,000 dilution) and GAR IgG-AP (Sigma, St. Louis, MO). Next, we determined the expression of the SIINFEKL epitope in sorted recombinant *M. smegmatis* bacilli by Western blot analysis. The SIINFEKL/DnaK expression ratio in scanned gels was analyzed with Quantity One software (Bio-Rad, CA).

Tetramer staining and flow cytometric analysis. Mice were anesthetized with isoflurane, and blood was collected in RPMI 1640 containing 40 U of heparin (American Pharmaceutical Partners, Schaumburg, IL) per ml. 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 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 PBS containing 2% fetal bovine serum and fixed with PBS containing 2% ultrapure methanol-free formaldehyde. CD8⁺ T cells were analyzed for tetramer staining using two-color flow cytometry analysis with a BD LSR II cytometer (BD Biosciences, CA) and FlowJo software (Tree Star, Ashland, OR).

IFN- γ ELISPOT assays. Enzyme-linked immunosorbent (ELISPOT) assays were performed as described previously (21, 22). 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 MHC class II E-alpha peptides (ASFEAQGALANIAVDKA) (17) (these peptides are immunogenic in C57BL/6 mice) and then incubated for 24 h at 37°C in 5% CO₂. 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. 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 Student's *t* test. Data are expressed as the means \pm standard errors of the means.

RESULTS

Study of mixtures of labeled and unlabeled bacteria using anti-BCG serum for ability to identify antibody-coated bacteria. Our initial attempts to stain and sort recombinant BCG

(rBCG) expressing high levels of foreign inserts were limited by high background staining of anti-immunoglobulin reagents. Therefore, to increase sensitivity, we used polyclonal anti-BCG antibody obtained from mice immunized i.p. with 1×10^7 CFU of BCG-Danish two times. In order to optimize the flow cytometry-based assay of surface staining of BCG-Danish cells, we incubated rBCG with polyclonal murine anti-BCG serum followed by GAM IgG-PE. We observed an effect of unlabeled BCG decreasing the fluorescence intensity of fluorescently labeled BCG when labeled BCG was mixed with unlabeled BCG. That is, the observed fluorescence of labeled bacteria in labeled plus unlabeled bacterial mixtures was markedly decreased compared to the expected fluorescence (Fig. 1A). When we performed surface staining with mixed BCG-Danish and *Escherichia coli* with anti-BCG serum-GAM IgG-PE, BCG cells were also not stained due to the quenching effect of unlabeled *E. coli* bacterial cells (Fig. 1B). Similar results were observed when BCG-Danish cells were reacted with anti-BCG serum-GAM IgG-PE and mixed with BCG cells stained with a 15 mM dimethylformamide solution of 5-*N*-hexadecanoyl aminofluorescein (HAF) (Invitrogen, CA) (10) after surface staining (Fig. 1C). We also used anti-BCG serum with GAM IgG-fluorescein isothiocyanate (FITC) and GAM IgG-APC for bacterial surface staining, but the results showed quenching patterns similar to those when GAM IgG-PE was used (not shown). Figure 1D shows results for GAM IgG-AF647-labeled antibody-coated mycobacterial mixtures without quenching.

Next, we stained the mycobacterial surface with anti-BCG rabbit serum and GAR IgG-PE, GAR IgG-FITC, or GAR IgG-AF647. We found that GAR IgG-AF647 stained the bacterial surface strongly both in the presence of polyclonal rabbit anti-BCG serum and in the presence of polyclonal rabbit anti-SIINFEKL serum (Fig. 2).

Development of double-label bacterial surface staining with recombinant mycobacteria expressing the SIINFEKL epitope for sorting subpopulations of recombinant mycobacteria. In order to screen for mutants of recombinant mycobacteria with higher levels of surface expression of insert proteins on the surface, we analyzed surface staining of recombinant *M. smegmatis* mc²155 expressing the *M. tuberculosis* 19-kDa ovalbumin SIINFEKL-E α ₍₅₂₋₆₈₎ fusion protein (*M. smegmatis*/pMP10) regulated by an *hsp60* expression cassette.

We compared the results of incubating *M. smegmatis*/pMP10 with anti-SIINFEKL rabbit serum and GAR IgG-AF647, GAR IgG-AF488, or GAR IgG-AF647 and GAR IgG-AF488 (Fig. 3). With single staining with anti-SIINFEKL serum and GAR IgG-AF647 or GAR IgG-AF488 and sorting of AF647- or AF488-positive recombinant mycobacteria, bacterial cells were not separated effectively (data not shown). However, double staining of recombinant mycobacteria with anti-SIINFEKL rabbit serum with both anti-IgG-AF647 and anti-IgG-AF488 effectively decreased the background staining for flow cytometry sorting by allowing the isolation of double-positive (i.e., AF488 and AF647 surface-stained SIINFEKL⁺) cells (16). That is, each fluor has its own background such that the most specific staining was where GAR IgG-AF647 and GAR IgG-AF488 both stained mycobacteria. After mixing *M. smegmatis*/pMP10 (to give rise to white, SIINFEKL⁺ colonies) and *M. smegmatis* dsRed (to give rise to red, SIINFEKL⁻ colonies), the mixed cells were stained with anti-SIINFEKL rabbit serum

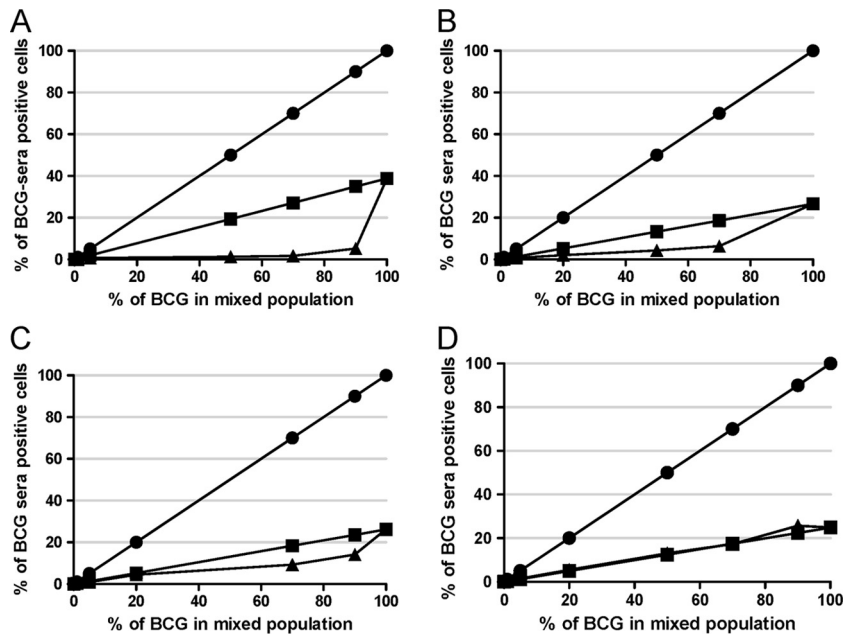


FIG. 1. BCG surface staining with anti-BCG serum. (A) Results for mixed BCG reacted with murine anti-BCG serum and unlabeled BCG. (B) Results for mycobacteria stained with anti-BCG serum in mixtures of BCG-Danish and *E. coli*. (C) Results for mixed BCG-Danish reacted with anti-BCG serum and BCG with the lipophilic dye 5-*N*-hexadecanoyl aminofluorescein (Invitrogen, CA) after surface staining. (A, B, and C) We observed a quenching effect when BCG was stained with anti-BCG serum and goat anti-mouse IgG phycoerythrin (PE) and mixed with unlabeled BCG or *E. coli* cells. (D) Results for BCG reacted with anti-BCG serum and with goat anti-mouse IgG-Alexa Fluor 647 and then mixed with unstained BCG after surface staining. *x* axis is % of BCG-serum-positive cells and *y* axis is % of labeled BCG in mixed population. We assumed that anti-BCG would stain 100% of bacteria under ideal conditions (A, closed circles). Thus, the closed-circle curve shows the overall expected values assuming that all bacteria that are positive for a particular marker are indeed positive, and the line is a straight line on the diagonal. The data represented by the closed squares show expected values based on actual BCG staining with anti-BCG serum, where only 39% stain positive with the anti-BCG serum. Therefore, in 50:50 mixtures of labeled BCG versus unlabeled BCG, the anti-BCG-positive cells should be 19.5% of the total (A, closed squares). However, experimentally, 50:50 mixtures of labeled-BCG and unlabeled-BCG showed only 1.3% of BCG labeled with anti-BCG serum, thus demonstrating quenching of the fluorescence from the anti-BCG label (A, closed triangles). We studied 7 different ratios of labeled BCG versus unlabeled BCG as follows: 0:100, 1:99, 5:95, 50:50, 70:30, 90:10, and 100:0.

with GAR IgG-AF647 and GAR IgG-AF488, and then sorted cells that were in the double-positive-labeled gate were isolated (Fig. 4). When sorted cells were grown on 7H10 plates containing kanamycin and hygromycin, sorted cells with

AF488^{hi} AF647^{hi} gating generated 74% white SIINFEKL⁺ colonies (26% red colonies), while sorted cells with AF488^{lo} AF647^{lo} gating contained only ~5% white colonies (~95% red colonies) (Fig. 4, average of counts from 3 plates). Therefore,

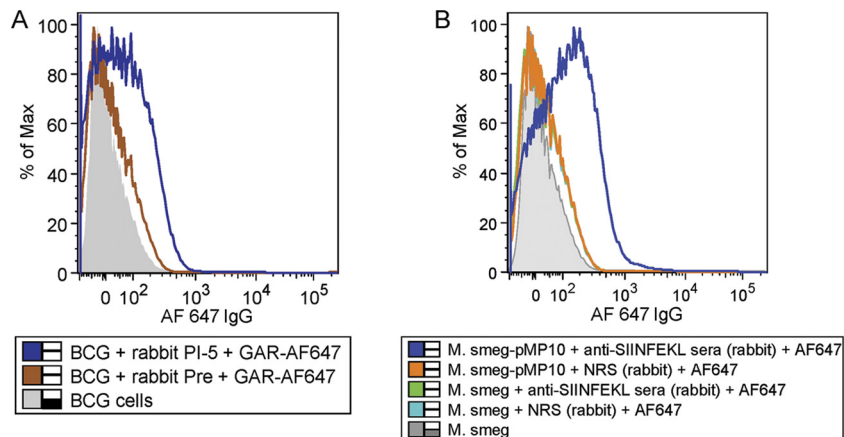


FIG. 2. Polyclonal rabbit serum with goat anti-rabbit IgG-Alexa Fluor 647 (GAR-AF647) strongly stained the mycobacterial surface. We stained mycobacterial surface antigens with anti-BCG rabbit serum and GAR IgG-AF647. (A) GAR IgG-AF647 strongly labeled the bacterial surface. “Pre” indicates preimmune rabbit serum (1:5,000 dilution), and “PI-5” indicates rabbit immune serum (1:5,000 dilution). (B) In addition, rabbit anti-SIINFEKL serum strongly stained *M. smegmatis*/pMP10 conjugated with GAR IgG-AF647. “*M. smegmatis*-pMP10” is the *M. smegmatis* mc²155 strain expressing the ovalbumin SIINFEKL epitope, and “*M. smegmatis*” indicates empty *M. smegmatis* mc²155. “NRS” indicates normal rabbit serum.

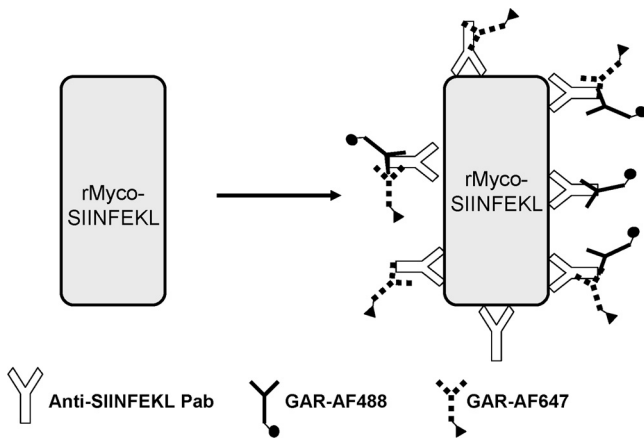


FIG. 3. Double staining of recombinant mycobacteria (rMyco) with anti-SIINFEKL rabbit serum (Pab [polyclonal antibody]) and goat anti-rabbit IgG-Alexa Fluor 647 (GAR-AF647)/GAR-AF488. Recombinant *M. smegmatis* mc²155 or BCG that expressed the SIINFEKL epitope was surface stained with anti-SIINFEKL rabbit serum and GAR IgG-AF647 and GAR IgG-AF488.

we concluded that the flow cytometry-based sorting technique can effectively isolate surface-labeled bacterial cells from a mixed recombinant mycobacterial culture.

Flow cytometry sorting and detection of recombinant mycobacterial mutants with enhanced SIINFEKL epitope expression in *M. smegmatis* mc²155. To isolate high expressers, we stained and sorted bacteria from an *M. smegmatis*/pMP10 transposon mutant library expressing SIINFEKL. Sorted double-positive bacterial populations were plated onto 7H10 medium containing kanamycin and hygromycin. Colonies were collected and resuspended in 7H9 medium with kanamycin and hygromycin and grown for 2 to 3 h, and bacterial surface

staining and flow cytometry sorting performed as described in Materials and Methods. SIINFEKL double-positive bacteria comprised 0.017% (AF488^{hi} AF647^{hi}) of the primary sort, while double-positive populations were enriched ~17-fold, to 0.29% (Fig. 5A). We also sorted double-negative populations (AF488^{lo} AF647^{lo}) as negative controls and found that they expressed the SIINFEKL epitope at lower levels than double-positive populations (Fig. 5B).

To determine the level of expression of the SIINFEKL epitope, individual colonies of sorted recombinant *M. smegmatis* were grown and the lysate of recombinant *M. smegmatis* was fractionated on 4 to 20% SDS-PAGE gels and blotted onto nitrocellulose filters (Invitrogen, CA). As expected, there were no detectable protein bands at 19 kDa in lanes with the parent strain not expressing the SIINFEKL epitope (empty *M. smegmatis* mc²155) (Fig. 5B). DnaK is a major heat shock protein in *M. smegmatis* mc²155 that is induced in response to oxidative stress, heat shock, and ethanol challenge (7, 9). We did see 75-kDa DnaK protein bands in every lane, including lanes with empty *M. smegmatis* mc²155. The average expression ratio (SIINFEKL/DnaK) of double-negative populations (AF488^{lo} AF647^{lo}) was 1.67, while the average ratio of double-positive populations (AF488^{hi} AF647^{hi}) was 2.19 ($P = 0.047$) (Fig. 5B). Therefore, the expression level of the SIINFEKL epitope in double-positive (SIINFEKL⁺) populations was higher than the SIINFEKL expression level in double-negative (SIINFEKL⁻) populations.

Sorted *M. smegmatis*/pMP10 elicited SIINFEKL-specific CD8⁺ T cell responses. We next tested the sorted *M. smegmatis*/pMP10 from the transposon mutagenesis library for immunogenicity in mice. Immune responses were monitored by measuring CD8⁺ T cell responses to the MHC class I H-2-restricted SIINFEKL epitope in peripheral blood of immunized mice. *M. smegmatis* constructs expressing SIIN

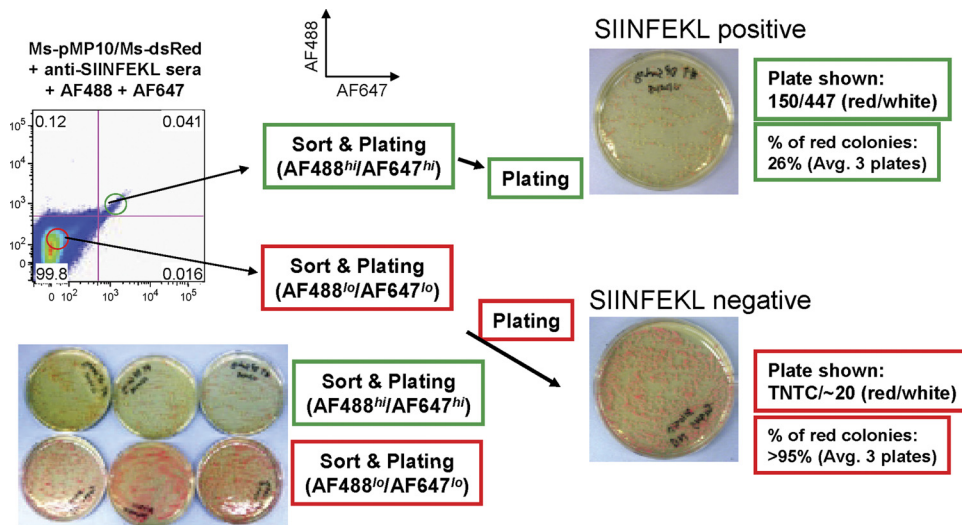


FIG. 4. Sorting and postsorting analysis of mixed cultures. Mixed cultures of *M. smegmatis* mc²155 expressing the SIINFEKL epitope (Ms-pMP10), producing white colonies, and *M. smegmatis* mc²155 (Ms-dsRed), producing red colonies, were stained with anti-SIINFEKL serum followed by goat anti-rabbit IgG-Alexa Fluor 647 (AF647) and goat anti-rabbit IgG-AF488. Bacteria with double-negative (AF488^{lo} AF647^{lo}) and double-positive (AF488^{hi} AF647^{hi}) staining were sorted and then plated onto 7H10 plates containing kanamycin. After growth, the percentage of red colonies in each plate was measured. "TNTC" indicates that colonies were too numerous to count. We performed sorting and plating experiments three times with the same results.

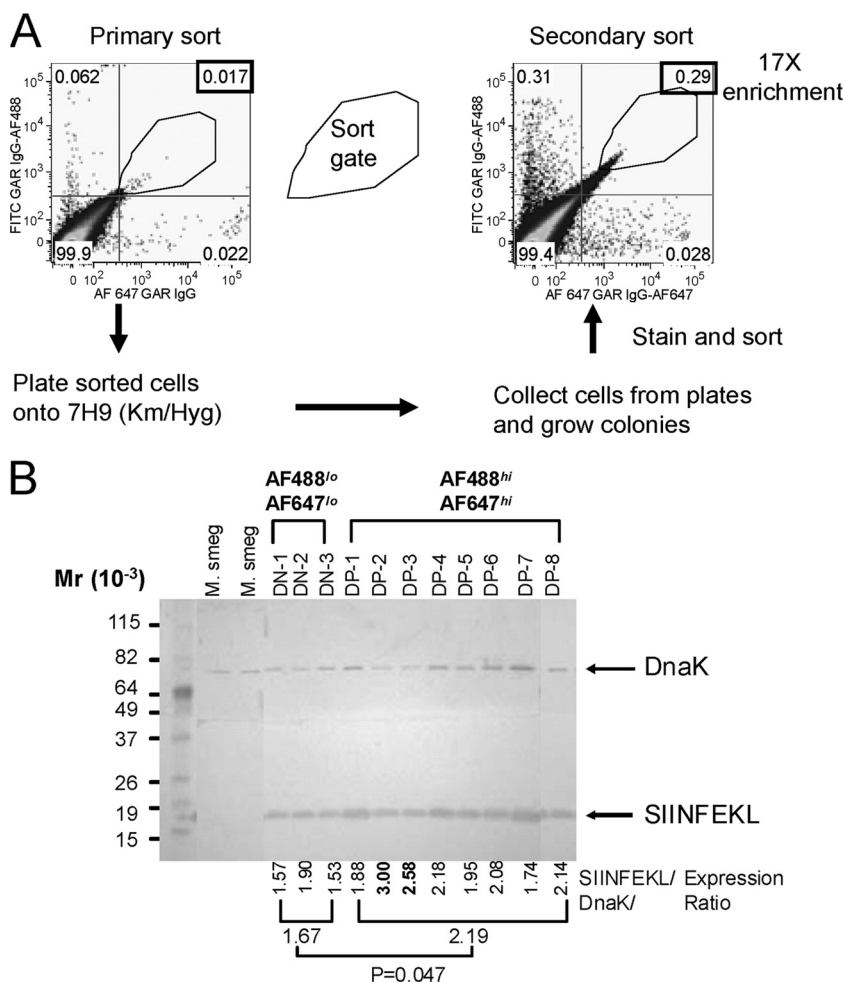


FIG. 5. Enrichment of *M. smegmatis*/pMP10 transposon library with primary and secondary recombinant *M. smegmatis* sorts. (A) An *M. smegmatis*/pMP10 transposon library was constructed by specialized transduction in recombinant *M. smegmatis* mc²155 expressing the ovalbumin SIINFEKL epitope. The double-positive population was sorted, plated onto 7H10 plates containing kanamycin and hygromycin (Km/Hyg), and grown. Then, a secondary sort was performed with recombinant *M. smegmatis* cells from the primary sort plates. The double-positive population was enriched from 0.017% (primary sort) to 0.29% (secondary sort). (B) Expression of the SIINFEKL epitope in sorted recombinant *M. smegmatis* bacilli as shown by Western blot analysis. The SIINFEKL/DnaK expression ratio in scanned gels was analyzed with Quantity One software (Bio-Rad, CA). Molecular weight standards in thousands (Bio-Rad, CA) are indicated. “DN” (double negative) indicates sorted population with AF488^{lo} AF647^{lo} staining and “DP” (double positive) indicates sorted population with AF488^{hi} AF647^{hi} staining. “M. smeg” indicates empty *M. smegmatis* mc²155 as negative controls. The immunization groups and doses are shown on the x axis. Statistical significance was assessed by comparing results for double-negative groups (AF488^{lo} AF647^{lo}) to those for double-positive groups (AF488^{hi} AF647^{hi}) ($P = 0.047$).

FEKL elicited peripheral blood SIINFEKL-specific CD8⁺ T cell responses in mice immunized intraperitoneally with 1×10^7 CFU bacilli (Fig. 6A). Empty *M. smegmatis* mc²155 did not induce any detectable CD8⁺ T cell responses. Double-negative populations (AF488^{lo} AF647^{lo}) generated 0.3% (DN-1) and 0.36% (DN-2) SIINFEKL tetramer-positive CD8⁺ T cells, while the highest-staining double-positive (AF488^{hi} AF647^{hi}) recombinant *M. smegmatis* clone (DP-2) induced 0.65% SIINFEKL tetramer-positive CD8⁺ T cells ($P < 0.05$).

Detection of class II IFN- γ SFCs generated in spleen by sorted *M. smegmatis*/pMP10 prime-boost. We previously observed that HIV-1 CON6 gp120-specific T cell responses were easily detected by the IFN- γ SFC assay when rBCG or recombinant *M. smegmatis* expressing CON6 gp120 was boosted at 8 weeks following the priming immunization (21, 22). In this study, mice were similarly primed and boosted with 1×10^7

CFU double-positive-sorted *M. smegmatis* cells. For determination of T cell responses after prime-boost with sorted *M. smegmatis*/pMP10 cells, lymphocytes were isolated from splenocytes of all immunization groups of mice and assayed for allogeneic E $\alpha_{(52-68)}$ epitope-specific MHC class II T cell responses in IFN- γ ELISPOT assays (17). C57BL/6 (E β) mice were immunized i.p. with double-positive-sorted *M. smegmatis*/pMP10 at 1×10^7 CFU in week 0 and week 8. Unimmunized mice and double-negative-sorted *M. smegmatis*/pMP10 (DN clones) served as negative controls. Mice primed and boosted with 1×10^7 CFU double-positive-sorted *M. smegmatis*/pMP10 (DP-3) induced 51 ± 5 SFCs/ 10^6 splenocytes, a significantly greater number of SFCs than in mice primed and boosted with double-negative-sorted *M. smegmatis*/pMP10 (DN-2, 27 ± 7 SFC) ($P = 0.035$) or mice immunized with empty *M. smegmatis* mc²155 (3 ± 2 SFC) ($P < 0.0001$),

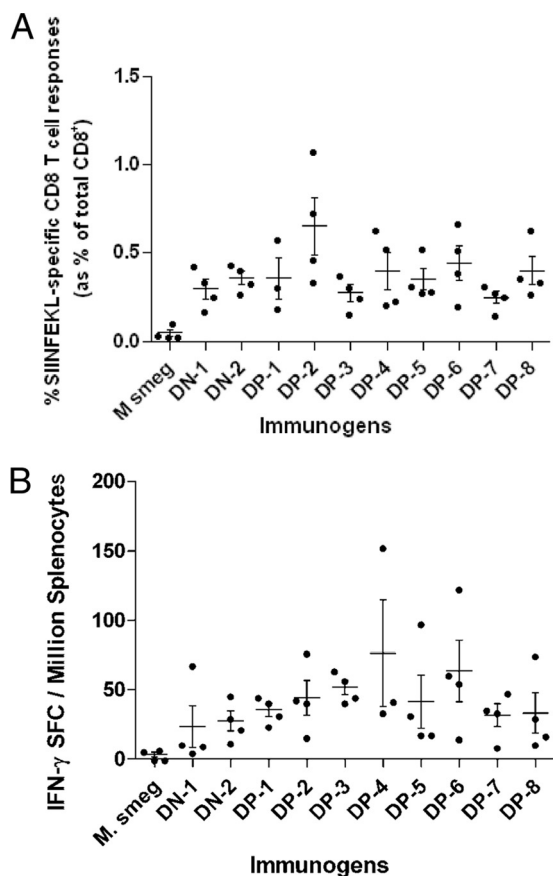


FIG. 6. Sorted recombinant *M. smegmatis*/pMP10 elicited SIINFEKL-specific T cell responses in mice. C57BL/6 mice were inoculated i.p. with approximately 1×10^7 CFU of SIINFEKL-expressing recombinant *M. smegmatis* mc²155 that had been sorted with double-positive gating. (A) The mean (\pm standard error of the mean) percentages of SIINFEKL tetramer-positive CD8⁺ T cells from PBMCs collected at week 1 are shown ($n = 4$ per group). (B) MHC II-specific T cell responses in spleen were assessed by using IFN- γ ELISPOT assays; mean (\pm standard error of the mean) results are shown. Mice were primed and boosted with 1×10^7 CFU recombinant *M. smegmatis*. Statistical significance was assessed by comparing ELISPOT assay results for DN-2 groups to those for DP-3 groups ($P = 0.035$) ($n = 4$ per group). As a negative control, mice were inoculated with the same dose of mycobacteria sorted with double-negative gating. "M. smeg" indicates empty *M. smegmatis* mc²155. "DN" (double negative) indicates sorted populations with AF488^{lo} AF647^{lo} staining, and "DP" (double positive) indicates sorted populations with AF488^{hi} AF647^{hi} staining. The indicated immunization groups and doses are shown on the x axis.

indicating that double-positive-sorted *M. smegmatis*/pMP10 induced greater MHC class II T cell responses (Fig. 6B).

DISCUSSION

In this paper, we demonstrate a strategy for FACS-based isolation of recombinant mycobacteria that have enhanced surface expression of the inserts. Populations of recombinant mycobacteria that expressed the SIINFEKL epitope were identified as expressing either high or low levels of SIINFEKL and collected for expansion and reanalysis after sorting. Flow cytometry-based assays for the rapid identification of *Mycobacterium* species using fluorescein-labeled antibodies (20), for

fluorescence-based detection of reporter gene expression in *Mycobacterium* species (13), for detection of selected surface-exposed antigens of mycobacteria (10), and for susceptibility of *M. tuberculosis* to antimicrobials with a nucleic acid stain (11) have been previously reported. Another report demonstrated flow cytometric detection of polyclonal antibody binding to *Lactococcus lactis* with surface expression of HIV-1 Env V2-V4 (18). However, there have been no reports on the use of this method for surface staining and sorting of recombinant mycobacteria for isolation of those expressing inserts at high levels.

Even though flow cytometry has been broadly used as an analytical tool in cell biology since the 1970s, its application for bacterial cells has been limited, mainly due to the difficulty of using flow cytometry to study small particles (1, 2). We have overcome this problem by using bead sizing standards and double labeling of our detection reagents to decrease the fluorescent background and enhance detection sensitivity. Fluorescence *in situ* hybridization (FISH)-FACS has been applied to target and sort previously uncultivated microorganisms from complex environments for genome analyses with whole-genome amplification techniques (5, 12). One study reported applying FISH-FACS to isolate microorganisms presenting at low frequencies in the environment for whole-genome sequencing (12). Others have shown successful mRNA-based sorting of cells in laboratory cultures, but attempts to sort cells from live bacterial cultures have been unsuccessful due to low sensitivity (5). The difference between these previous studies and our studies is that our studies sorted unfixed bacteria for subsequent growth.

In our study, we observed that unlabeled BCG and *E. coli* cells quenched fluorescence-labeled BCG cells such as those labeled with PE but that there was no interference with Alexa Fluor 647 and 488. Similar fluorescence quenching by bacteria has been previously reported (3, 8, 19). However, we overcame this problem by surface staining with rabbit anti-BCG serum conjugated with Alexa Fluor 647 and 488. In addition, we showed that target recombinant mycobacterial cells sorted with AF488^{hi} AF647^{hi} gating were enriched to 74% white colonies from a more than 1:20 mixed culture (i.e., red colonies at $\geq 95\%$) (Fig. 4).

The experiments performed here also asked the question, "Can we screen for mutants of recombinant mycobacteria with higher levels of surface expression of the SIINFEKL epitope using SIINFEKL-specific antibody and flow cytometry analysis?" We generated a random transposon mutant library of *M. smegmatis*/pMP10. This transposon library contained high expressors and low expressors of the SIINFEKL epitope. We showed that we were able to screen for recombinant mycobacterial mutants with higher levels of surface expression of the SIINFEKL epitope using SIINFEKL-specific antibody and flow cytometry analysis. Our next question was, "Can enriched recombinant mycobacteria with higher levels of surface expression of the SIINFEKL epitope induce higher levels of immunogenicity in mice than unsorted recombinant mycobacteria?" More importantly, we demonstrated that the bacilli that sorted with double-positive gating, which expressed higher levels of the SIINFEKL epitope, induced higher levels of immunogenicity than double-negative-sorted bacilli (Fig. 6).

In conclusion, our data are proof of concept that a FACS-

based sorting technique can isolate recombinant mycobacteria and enrich for both higher levels of insert expression and immunogenicity. These techniques should be useful for screening live vaccine vectors for tuberculosis and other pathogens.

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