Generation of Mucosal Anti-Human Immunodeficiency Virus Type 1 T-Cell Responses by Recombinant Mycobacterium smegmatis

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A successful vaccine vector for human immunodeficiency virus type 1 (HIV-1) should induce anti-HIV-1 immune responses at mucosal sites. We have generated recombinant Mycobacterium smegmatis vectors that express the HIV-1 group M consensus envelope protein (Env) as a surface, intracellular, or secreted protein and have tested them in animals for induction of both anti-HIV-1 T-cell and antibody responses. Recombinant M. smegmatis engineered for expression of secreted protein induced optimal T-cell gamma interferon enzyme-linked immunospot assay responses to HIV-1 envelope in the spleen, female reproductive tract, and lungs. Unlike with the induction of T-cell responses, priming and boosting with recombinant M. smegmatis did not induce anti-HIV-1 envelope antibody responses, due primarily to insufficient protein expression of the insert. However, immunization with recombinant M. smegmatis expressing HIV-1 Env was able to prime for an HIV-1 Env protein boost for the induction of anti-HIV-1 antibody responses.

A vector that induces mucosal immunity will be an important component of an effective human immunodeficiency virus type 1 (HIV-1) vaccine. Recombinant bacillus Calmette-Guérin (rBCG) has been shown to induce systemic anti-HIV-1 T-cell responses in animals and humans (1, 11–13, 15, 24). However, in patients with immune deficiency, BCG can cause, albeit rarely, a clinically significant mycobacterial infection (4, 30). We have previously demonstrated that recombinant Mycobacterium smegmatis can induce systemic anti-HIV-1 T-cell responses to the wild-type HIV-1 group M consensus envelope (Env) epitope expressed as a surface protein (2).

A strategy to address HIV-1 diversity for the induction of broadly reactive anti-HIV-1 T-cell responses is the design of artificial group M consensus genes (6). As we have previously described, the HIV-1 group M consensus envo gene CON6 induces anti-HIV-1 T-cell responses with a breadth equal to that of polyvalent HIV-1 immunogens (5, 28).

In this paper, we describe recombinant M. smegmatis vectors constructed to express the HIV-1 group M consensus envo gene CON6 either as a surface, intracellular, or secreted protein and determine the insert expression and immunogenicity of these constructs. Moreover, we have determined the ability of HIV-1 Env expressed in recombinant M. smegmatis to induce mucosal T-cell immune responses after systemic immunizations. We found that recombinant M. smegmatis induced lower anti-HIV-1 T-cell responses than did a recombinant adenosine virus (rAd)-recombinant vaccinia virus (rVV) prime/boost in the spleen but induced similar gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay responses to rAd-rVV in the female reproductive tract (FRT) and lungs. These data demonstrate that recombinant M. smegmatis is immunogenic for the induction of HIV-1 T-cell responses at mucosal surfaces.

MATERIALS AND METHODS

Bacterial strains and plasmids. M. smegmatis mc2 155 was used for the generation of recombinant M. smegmatis. Table 1 summarizes the mycobacterial plasmids that were used to transform M. smegmatis mc2 155 for expression of the insert HIV-1 envelope (env) CON6-gp120 or CON6-gp140CF gene (23). Expression of the HIV-1 CON6 env gene in the pMV261 and pMV361 vectors is regulated by the Mycobacterium tuberculosis heat shock protein 60 (hsp60) expression cassette (26). pMV261 is a constitutive expression plasmid, while expression of Hsp60 fusion protein in pMV361 was increased in response to stress with heat, acid, and peroxide (26). pH7 plasmids are kanamycin resistant and were designed to express foreign proteins in different locations under regulation of the M. tuberculosis α-antigen promoter. Plasmid pH152 was used to express the CON6 env gene fused to the M. tuberculosis 19-kDa signal sequence as a surface protein. Plasmid pH153 was used to express the CON6 env gene as an intracellular protein. Plasmid pH154 was used to express the CON6 env gene fused to an M. tuberculosis α-antigen export signal sequence as a secreted protein. Plasmids pYUB2051, pYUB2052, and pYUB2053 are the same as plasmids pH152, pH153, and pH154, respectively, except that the pH7 plasmids contain the hygromycin resistance gene instead of the kanamycin resistance gene.

Construction of the HIV-1 env gene in mycobacterial expression vectors. To clone the group M consensus CON6 gp120 and CON6 gp140CF env genes (6) into various plasmids (Table 1) at the desired cloning sites, DNA fragments were generated by PCR with primer containing sequences of the corresponding restriction enzyme sites. Specifically, to clone the HIV-1 CON6 gp120 and gp140CF env genes in pMV261 and pMV361, CON6 gp120 and gp140CF env DNAs were amplified by PCR using Platinum Pfx polymerase (Invitrogen) with the forward primer 5'-ATGGCCGCGATCACAGATTCGATGGGTGAAGTGG C-3' and the backward primer set 5'-ATCGGCGCGGTATACACTAGCCCTTG GATGGG-3' (for amplifying gp120) and 5'-ATCGGCGCGGTATACACTAGCCCTTG GATGGG-3' (for amplifying gp140CF). For the construction of gp120 and gp140CF in the pH7 and pYUB series plasmids, the HIV-1 env DNAs...
were amplified by PCR using the forward primer 5'-ATGCCGCGCCGCACCCGCAC-TGAACCG-3' and the backward primers described above. Enzymatic manipulations and cloning of the CON6 gp120 and CON6 gp140CF env genes into various plasmids were performed as described previously (22). All constructs were confirmed by DNA sequencing.

Generation of recombinant *M. smegmatis* expressing HIV-1 envelope. All *M. smegmatis* cultures were grown in Middlebrook 7H9 broth (Difco) containing 10% albumin-dextrose saline (ADS)-0.5% glycerol-0.15% Tween 80 (7). For generation of recombinant *M. smegmatis*, *M. smegmatis* mc^2^ 155 prepared in 10% glycerol was transformed with the constructed plasmids (Table 1) by electroporation with a Gene Pulser (Bio-Rad) set at 2.5 kV and 25 μF and with the pulse controller resistance set at 1.00 Ω (23, 27). Transformed *M. smegmatis* organisms were selected on Middlebrook 7H10 (Difco, Sparks, MD) agar plates supplemented with 10% ADS containing either 30 μg/ml kanamycin or 20 μg/ml hygromycin. To monitor the expression of HIV-1 gp120 or gp140CF, individual colonies of recombinant *M. smegmatis* grown in Middlebrook 7H9-ADS-Tween broth in the presence of 30 μg/ml of kanamycin or hygromycin were harvested by centrifugation. After a rinse with sterile phosphate-buffered saline, mycobacterial cells were lysed by using the modified extraction buffer with 106-μm glass beads (Sigma) (7) and cell lysates were cleared by centrifugation. The lysate of recombinant *M. smegmatis* was fractionated by 4 to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose filters (Schleicher & Schuell, Germany). HIV-1 Env protein bands were detected by incubation of the filters with the monoclonal antibody (MAb) T8, 7B9, or 7B2 at 1 μg/ml followed by incubation with goat anti-mouse immunoglobulin G (IgG)-or goat anti-human IgG (Sigma, St. Louis, MO)-alkaline phosphatase. T8 is a mouse MAb that binds to the HIV-1 Env gp120 C1 region (a gift from Pat Earl, NIH, Bethesda, MD). 7B2 is a human MAb against the immuno-dominant region of the HIV-1 Env gp41 region and was a gift from James Robinson (Tulane Medical School, New Orleans, LA). 7B9 is a mouse IgG1 MAb made from murine B cells immunized with the 6219 HIV-1 clade B V3 loop sequence (RPNNYTRK SHICPGPRAFT) (18) that reacts with the CON6 gp120 V3 loop.

Mouse immunizations. Female BALB/c mice (6 to 8 weeks of age) were purchased from Charles River Laboratory (Raleigh, NC) and housed in the Duke Cancer Center Isolation Facility under AALAC guidelines with animal use and care protocols approved by the Duke University Animal Use and Care Committee. The Duke Cancer Center Isolation Facility is a Level 4 facility with a Biosafety Level 4 cabinet (BSC) and a Biosafety Level 3 cabinet (BSC) for the handling of dangerous materials. For immunization, mice were inoculated subcutaneously with 10^5 CFU/mouse in 100 μl of PBS containing 10% FBS. The mice were boosted with 10^6 CFU/mouse 2 weeks later. The immunizations were repeated three times. After the final immunization, sera were collected from the mice and stored at −80°C until further analysis.

ELISA and antibody isotype analysis. Serum antibody titers against HIV-1 CON6-gp120 or *M. tuberculosis* whole-cell lysate (WCL) (Colorado State University, Fort Collins, CO) were measured in a standard enzyme-linked immunosorbent assay (ELISA). For isotype analysis of serum antibodies, we used goat anti-mouse IgG (heavy chain specific), IgG1, IgG2a, IgG2b, and IgM (Southern Biotechnology) as alkaline phosphatase-conjugated antibodies. Antibody endpoint titers were determined as the reciprocal of the highest dilution of the serum assayed against recombinant HIV-1 envelope protein or *M. tuberculosis* WCL giving an optical density reading of experiment/control of ≥3.5 times over the background activity of prebleed serum samples. Geometric mean titers were determined based on the endpoint titer of each mouse serum. Statistical significance was assessed by Student's t test.

### IBN-γ ELISPOT assays

ELISPOT assays were performed as described previously (20). Briefly, 96-well flat-bottom plates (Millipore) were coated with an anti-mouse IBN-γ capture antibody (Pharmingen). After the plates were blocked and washed, lymphocytes freshly isolated from the spleen, lungs, and FRT of immunized mice were added and incubated with a mixture of either 10 CON6-gp140 Env 15-mer peptides (for determining T-cell responses to the HIV-1 Env insert) that have been previously shown to represent CON6 Env epitopes recognized in BALB/c mice (6, 28; E. Weaver, Z. Lu, H. X. Liao, B. Ma, M. S. Alam, R. M. Scaife, L. L. Sutherland, J. M. Decker, Z. Hartman, A. Amalfitano, B. T. Korber, R. H. Hahn, D. C. Montefiori, and B. Hayes, AIDS Vaccine 2003 Meet., abstr. 43, 2003) or *M. tuberculosis* WCL (for determining T-cell responses to the *M. smegmatis* vector itself) for 24 h at 37°C in a 5% CO2 incubator. For the counting of spot-forming cells (SFC), ELISPOT plates were scanned into an ImmunoSpot Series I analyzer and spots were quantitated with ImmunoSpot 2.1 software (CTL Analyzers, Cleveland, OH). Cells cultured in medium in the absence of peptides or *M. tuberculosis* WCL were used as controls. For analysis of mucosal T-cell responses, extracted lungs or FRT were pooled from three to four mice and assayed as one data point. At least three data points representing three groups of mice were analyzed. Statistical significance was assessed by Student's t test.

### RESULTS

Expression of CON6 gp120 and gp140CF HIV-1 group M consensus Env proteins in *M. smegmatis* mc^2^ 155. We used various mycobacterial expression vectors for the expression of CON6 gp120 Env or gp140CF Env as a surface, intracellular, or secreted protein. HIV-1 CON6 Env protein expression in *M. smegmatis* was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis (Fig. 1A). No HIV-1-specific protein bands were detected in untransformed *M. smegmatis* (Fig. 1A, lane 2) or recombinant *M. smegmatis* transformed with empty plasmid (lane 3). Figure 1A shows that the expression of the intact CON6 gp120 was detected in recombinant *M. smegmatis* transformed with either the surface expression plasmid pJH152 (Fig. 1A, lane 4), the intracellular expression plasmid pJH153 (lane 5), or the secreted expression plasmid pJH154 (lane 6). Expression of CON6 gp140CF was also detected in recombinant *M. smegmatis* transformed with the surface expression plasmid pJH152 (lane 7) or pJH222 (lane 8). Both the intact and partially cleaved gp140 products were identified with the gp120-specific MAB T8 (anti-C1 gp120 region), the gp41-specific MAB 7B2, and V3 loop-specific MAB 7B9. The calculated nonglycosylated molecular masses based on amino acid sequences were 53 kDa for CON6 gp120 and 71 kDa for CON6 gp140CF. However, the protein bands of CON6 gp120 and CON6 gp140CF expressed in recombinant *M. smegmatis* were approximately 80 kDa and 90 kDa, respectively, suggesting that the CON6 gp120 and CON6 gp140CF proteins expressed in recombinant *M. smegmatis* were partially glycosylated.
Figure 1B shows schematic representations of the full-length (top) and cleaved (bottom) CON6 gp140, with indications of the binding locations of MAbs T8, 7B2, and 7B9. In the cleaved gp140, MAb T8 reacted only with the 34-kDa protein band, while MAbs 7B2 and 7B9 reacted with the 49-kDa protein band (Fig. 1B). These data suggest that the 49-kDa cleaved protein contained portions of the V3 loop region and the gp41 domain. In addition, analysis of the supernatant of recombinant M. smegmatis expressing secreted CON6 gp140 that was grown in Sauton liquid medium revealed that HIV-1 CON6 Env was produced as a truncated soluble protein containing the V3 loop in tissue culture supernatant, as detected by MAb 7B9 (Fig. 1C).

Detection of HIV-1 envelope-specific IFN-γ SFC in the spleen. Mice were immunized with recombinant M. smegmatis expressing HIV-1 gp120 as a secreted insert at doses ranging from $10^7$ to $10^{10}$ CFU at week 0 and week 8 via i.p. injections. Unimmunized mice were used as negative controls. Animals that were primed with rAd5 expressing CON6 Env (rAdCON6) and boosted with rVV expressing CON6 Env (rVV-CON6) were used as positive controls. Splenocytes from animals primed once with recombinant M. smegmatis i.p. yielded no CON6 gp120-specific responses ($<5$ SFC/10^6 cells). The lack of response to a single immunization with recombinant M. smegmatis was consistent regardless of the concentration of recombinant M. smegmatis used ($10^8$, $10^9$, $10^7$, or $10^6$ CFU). When mice were boosted i.p. with recombinant M. smegmatis 8 weeks following the recombinant M. smegmatis prime, HIV-1 CON6 gp120-specific responses in spleens were easily detectable (Fig. 2A). Mice primed and boosted with $10^9$ CFU of recombinant M. smegmatis produced 66 ± 6 SFC/10^6 cells, a significantly greater ($P < 0.01$) number of SFC than mice primed and boosted with lower doses ($10^7$, $10^8$, or $10^6$ CFU). Mouse splenocytes in positive-control mice primed with rAdCON6 and boosted with rVV-CON6 Env produced 358 ± 155 IFN-γ SFC/10^6 cells.

Mucosal anti-HIV-1 T-cell responses induced by recombinant M. smegmatis. In addition to antigen-specific responses to CON6 Env peptides in splenocytes, we also examined antigen-specific T-cell responses in lungs and FRT of immunized mice. Mice immunized once with recombinant M. smegmatis produced no mucosal CON6 gp140 peptide-specific IFN-γ SFC regardless of the number of doses. However, upon boosting of mice with the same initial dose of recombinant M. smegmatis, IFN-γ ELISPOT assay responses were detected 2 weeks following boosting. In the lungs, a prime/boost with $10^8$ CFU of recombinant M. smegmatis gave the highest frequency ($89 ± 2$ SFC/10^6 cells) (Fig. 2B) of IFN-γ SFC, a significantly greater number than that seen with $10^9$ CFU (52 ± 2 SFC/10^6 cells), $10^7$ CFU (8 ± 4 SFC/10^6 cells), or $10^6$ CFU (6 ± 2 SFC/10^6 cells) ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively). Mice primed with rAdCON6 and boosted with rVV-CON6, produced 201 ± 89.6 SFC/10^6 cells in lungs.

We also found that after a prime and boost of recombinant M. smegmatis, CON6 peptide-specific responses were detectable in the mouse FRT (Fig. 2C). Lymphocytes isolated from the FRT of mice that were primed and boosted with $10^9$ CFU of recombinant M. smegmatis produced 63 ± 4 IFN-γ SFC/10^6 cells, a response comparable to that induced by the rAd prime/ rVV boost immunizations (77 ± 36 IFN-γ SFC/10^6 cells).
Vector-specific T-cell responses. Responses to the *M. smegmatis* vector were also monitored in the spleen, lungs, and FRT. Immunizations of mice with 10^9, 10^8, 10^7, and 10^6 CFU of recombinant *M. smegmatis* pJH154-gp120 induced 832 ± 110, 738 ± 75, 591 ± 142, and 154 ± 44 SFC/10^6 cells in splenocytes (Fig. 2D), respectively. Optimal concentrations for the induction of *M. tuberculosis* WCL-specific immunity were 10^8 CFU in FRT (923 ± 212 SFC/10^6 cells) and 10^7 CFU in the lungs (1,169 ± 102 SFC/10^6 cells) (Fig. 2E and F). Unlike the HIV Env-specific T-cell responses, detectable T-cell responses to the vector in the spleen, lungs and FRT were induced with a single priming immunization with recombinant *M. smegmatis*.

IFN-γ SFC responses were optimal in mice immunized with constructs that secreted the HIV-1 CON6 protein. We next compared the immunogenicity of recombinant *M. smegmatis* constructs designed to express HIV-1 Env as a surface, intracellular, or secreted antigen. Mice were first immunized with recombinant *M. smegmatis* at doses ranging from 10^7 to 10^10 CFU at week 0 and week 10 by i.p. or i.d. immunization. Untransformed *M. smegmatis* and recombinant *M. smegmatis* transformed with empty plasmid (pJH222) were used as negative controls. Immunogen groups were recombinant *M. smegmatis* expressing HIV-1 gp120 as full-length proteins (pJH152-gp120, pJH153-gp120, and pJH154-gp120) and recombinant *M. smegmatis* expressing HIV-1 gp140CF as full-length proteins and in cleaved forms (pJH152-gp140CF, pJH222-gp140CF, and pJH154-gp140CF). We found that immunization via the i.p. route (Fig. 3A and C) was optimal for the induction of T-cell responses in comparison with i.d. immunization (Fig. 3B and D). In the spleen, optimal IFN-γ SFC responses were seen in mice immunized i.p. with pJH154-gp120 or pJH154-gp140CF constructs, both engineered to secrete insert protein (Fig. 3A and B). An rAd-CON6 prime/rVV-CON6 boost was used for comparison and showed an approximately fourfold-greater IFN-γ ELISPOT response than did recombinant *M. smegmatis* pJH154-gp120 or pJH154-gp140 (Fig. 3A and B). In addition to this positive control, one group of mice was immunized with only the CON6 Env protein at the time all other groups were boosted with the CON6 Env protein. We found that protein immunization did not augment T-cell responses to the recombinant *M. smegmatis* prime and boost.

Responses to the recombinant *M. smegmatis* vector itself in the spleen were also monitored and showed that i.p. immunization was superior to the i.d. route for induction of vector responses (Fig. 3C and D). While boosting once with *M. smeg-
was effective in boosting an *M. smegmatis* prime, we found that boosting with recombinant *M. smegmatis* a second time had no effect on either anti-Env antibody levels or IFN-γ/H9253 T-cell responses (not shown). Recombinant *M. smegmatis* does not induce anti-HIV-1 Env antibody. Serum samples were collected 2 weeks after each recombinant *M. smegmatis* immunization and assayed by ELISA for antibody titers against recombinant CON6 gp140CF. We found that neither a prime nor a boost of recombinant *M. smegmatis* alone could induce any detectable anti-HIV-1 Env antibody (not shown).

To determine whether recombinant *M. smegmatis* gp120 or recombinant *M. smegmatis* gp140CF could prime for an Env protein boost of antibody responses to the HIV-1 envelope, mice were boosted once with recombinant CON6 gp140CF protein 4 weeks after the second immunization with recombinant *M. smegmatis*. We found that neither a prime nor a boost of recombinant *M. smegmatis* alone could induce any detectable anti-HIV-1 Env antibody (not shown).

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**TABLE 2. Antibody responses to CON6 envelope protein after recombinant *M. smegmatis* CON6 prime and recombinant HIV-1 CON6-gp140 protein boost**

| Prime | Expression | Boost | GMT |
|-------|------------|-------|-----|
| M. smegmatis | Control | CON6-gp140 | 667 |
| Empty pH222 | Control | CON6-gp140 | 560 |
| pH152-gp120 | Surface, full length | CON6-gp140 | 368 |
| pH153-gp120 | Intracellular, full length | CON6-gp140 | 5,164 |
| pH154-gp120 | Secreted, full length | CON6-gp140 | 7,211 |
| pH152-gp140CF | Surface, full length | CON6-gp140 | 700 |
| pH222-gp140CF | Surface, cleaved form | CON6-gp140 | 1,622 |
| pH154-gp140CF | Secreted, cleaved form | CON6-gp140 | 4,498 |
| pH154-gp140CF | Secreted, cleaved form | CON6-gp140 | 759 |

* Dose of recombinant *M. smegmatis*, 10⁸ or 10⁹ CFU.
* pH152-gp140CF protein with Ribi adjuvant.
* GMT, anti-HIV-1 gp140 geometric mean titer (M. smegmatis–M. smegmatis–CON6-gp140CF) ($n = 5$).
* 1× CON6.
other modes of Env expression in recombinant *M. smegmatis* (P < 0.05) (Table 2).

Next, HIV-1-specific IgG subclass responses to recombinant *M. smegmatis* were determined. Immunization of mice with both rAd-rVV and CON6 protein alone induced predominantly IgG1 antibodies, characteristic of a Th2-type response: the IgG1/IgG2a ratios were 4.44 and 3.59, respectively (Fig. 4). Untransformed (empty) *M. smegmatis* plus CON6 Env oligomer boosting also resulted in antibody responses that were biased toward IgG1, with an IgG1/IgG2a ratio of 2.20 (Fig. 4). Interestingly, antibody responses primed by immunizations with recombinant *M. smegmatis* pJH154-gp120 or pJH154-gp140CF showed a dominance of IgG2a over IgG1 antibodies (IgG1/IgG2a ratios of 0.82 and 0.49, respectively) (Fig. 4).

Thus, priming with recombinant *M. smegmatis* expressing HIV-1 Env or Env protein alone changed the HIV-1 isotype profile from IgG1 predominant to IgG2a predominant.

**Mechanism of limitations of recombinant *M. smegmatis* for induction of anti-HIV-1 antibody.** To determine the factors associated with the lack of induction of anti-Env antibodies by recombinant *M. smegmatis*, we first determined whether live recombinant *M. smegmatis* was immunosuppressive for Env insert antibody responses. Live recombinant *M. smegmatis* was mixed with 50 μg of CON6 Env oligomer protein and used to immunize mice. This group of mice was compared to groups that were immunized either with 50 μg CON6 Env oligomer protein alone or with heat-killed recombinant *M. smegmatis* plus 50 μg CON6Env. We found that recombinant *M. smegmatis* did not suppress anti-Env antibody responses, as the three immunogens induced similar levels of antibody responses (Fig. 5). Immunization with recombinant *M. smegmatis* formulated in incomplete Freund’s adjuvant (IFA) did not induce anti-HIV antibody responses, while the formulation of 50 μg CON6 Env in IFA did induce anti-Env antibody. Finally, killed recombinant *M. smegmatis* either alone or with CON6 Env oligomer immunization did not augment HIV-1 Env antibody induction. Thus, recombinant *M. smegmatis* was not suppressive of antibody responses; rather, HIV-1 Env was not expressed at sufficiently high levels in recombinant *M. smegmatis* for an induction of anti-Env antibody.

**DISCUSSION**

In this study, we have shown that recombinant *M. smegmatis* can express full-length hypoglycosylated HIV-1 Env and that, when engineered for insert secretion, it can induce HIV-1 IFN-γ T-cell responses in the spleen, FRT, and lungs. While unable to directly induce anti-HIV-1 Env B-cell responses, recombinant *M. smegmatis* was able to prime for an Env protein boost.

*M. smegmatis* is a rapidly growing nonpathogenic mycobacterial species that has properties that may make it a practical vector, particularly for priming for heterologous recombinant vectors or for protein boosting. *M. smegmatis* has been used as a model system for *M. tuberculosis* and for high-level expression of foreign genes (23). Unlike other mycobacterial species, such as BCG, that survive in host cells by inhibiting phagosome maturation, *M. smegmatis* is rapidly destroyed by phagolysosomal proteases in the phagosomes of infected cells (16, 19), facilitating rapid uptake of expressed antigens in *M. smegmatis* and cross-presentation of antigen. Recombinant *M. smegmatis* has been tested experimentally as a vaccine candidate for *M. tuberculosis* (21), as an alternative gene expression system to BCG or *M. tuberculosis* (3), and as an anticancer immunotherapy with cytokine-expressing *M. smegmatis* (29). Recently, we demonstrated that recombinant *M. smegmatis* expressing HIV-1 HXBc2 env can generate effector, effector memory, and central memory HIV-specific CD8 T cells (2). We also found that preexisting immunity to BCG may have only a marginal effect on the immunogenicity of recombinant *M. smegmatis* (2).

Others have fused the V3 epitope of HIV-1 Env with mycobacterial antigen 85B and expressed the V3-85B fusion pro-
tein in BCG as an HIV immunogen (11, 15). The anti-HIV-1 envelope V3 neutralizing antibody induced by immunization of rhesus macaques with BCG expressing HIV-1 envelope V3 antigen was able to protect against homologous T-cell-line-adapted simian-human immunodeficiency virus type 1 (SHIV-1) but not against heterologous SHIV challenge (24). Moreover, we and others have recently shown the limitation of anti-V3 loop immunogens for practical use as a HIV-1 vaccine immunogen in vivo (8). We found that insufficient levels of HIV-1 CON6 envelope protein expression in M. smegmatis were likely the cause of the lack of induction by a recombinant M. smegmatis prime and boost of anti-Env antibody responses. Thus, our current efforts are targeted at enhancing the expression of HIV-1 Env by incorporation of novel promoters in recombinant M. smegmatis and by insertion of new HIV-1 env genes in recombinant M. smegmatis and screening for optimal Env expression.

To address the issue of genetic diversity in HIV-1 vaccine design, we have begun to explore the immunogenicity of artificial consensus HIV-1 genes (6, 28; Weaver et al., AIDS Vaccine 2003 Meet.). We have previously described the 1999 CON6 envelope and shown that it induced a greater breadth of cross-clade T-cell responses than did a polyclonal subtype A, B, and C envelope immunogen (5, 6). In addition, CON6 protein was equally well recognized by patient sera of multiple HIV-1 subtypes but induced a limited spectrum of anti-HIV neutralizing antibodies to subtype B primary isolates (5, 6).

We have now produced a second-generation HIV-1 consensus Env protein, CON-S, that has shortened variable loops and induces a greater breadth of neutralizing antibodies than CON6 Env to subtype A, B, and C primary isolates (18). Our present study was undertaken to determine whether full-length consensus CON6 Env could be expressed in recombinant M. smegmatis and, if so, whether the expressed Env was immunogenic. While anti-HIV-1 antibodies were not induced by immunization with recombinant M. smegmatis alone, it is important that recombinant M. smegmatis was able to prime for an Env oligomer boost. The experiment performed here sought to answer the question of whether fully glycosylated Env protein can boost antibody responses to conserved Env regions in hypoglycosylated Env expressed in recombinant M. smegmatis. HIV-1 neutralization assays are technically difficult to perform with mouse sera due to serum volumes and natural inhibitors of HIV-1 in mouse sera. It will be of interest to test the breadth of neutralizing antibodies induced by an M. smegmatis prime protein boost in similar experiments with guinea pigs.

To address the issue of the optimal mode of insert expression in recombinant M. smegmatis, we have tested the HIV-1 CON6 env gene that was expressed either as a surface, intracellular, or secreted antigen and determined the comparative expression levels and immunogenicities of these constructs. Differential localization of HIV envelopes within mycobacteria could affect both the timing of the immune response and the pathway of presenting antigen to the immune system. Since mycobacteria are intraphagosomal, expressed antigens in mycobacteria can be delivered into phagolysosomes and preferentially activate CD4+ T cells via the major histocompatibility complex class II pathway (9). Proteins produced within the mycobacterial cytoplasm could remain encapsulated inside the bacterium and their presentation to the host immune system delayed as long as the bacterium persists within the macrophage (14). However, membrane-anchored and secreted antigens from M. smegmatis in the phagosome could result in earlier presentation to the immune system. The most commonly studied secreted sequence is the mycobacterial α-antigen (also known as MPT59 or antigen 85B), which we used to fuse the HIV-1 envelope. The other common sequence is a 19-kDa lipoprotein signal sequence that has been used to deliver the outer surface protein A (OspA) antigen of Borrelia burgdorferi to a membrane-associated lipoprotein (25). Others have shown that protein export via α-antigen is inefficient and that the majority of proteins fused with α-antigen are associated with cell membranes (10, 17). In our study, we demonstrated that anti-HIV-1 T-cell responses in splenocytes were optimal in mice immunized with recombinant M. smegmatis expressing HIV-1 CON6 Env engineered as secreted antigens (Fig. 3). In addition, antibody responses against HIV-1 envelope induced by priming with recombinant M. smegmatis and boosting with CON6 Env protein were also optimal when HIV gp120 and gp140CF were expressed by recombinant M. smegmatis as secreted antigens (Table 2).

Taken together, our data are proof of the concept that recombinant M. smegmatis vectors can induce mucosal cellular responses and prime for protein boosts of serum antibody. If the expression levels of recombinant M. smegmatis vector inserts are optimized, increased levels of immunogenicity should be attainable, and recombinant M. smegmatis may be a candidate to serve as a prime for a heterologous vector boost in an HIV-1 candidate vaccine formulation.

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