Generation of CD8\(^+\) T-Cell Responses by a Recombinant Nonpathogenic \textit{Mycobacterium smegmatis} Vaccine Vector Expressing Human Immunodeficiency Virus Type 1 Env

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Because the vaccine vectors currently being evaluated in human populations all have significant limitations in their immunogenicity, novel vaccine strategies are needed for the elicitation of cell-mediated immunity. The nonpathogenic, rapidly growing mycobacterium \textit{Mycobacterium smegmatis} was engineered as a vector expressing full-length human immunodeficiency virus type 1 (HIV-1) envelope protein. Immunization of mice with recombinant \textit{M. smegmatis} led to the expansion of major histocompatibility complex class I-restricted HIV-1 epitope-specific CD8\(^+\) T cells that were cytolytic and secreted gamma interferon. Effector and memory T lymphocytes were elicited, and repeated immunization generated a stable central memory pool of virus-specific cells. Importantly, preexisting immunity to \textit{Mycobacterium bovis} BCG had only a marginal effect on the immunogenicity of recombinant \textit{M. smegmatis}. This mycobacterium may therefore be a useful vaccine vector.

An effective human immunodeficiency virus (HIV)-AIDS vaccine will likely need to elicit virus-specific neutralizing antibodies and cytotoxic T-lymphocyte (CTL) responses. Although an immunogen that induces antibodies that neutralize a diversity of primary HIV type 1 (HIV-1) isolates has not yet been defined, a number of strategies are being developed for generating HIV-1-specific CTL (31). However, there are problems associated with each of these approaches for eliciting CTL that will likely limit their ultimate effectiveness. Plasmid DNA has not proven nearly as immunogenic in humans as it has in laboratory animals (9, 33, 57). The immunogenicity of replication-defective adenovirus serotype 5 is limited in human populations by preexisting serotype-specific anti-adenovirus antibodies (37). Pox-vectored vaccines only elicit very short-lived immunity in humans (Andrew McMichael, personal communication), and production problems have slowed the development of alphavirus-based vaccine vectors (59). Better vector systems will therefore be needed to induce anti-HIV-1 cellular immunity and prime for broadly neutralizing antibody responses.

Mycobacteria have features that make them attractive as potential HIV-1 vaccine vectors. They can be readily engineered to stably express transgenes and can elicit long-lasting cellular and mucosal immune responses (28, 36). Most importantly, they have been used successfully as vaccines. The attenuated, nonpathogenic \textit{Mycobacterium bovis} BCG is widely used as a vaccine for tuberculosis (TB) and leprosy (21, 27). Recombinant BCG (rBCG) vaccine constructs have shown immunogenicity and protection in murine models against various infectious agents, including \textit{Borrelia burgdorferi}, \textit{Streptococcus pneumoniae}, \textit{Bordetella pertussis}, rodent malaria, leishmania, and measles virus (11, 15, 29, 34, 38, 50). In murine and monkey studies, we and others have shown that rBCG elicited antibody and cell-mediated responses against HIV-1 and simian immunodeficiency virus antigens (2, 20, 51, 65).

\textit{Mycobacterium smegmatis} has a number of properties that may make it an effective vaccine vector. Some \textit{M. smegmatis} strains are nonpathogenic and commensal in humans (5, 39, 45). Unlike other mycobacterial strains such as BCG that survive in host cells for months by inhibiting phagosome maturation, \textit{M. smegmatis} is rapidly destroyed by phagolysosomal proteases in the phagosomes of infected cells (26, 32, 55, 56). Nevertheless, \textit{M. smegmatis} can induce cytokine production by macrophages better than pathogenic mycobacterial species (8, 64) and can activate and induce the maturation of dendritic cells better than BCG by upregulation of major histocompatibility complex (MHC) class I and costimulatory molecules (10). \textit{M. smegmatis} can also access the MHC class I pathway for presentation of mycobacterial antigens more efficiently than BCG (40). The present studies were initiated to assess the ability of recombinant \textit{M. smegmatis} to elicit HIV-1 envelope-specific CD8\(^+\) T-cell responses.

\textbf{MATERIALS AND METHODS}

\textit{Generation of recombinant mycobacteria.} \textit{Mycobacterium smegmatis} MC\(^{\#}155\) was grown in Middlebrook 7H9 (Difco) supplemented with 10% albumin-dex-
trose saline and 0.05% Tween 80 (Fisher Scientific). *Mycobacterium bovis* BCG (Pasteur) was grown in 7H9 media supplemented with 10% oleic acid-albumin-dextrose-catalase (Difco) and 0.05% Tween 80. A human codon-optimized HIV-1 IIB gp120 envelope gene (HXB2) was cloned into the multicopy pH1H222 and single-copy integrative pH1H223 Escherichia coli/mycobacteria shuttle plasmids. A synthetic operon was constructed containing the viral envelope gene, which is regulated by the *M. tuberculosis* α antigen promoter and the *M. tuberculo-* tis 19-kDa signal sequence. For detection of the HIV-1 envelope protein, a hemagglutinin (HA) tag was fused to the C-terminal end of the envelope. Within the operon, a kanamycin resistance gene was cloned downstream of the viral gene. The multicopy and integrative plasmids with the HXB2 envelope gene insert were transformed into the *M. smegmatis* MCΔ155 strain. Recombinant mycobacterial clones were selected for kanamycin resistance on 7H10 agar containing 20 μg/ml of kanamycin (Sigma). Single colonies were grown in 7H9 medium containing 20 μg/ml of kanamycin and grown by shaking for 2 to 3 days until an optical density at 600 nm (OD600) approximately equal to 1. Mycobacteria were then harvested and washed twice in ice-cold phosphate-buffered saline (PBS). Expression of the viral gp120 protein was assessed by Western blotting of mycobacterial lysates (1 μg of total protein) using an anti-HA monoclonal antibody (MAB) (clone 3F10) and a chemiluminescence detection kit according to the manufacturer’s protocol (Roche Applied Science).

**Mice and immunizations.** Eight- to 12-week-old female BALB/c mice were purchased from Taconic and Charles River laboratories. Mice were housed in a biosafety level 3 facility under specific-pathogen-free conditions at the Center for AIDS Research Animal Biohazard Containment Core Site (Dana-Farber Cancer Institute). Research on mice was approved by the Dana-Farber Cancer Institute Animal Care and Use Committee. Recombinant *M. smegmatis* and BCG bacilli were grown in 7H9 medium until an OD600 approximately equal to 1. Mycobacteria were then harvested and washed twice in ice-cold phosphate-buffered saline (PBS). Expression of the viral gp120 protein was assessed by Western blotting of mycobacterial lysates (1 μg of total protein) using an anti-HA monoclonal antibody (MAB) (clone 3F10) and a chemiluminescence detection kit according to the manufacturer’s protocol (Roche Applied Science).

**RESULTS**

**Generation of recombinant *Mycobacterium smegmatis* expressing HIV-1 gp120 envelope protein.** The pHJ222 and pHJ223 E. coli/mycobacteria shuttle plasmids were used to express a HIV-1 HXB2 envelope gene codon-optimized for human cell expression. A human codon-optimized HIV-1 env was used, since the codons used by human cells are similar to those used by mycobacteria (3, 14, 44). pHJ222 is a multicopy plasmid that replicates episomally in mycobacteria (47); pHJ223 is an integration-proficient plasmid (30) that integrates as a single-copy DNA in the mycobacterial genome (Fig. 1A). Both plasmids contained the *M. tuberculosis* α antigen promoter to drive expression of HXB2 env. The HIV-1 envelope was fused to an HA tag at the C terminus. The N terminus of the HIV-1 envelope envelope protein was fused to the 19-kDa signal sequence to facilitate expression of the viral protein in the mycobacterial membrane. Fusion with the 19-kDa signal sequence has been shown to increase immunogenicity of a heterologous protein (50).

Both env-containing plasmids were transformed into the efficient plasmid transformation mutant *M. smegmatis* MCΔ155 (48). Western blot analysis using an anti-HA MAB showed that recombinant *M. smegmatis* clones transformed with the multicopy plasmid had higher expression levels of the viral protein than those transformed with the integrating plasmid (Fig. 1B). Control *M. smegmatis* constructs, which were transformed with either pHJ222 or pHJ223 containing the malaria msp1 gene, did not express the viral antigen. The predicted molecular weight of the envelope protein expressed by the *M. smegmatis* constructs suggested an absence of the heavy glycosylation seen in the mature gp120 envelope of HIV-1 (63). These results show the successful expression of the full-length HIV-1 gp120 protein in the rapidly growing, nonpathogenic *M. smegmatis*.

**rM. smegmatis** immunization elicited functional HIV-1-specific CD8\(^+\) T cells. While BCG and *Mycobacterium vaccae* have been assessed as potential vaccine vectors, other nonpathogenic species of mycobacteria have not been evaluated for this application. We therefore tested the *rM. smegmatis* constructs for their immunogenicity in mice. Immune responses were monitored in BALB/c mice using a tetramer assay, measuring CD8\(^+\) T-cell responses to the H-2D\(^b\)-restricted P18 epitope from the V3 loop of the HXB2 envelope protein. Both the multicopy and the single-copy *rM. smegmatis*
constructs expressing gp120 elicited peripheral blood HIV-specific CD8+ T-cell responses in mice immunized intraperitoneally with either 10^6 or 10^8 CFU bacilli (P < 0.05 versus control groups at weeks 1, 2, and 3 postimmunization) (Fig. 2A and B). Higher frequency responses were seen in mice immunized with 10^8 CFU than those immunized with 10^6 CFU bacilli. Interestingly, despite significantly lower viral antigen expression by rM. smegmatis containing the highly stable single-copy plasmid than rM. smegmatis containing the multicopy plasmid (Fig. 1B), the magnitudes of the immune responses elicited by both constructs were comparable. CD8+ T-cell responses peaked at week 1, rapidly declined thereafter, and were undetectable in the peripheral blood by week 4.

Mice were inoculated with rM. smegmatis expressing gp120 twice, at an interval of 10 weeks, to determine whether the T-cell responses could be boosted. P18-specific responses increased in magnitude and were seen 1 week after immunization (P < 0.05 versus control groups). However, these peak responses were not greater than those seen following a single inoculation (Fig. 2C). Responses in the boosted mice declined thereafter but remained detectable even 1 year following the initial immunization (data not shown). These results demonstrate that rM. smegmatis is capable of eliciting MHC class I-restricted CD8+ T cells specific for the HIV-1 envelope.

The functional capacity of the rM. smegmatis-induced HIV-1 envelope-specific CD8+ T cells was then determined. Splenocytes were harvested 1 week after immunization of mice with 10^7 CFU of the single-copy rM. smegmatis construct and evaluated in IFN-γ ELISPOT and 51Cr release assays. The splenocytes secreted IFN-γ after overnight stimulation with the dominant CD8+ T-cell P18 epitope peptide or a pool of overlapping peptides spanning the entire gp120 protein (P < 0.001 versus control groups) (Fig. 3). The cytotoxic activity of the rM. smegmatis-elicited CD8+ T cells was also assessed. Splenocytes were stimulated with the P18 peptide for 1 week in the presence of IL-2 and evaluated as effector cells in a 51Cr release assay. These effector cells were able to kill efficiently
P815 target cells pulsed with the P18 peptide (P < 0.01 versus control groups at effector-to-target ratios 50:1, 12:1, and 3:1) (Fig. 4). Thus, the rM. smegmatis-elicted HIV-1-specific CD8+ T cells were capable of secreting IFN-γ and mediating cytolytic activity in response to HIV-1 envelope peptide stimulation.

rM. smegmatis immunization elicited both effector and memory HIV-1 Env-specific CD8+ T cells. To further characterize the rM. smegmatis-induced CD8+ T cells, we evaluated these Env-specific T cells for their state of maturation and functional commitment by assessing their expression of CD62L, CD127, and CD44 using surface staining with monoclonal antibodies.

FIG. 3. rM. smegmatis-elicted HIV-1-specific CD8+ T cells secreted IFN-γ. Day 7 splenocytes from mice immunized with 10^7 CFU recombinant mycobacteria expressing gp120 (integrative) were exposed to no peptide, P18, or a gp120 peptide pool and evaluated in an ELISPOT assay. Splenocytes from mice immunized with rM. smegmatis expressing Msp1 were used as a control. The mean (± SEM) spot-forming cells (SFC) per 10^6 splenocytes for each group of mice (n = 4 per group) is shown.

To further characterize the rM. smegmatis-induced CD8+ T cells, we evaluated these Env-specific T cells for their state of maturation and functional commitment by assessing their expression of CD62L, CD127, and CD44 using surface staining with monoclonal antibodies and flow cytometric analysis. Tetramer-positive CD8+ T cells were found in both the spleen and the peripheral blood 1 week after immunization with rM. smegmatis expressing gp120. In contrast, HIV-1-specific CD8+ T cells were not generated in mice immunized with the control mycobacteria construct. All the tetramer-positive CD8+ cells expressed CD44, indicating that they were activated (Fig. 5A). Moreover, the majority of these CD8+ T cells were effector cells (tetramer positive, CD44hi, CD127−, and CD62Llo), and a small proportion was either effector memory (tetramer positive, CD44hi, CD127+, and CD62Llo) or central memory cells (tetramer positive, CD44hi, CD127+, and CD62Lhi). This was seen both in the peripheral blood and spleen of the immunized mice (Fig. 5B). One year after immunization with rM. smegmatis, essentially all of the peripheral blood tetramer-positive CD8+ T cells were central memory cells (Fig. 5C). These data indicate that rM. smegmatis can generate effector, effector memory, and long-lived central memory HIV-specific CD8+ T cells.

rM. smegmatis elicited HIV-1 Env-specific CD8+ T cells in BCG immune mice. A large proportion of the human population has received BCG as a tuberculosis vaccine, and we were concerned that prior BCG exposure might substantially blunt the immunogenicity of an rM. smegmatis vaccine. We therefore evaluated whether anti-BCG immunity can affect the immunogenicity of rM. smegmatis constructs in mice. To induce anti-BCG immunity, mice were inoculated with 10^8 CFU wild-type BCG or PBS and 6 months later were inoculated with rM. smegmatis expressing gp120 (17). In fact, only a modest reduction in peak tetramer-positive CD8+ T-cell responses was observed in the BCG-preimmunized mice (Fig. 6). These results suggest that preexisting immunity to BCG may have only a marginal effect on the immunogenicity of rM. smegmatis.

FIG. 4. Cytotoxic activity of HIV-1-specific CD8+ T cells elicited by rM. smegmatis immunization. HIV-1-specific CTL were expanded in vitro by stimulating splenocytes isolated from mice at day 14 after a single inoculation with 10^6 CFU rM. smegmatis expressing gp120 (integrative) with 10 ng/ml p18 peptide in the presence of rat IL-2 for 7 days. Cytotoxic activity of the effector cells for P815 target cells pulsed with or without p18 was assessed in a 3^Cr chromium release assay. Effector-to-target (E:T) ratios used in the study are indicated.

DISCUSSION

We have thus demonstrated that a novel vaccine vector, a recombinant nonpathogenic Mycobacterium smegmatis MC2-155 (rM. smegmatis) expressing the entire HIV-1 HXBc2 gp120 envelope protein, was immunogenic in mice. The strain MC2-155 is a mutant of M. smegmatis (ATCC 607) that is transformable with pAL5000 plasmids at 7 orders of magnitude higher frequency than the parental strain (48). The efficient plasmid transformation phenotype has caused MC2-155 to be the surrogate host of choice for the analysis of genes from pathogenic mycobacteria (12, 13, 58) and has recently been sequenced by The Institute for Genomic Research (http://www.tigr.org). Moreover, this strain has been shown to be nonpathogenic following intravenous infections of SCID mice (5). We evaluated a variety of mycobacterial promoters and regulatory genes and found that the use of M. tuberculosis α antigen promoter and fusion of the transgene with the 19-kDa signal sequence optimized the immunogenicity of the vaccine construct. A number of factors probably contributed to this increased immunogenicity. This configuration clearly enhanced the expression of the HIV-1 gp120 envelope protein in mycobacteria (data not shown). Furthermore, fusion of the gp120 protein with the 19-kDa protein may have created a chimeric lipoprotein that is immunogenic, perhaps because of acylation of the signal sequence (66). Neyrolles et al. found that the acylated moiety was important for MHC class I antigen presentation of lipoproteins, perhaps because it facilitates lipoprotein interaction with Toll-like receptor 2 (TLR2) (18, 40).

It is interesting to speculate that the immunogenicity of the HIV-1 envelope 19-kDa fusion lipoprotein in the rapidly growing nonpathogenic rM. smegmatis mycobacteria may be associated with the inability of this recombinant vector to persist. Although lipoproteins are certainly immunogenic, persistent exposure to lipoproteins can lead to suppression of antigen.
presentation by macrophages (41, 53). Both \textit{M. bovis} BCG and \textit{M. tuberculosis} can persist in host cells and inhibit phagosome maturation (55, 56). Persisting \textit{M. tuberculosis} and BCG may exert immunosuppressive effects through ligation of lipoproteins to TLRs localized to the phagosome (1, 42, 43, 53, 54). On the other hand, \textit{M. smegmatis} does not inhibit phagosome maturation and is degraded rapidly by phagolysosomal proteases. An explanation for the robust immunogenicity of the \textit{rM. smegmatis} constructs will likely come from parallel studies of the immunogenicity of the HIV envelope chimeric lipoprotein in \textit{rBCG} and \textit{rM. smegmatis} as well as an evaluation of the roles of persistence and lipoprotein-TLR interactions in the generation of CD8$^+$ T-cell responses elicited by mycobacteria.

\textit{rM. smegmatis}-elicited HIV-1-specific CD8$^+$ T cells exhibited effector functions such as cytosis and production of IFN-$\gamma$. The ability of recombinant, nonpathogenic rapidly growing mycobacteria to elicit antigen-specific CTL responses has never been reported previously. However, \textit{M. tuberculosis} or recombinant \textit{M. bovis} BCG have been shown to elicit antigen-specific CTL (2, 24). The ability of HIV-1 vaccine vectors to elicit strong CTL responses is likely to be critical for vaccine-induced immune containment of HIV-1 replication and prevention of AIDS (31).

Recombinant \textit{M. smegmatis} was previously assessed as a vaccine in a mouse tumor model (10). Cheadle et al. showed that \textit{M. smegmatis} was better than BCG at promoting DC maturation. However, \textit{rM. smegmatis} expressing a CTL epitope of the ovalbumin (OVA) antigen did not protect against challenge with a tumor expressing this epitope, whereas BCG expressing the same epitope protected mice against the OVA epitope-expressing tumor (10). This absence of antitumor activity elicited by recombinant \textit{M. smegmatis} was asso-

![FIG. 5. Phenotype of HIV-1-specific CD8 T cells elicited by immunization with \textit{rM. smegmatis} expressing gp120 (integrative). (A) Flow cytometric analysis of week 1 PBMC and splenocytes from immunized mice revealed expression of CD44 on the surface of all \textit{rM. smegmatis}-elicited tetramer-positive cells. CD62L and CD127 were expressed on a subset of the tetramer-positive cells. (B) The proportions of effector (P18-tetramer positive, CD127$^+$, and CD62L$^+$), effector memory (P18-tetramer positive, CD127$^-$, and CD62L$^+$), and central memory (P18-tetramer positive, CD127$^-$, and CD62L$^-$) cells in the blood and spleen of mice immunized with \textit{rM. smegmatis} are shown. Effector, effector memory, and central memory cells are denoted E, EM, and CM, respectively. The mean (± SEM) percent E, EM, or CM for each group of mice (n = 4 per group) is shown. (C) Peripheral blood HIV-1-specific CD8$^+$ T cells from mice 1 year after immunization with \textit{rM. smegmatis} expressing gp120 were predominantly central memory cells. PBMC were pooled from 4 mice that were inoculated twice (10 weeks apart) with 10$^8$ CFU bacilli.](http://jvi.asm.org/ on August 29, 2017 by guest)
elicited by plasmid DNA (46). However, the OVA-specific T-cell responses were not measured after immunization with the recombinant mycobacteria in this study, the inability of recombinant M. smegmatis to confer protection against a tumor challenge could not be attributed to inefficient induction of tumor antigen-specific CTL responses in vivo. In contrast to the findings of Cheadle et al., recombinant M. smegmatis was shown to access the MHC class I pathway better than BCG for presentation of peptide antigens (40). Furthermore, a recombinant M. smegmatis expressing TNF-α was shown to have anti-tumor properties in mice (67). The conflicting findings in these studies may be explained by the nature of the antigen expressed by mycobacteria. Neyrolles et al. expressed the influenza NP CTL epitope fused to the 19-kDa lipoprotein in mycobacteria (40). In contrast, Cheadle et al. expressed a secreted OVA CTL epitope in mycobacteria (10). The kinetics of the rM. smegmatis-elicited T-cell responses differed from those of T-cell responses generated using other vaccine modalities. rM. smegmatis-elicited HIV-specific CD8+ T-cell responses were maximal 1 week after immunization. This peak T-cell response is earlier than responses elicited by plasmid DNA, adenoviral vectors, and vaccinia vectors, which generally are maximal 10 to 14 days postimmunization (6, 46). Interestingly, an early peak immune response has also been described in mice immunized with recombinant Listeria monocytogenes (23). rM. smegmatis also induced peak T-cell responses that were of lower magnitude than those induced by recombinant viral vectors but similar in magnitude to those elicited by plasmid DNA (46).

The maturation and differentiation status of the rM. smegmatis-elicited CD8+ T cells was defined using MAb specific for CD44, CD62L, and CD127 (22). In both the peripheral blood and spleen of rM. smegmatis-immunized mice, the majority of the HIV-1-specific CTL generated were effector cells (CD44hi, CD127−, CD62Llo), and very few effector memory (CD44hi, CD127+, CD62Llo) and central memory (CD44lo, CD127+, CD62Lhi) CTL were seen at the time of peak immune responses. Interestingly, in mice receiving two immunizations with rM. smegmatis, we also found a small but stable population of HIV-1-specific central memory CD8+ T cells. These data therefore suggest that rM. smegmatis is capable of generating both HIV-1-specific effector and memory cells in vivo. The ability of the rM. smegmatis vector to generate central memory cells is particularly important, since these cells have been shown to expand in vivo and mediate protective immunity following a challenge with a pathogenic organism (62).

There is growing evidence that vector or pathogen persistence may have an adverse effect on the generation of T-cell memory. Persistent lymphocytic choriomeningitis virus and lentiviral infections result in the generation of T cells that have lost the ability to perform some important effector functions (4, 16, 35, 61, 68). Persistent mycobacterial infections by slow-growing M. tuberculosis and BCG may also adversely affect T-cell memory responses. On the other hand, vectors that do not persist can generate good T-cell memory (60). Therefore, we speculate that the rapidly growing M. smegmatis vector may be better at eliciting memory T-cell responses than persistent mycobacterial vectors because M. smegmatis is eliminated rapidly in the host.

Interestingly, we found that the multicopy and the single-copy vectors elicited comparable tetramer responses despite the fact that the multicopy rM. smegmatis construct expressed significantly more HIV-1 envelope protein. High levels of gp120 expression have been shown to be toxic to mycobacteria (51). Consistent with this finding, we observed that the in vivo growth of multicopy rM. smegmatis was slower than that of the single-copy vector (data not shown). Moreover, studies have shown that recombinant mycobacteria containing an integrated HIV transgene stably express that transgene and are highly immunogenic (36, 51). Thus, recombinant mycobacteria with integrated transgenes appear to be useful vaccine vectors.

A major limitation of the clinical utility of a number of vaccine vectors currently in development is the inhibition of vector immunogenicity by preexisting anti-vector immunity. For example, immunity to the HIV-1 vaccine vector adenovirus serotype 5 (rAd5) has been shown to blunt the immunogenicity of rAd5 vaccines (7, 37). Since BCG is administered to a large proportion of the human population as a TB vaccine, ant mycobacterial immunity might diminish the immunogenicity of recombinant mycobacterial vectors such as rM. smegmatis. However, our data indicate that BCG immunity affects the immunogenicity of rM. smegmatis only modestly. Consistent with this observation, it was previously reported that mice immunized with wild-type BCG still developed T-cell and antibody responses to the HIV-1 Nef and β-galactosidase transgenes expressed in recombinant BCG (17). Preexisting immunologic memory responses to BCG could result in the rapid destruction of recombinant M. smegmatis, which might favor cross-priming of the heterologous HIV-1 gp120 antigen (25). Hence, recombinant mycobacterial vaccines may be useful in BCG-immunized individuals.

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