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Generation of CD8$^+$ T-Cell Responses by a Recombinant Nonpathogenic *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 *Mycobacterium smegmatis* was engineered as a vector expressing full-length human immunodeficiency virus type 1 (HIV-1) HXBc2 envelope protein. Immunization of mice with recombinant *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 *Mycobacterium bovis* BCG had only a marginal effect on the immunogenicity of recombinant *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 *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 *Borrelia burgdorferi*, *Streptococcus pneumoniae*, *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).

Mycobacterium smegmatis has a number of properties that may make it an effective vaccine vector. Some *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, *M. smegmatis* is rapidly destroyed by phagolysosomal proteases in the phagosomes of infected cells (26, 32, 55, 56). Nevertheless, *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). *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 *M. smegmatis* to elicit HIV-1 envelope-specific CD8$^+$ T-cell responses.

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MATERIALS AND METHODS

Generation of recombinant mycobacteria. *Mycobacterium smegmatis* MC^{2}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 IIIB gp120 envelope gene (HXBc2) was cloned into the multicopy pJH222 and single-copy integrative pJH223 *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. tuberculosis* 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 HXBc2 envelope gene insert were transformed into the *M. smegmatis* MC^2155 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 (OD_{600}) 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 Suite (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 were grown in 7H9 medium until an OD_{600} approximately equal to 1. We estimated that bacterial growth to an OD value of 1 is equal to 5×10^8 CFU. For recombinant nonpathogenic *Mycobacterium smegmatis* MC^2155 (r*M. smegmatis*) immunizations, approximately 10^6 or 10^8 CFU bacilli were injected via the intraperitoneal route in 200 μl of sterile PBS, 0.02% Tween. Approximately 10^6 CFU bacilli were injected subcutaneously for BCG immunization.

Tetramer staining and flow cytometric analysis. H-$2D^d$ tetrameric complexes folded with the P18 peptide (RGPGRAFVTI) (52), a sequence found in the V3 loop of HIV-1 HXBc2 envelope protein, were prepared as described previously (49). Mice were anesthetized with Isoflurane and bled retro-orbitally. Blood was collected in RPMI 1640 containing 40 U of heparin (American Pharmaceutical Partners) per ml. Peripheral blood mononuclear cells (PBMCs) were isolated using lympholyte-M (Cedarlane) and stained with the P18 tetramer conjugated with phycoerythrin (PE) and anti-CD8α MAb (Ly-2; Caltag) conjugated with allophycocyanin (APC) to detect P18-specific $CD8^+$ T cells. The cells were washed in PBS containing 2% fetal bovine serum (FBS) and fixed with PBS containing 2% formaldehyde (Polysciences). $CD8^+$ T cells were analyzed for tetramer staining using two-color flow cytometry on a FACS Array (BD Pharmingen). For phenotyping the P18-specific $CD8^+$ T cells, splenocytes and PBMC were sampled 1 week after immunization of mice with recombinant mycobacteria and stained with anti-CD8α MAb (53-6.7; BD Pharmingen) conjugated with peridinin chlorophyll protein-Cy5.5, anti-CD62L MAb (MEL-14; BD Pharmingen) conjugated with APC, anti-CD44 MAb (IM-7; eBiosciences) conjugated with APC-Cy7, anti-CD127 MAb (A7R34; eBiosciences) conjugated with PE-Cy7, and the P18 tetramer conjugated with PE. Multicolor flow analysis was performed using the BD LSRII Cytometer (BD Biosciences) and the FlowJo software (Tree Star).

^{51}Chromium release assay. Splenocytes were harvested from mice 1 week after immunization with 10^7 CFU recombinant mycobacteria. The cells were resuspended in RPMI 1640 containing 10% FBS and cultured in a 24-well plate (8×10^6/well) with 10 ng of P18 epitope peptide per ml. Interleukin-2 (IL-2) (Sigma) was added to cultures on day 2 to a final concentration of 10 U/ml. On day 7, cells were harvested, washed once, and used as effectors in a ^{51}Cr release assay with P815 target cells (American Type Culture Collection). P815 cells were cultured overnight in the presence of medium alone or with 100 ng of P18 peptide per ml. Cells (2×10^6) were labeled with 150 μCi of ^{51}Cr for 1 h at 37°C, washed twice, and added to a 96-well round-bottom plate at 10^4/well in 100 μl of 10% RPMI medium. Titrations of effector cells were added to triplicate wells in 100 μl of medium. Lytic activity was assessed in a 4-h ^{51}Cr release assay as previously described (46). Percent specific lysis was calculated as follows: 100 × (experimental − spontaneous release)/(maximum − spontaneous release).

IFN-γ ELISPOT assay. An enzyme-linked immunospot (ELISPOT) assay was performed to measure gamma interferon (IFN-γ) production as previously described (46). Briefly, 96-well multiscreen HA plates (Millipore) were coated by overnight incubation (100 μl/well) at 4°C with rat anti-mouse IFN-γ MAb (clone R4-6A2; BD Pharmingen) at 10 μg/ml in PBS. Splenocytes were harvested from individual mice 1 week after immunization with 10^7 CFU recombinant mycobacteria. Effector cells were plated in triplicate at 2×10^5/well in a 100-μl final volume with medium alone, 4 μg of p18 epitope peptide per ml, or 4 μg of Env peptide pool per ml. The pool consisted of 47 overlapping 15-mer peptides spanning the HIV-1 IIIB gp120 protein (Centralized Facility for AIDS Reagents, Potters Bar, United Kingdom) and was used such that each peptide was present at a concentration of 4 μg/ml. After a 24-h incubation at 37°C, the plates were washed free of cells with PBS–0.05% Tween 20 and incubated overnight at 4°C with 100 μl of biotinylated rat anti-mouse IFN-γ MAb (clone XMG1.2; BD Pharmingen) per well at 5 μg/ml. Plates were washed four times, and 75 μl of streptavidin-alkaline phosphatase (Southern Biotechnology Associates) was added at a 1/500 dilution. After a 2-h incubation, plates were washed four times and developed with Nitro Blue Tetrazolium-5-bromo-4-chloro-3-indolylphosphate chromogen (Pierce). Plates were analyzed with an ELISPOT reader (Hitech Instruments).

Statistical analysis. Data were expressed as means ± standard errors of the means (SEM). Statistical tests were performed using Student's *t* test. A *P* value of less than 0.05 was considered significant.

RESULTS

Generation of recombinant *Mycobacterium smegmatis* expressing HIV-1 gp120 envelope protein. The pJH222 and pJH223 *E. coli*/mycobacteria shuttle plasmids were used to express a HIV-1 HXBc2 *env* 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). pJH222 is a multicopy plasmid that replicates episomally in mycobacteria (47); pJH223 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 HXBc2 *env*. The HIV-1 protein was fused to an HA tag at the C terminus. The N terminus of the HIV-1 envelope 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* MC^2155 (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 r*M. smegmatis* constructs, which were transformed with either pJH222 or pJH223 containing the malaria *msp1* gene, did not express the viral antigen. The predicted molecular weight of the envelope protein expressed by the r*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*.

r*M. smegmatis* immunization elicited functional HIV-1-specific $CD8^+$ T cells. While BCG and *Mycobacterium vaccae* (19) have been assessed as potential vaccine vectors, other nonpathogenic species of mycobacteria have not been evaluated for this application. We therefore tested the r*M. 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-$2D^d$-restricted P18 epitope from the V3 loop of the HXBc2 envelope protein. Both the multicopy and the single-copy r*M. smegmatis*

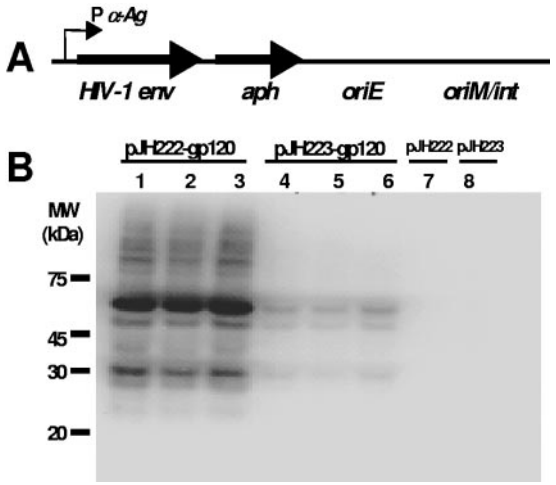

FIG. 1. Expression of HIV-1 HXBc2 gp120 envelope in nonpathogenic *Mycobacterium smegmatis.* (A) Codon-optimized HXBc2 gp120 *env* was cloned into the *E. coli*/mycobacteria shuttle plasmids pJH222 (multicopy) and pJH223 (integrative). The gp120 *env* in the plasmids is under the control of the *M. tuberculosis* α-*Ag* promoter (P α-*Ag*). A fusion protein was created in which the *M. tuberculosis* 19-kDa signal sequence was at the N terminus and an influenza hemagglutinin (HA) epitope tag was at the C terminus of the gp120 Env. Both plasmids contained the Tn*903*-derived *aph* gene conferring kanamycin resistance as a selectable marker and an *E. coli* origin of replication (*oriE*). The origin of replication (*oriM*) was inserted into the pJH222 plasmid, while the *attP* site and the *int* gene of mycobacteriophage L5 were included in pJH223. (B) Western blot analysis showed expression of the gp120 protein in recombinant *M. smegmatis* MC^2155. The gp120 expression of three independent clones of mycobacteria transformed with either pJH222-*gp120* (lanes 1 to 3) or pJH223-*gp120* (lanes 4 to 6) was determined using an anti-HA MAb (clone 3F10). Mycobacteria transformed with either mock pJH222 or pJH223 containing an irrelevant gene (malaria *msp1*) were utilized as negative controls (lanes 7 and 8). MW, molecular mass.

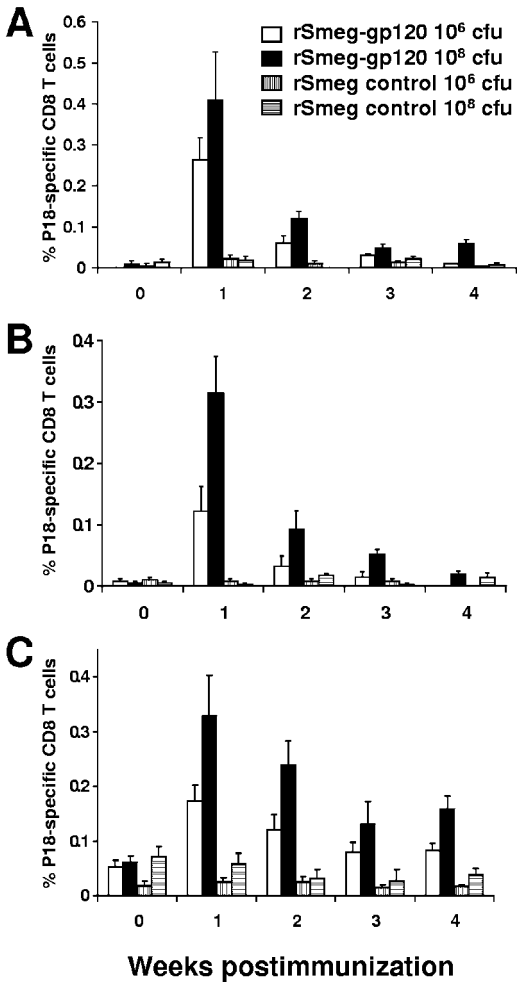

FIG. 2. Recombinant *M. smegmatis* elicited HIV-1-specific $CD8^+$ T-cell responses in mice. BALB/c mice were inoculated via the intraperitoneal route with approximately 10^6 CFU or 10^8 CFU gp120-expressing recombinant *M. smegmatis* (rSmeg-gp120) organisms transformed with either the integrative pJH223-*gp120* plasmid (A) or multicopy pJH222-*gp120* (B). As a negative control, mice were inoculated with the same dose of mycobacteria transformed with the control pJH222- and pJH223-*msp1* plasmids (rSmeg control). (C) Mice were inoculated twice (10 weeks apart) with the same dose of either the rSmeg-gp120 (integrative) construct or the rSmeg control. The mean (± SEM) percent HIV-1 HXBc2 gp120 P18 tetramer-positive CD8 T cells from PBMC collected at the indicated time points is shown for each group of mice ($n = 4$ per group).

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 r*M. smegmatis* containing the highly stable single-copy plasmid than r*M. 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 r*M. 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 r*M. smegmatis* is capable of eliciting MHC class I-restricted $CD8^+$ T cells specific for the HIV-1 envelope.

The functional capacity of the r*M. 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 r*M. smegmatis* construct and evaluated in IFN-γ ELISPOT and ^{51}Cr 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 r*M. 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 ^{51}Cr release assay. These effector cells were able to kill efficiently

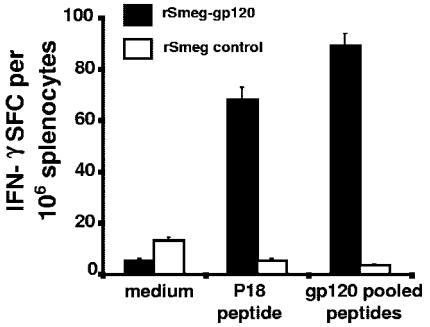

FIG. 3. r*M. smegmatis*-elicited HIV-1-specific $CD8^+$ T cells secreted IFN-γ. Day 7 splenocytes from mice immunized with 10^7 CFU recombinant mycobacteria expressing gp120 (integrative) were exposed to no peptide, P18, or a gp120 peptide pool and evaluated in an ELISPOT assay. Splenocytes from mice immunized with r*M. 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.

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 r*M. smegmatis*-elicited HIV-1-specific $CD8^+$ T cells were capable of secreting IFN-γ and mediating cytolytic activity in response to HIV-1 envelope peptide stimulation.

r*M. smegmatis* immunization elicited both effector and memory HIV-1 Env-specific $CD8^+$ T cells. To further characterize the r*M. 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 r*M. 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, $CD44^{hi}$, $CD127^-$, and $CD62L^{lo}$), and a small proportion was either effector memory (tetramer positive, $CD44^{hi}$, $CD127^+$, and $CD62L^{lo}$) or central memory cells (tetramer positive, $CD44^{hi}$, $CD127^+$, and $CD62L^{lo}$). This was seen both in the peripheral blood and spleen of the immunized mice (Fig. 5B). One year after immunization with r*M. smegmatis*, essentially all of the peripheral blood tetramer-positive $CD8^+$ T cells were central memory cells (Fig. 5C). These data indicate that r*M. smegmatis* can generate effector, effector memory, and long-lived central memory HIV-specific $CD8^+$ T cells.

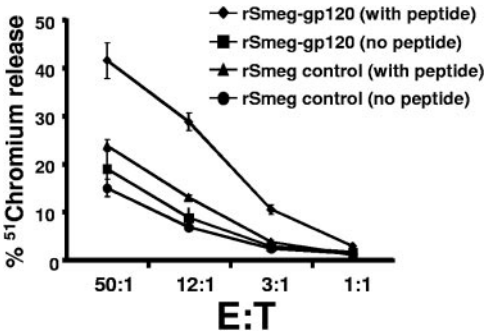

FIG. 4. Cytotoxic activity of HIV-1-specific $CD8^+$ T cells elicited by r*M. smegmatis* immunization. HIV-1-specific CTL were expanded in vitro by stimulating splenocytes isolated from mice at day 14 after a single inoculation with 10^8 CFU r*M. smegmatis* expressing gp120 (integrative) with 10 ng/ml 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 ^{51}chromium release assay. Effector-to-target (E:T) ratios used in the study are indicated.

r*M. 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 r*M. smegmatis* vaccine. We therefore evaluated whether anti-BCG immunity can affect the immunogenicity of r*M. smegmatis* constructs in mice. To induce anti-BCG immunity, mice were inoculated with 10^6 CFU wild-type BCG or PBS and 6 months later were inoculated with r*M. 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 r*M. smegmatis*.

DISCUSSION

We have thus demonstrated that a novel vaccine vector, a recombinant nonpathogenic *Mycobacterium smegmatis* MC^2155 (r*M. smegmatis*) expressing the entire HIV-1 HXBc2 gp120 envelope protein, was immunogenic in mice. The strain MC^2155 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 MC^2155 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 r*M. 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

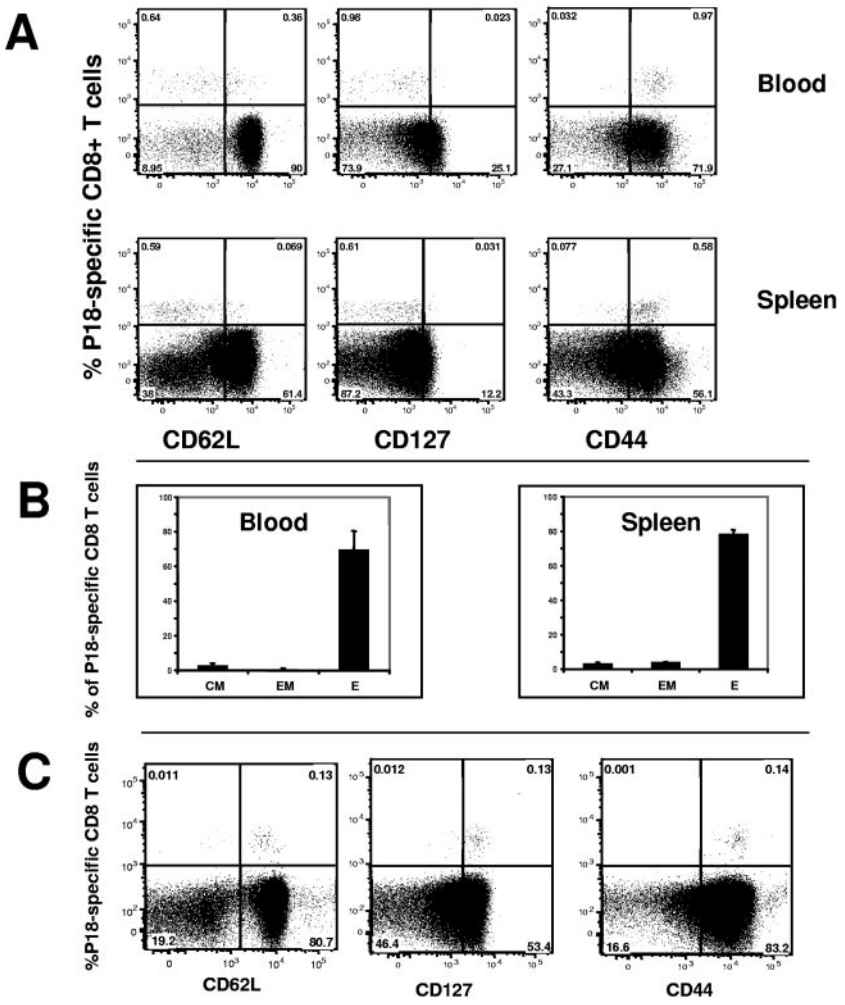

FIG. 5. Phenotype of HIV-1-specific CD8 T cells elicited by immunization with r*M. smegmatis*. Mice were immunized with 10^8 CFU r*M. smegmatis* expressing gp120 (integrative). (A) Flow cytometric analysis of week 1 PBMC and splenocytes from immunized mice revealed expression of CD44 on the surface of all r*M. 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 $CD62L^{lo}$), effector memory (P18-tetramer positive, $CD127^+$, and $CD62L^{lo}$), and central memory (P18-tetramer positive, $CD127^+$, and $CD62L^{hi}$) cells in the blood and spleen of mice immunized with r*M. 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 r*M. 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.

presentation by macrophages (41, 53). Both *M. bovis* BCG and *M. tuberculosis* can persist in host cells and inhibit phagosome maturation (55, 56). Persisting *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, *M. smegmatis* does not inhibit phagosome maturation and is degraded rapidly by phagolysosomal proteases. An explanation for the robust immunogenicity of the r*M. smegmatis* constructs will likely come from parallel studies of the immunogenicity of the HIV envelope chimeric lipoprotein in rBCG and r*M. 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.

r*M. smegmatis*-elicited HIV-1-specific $CD8^+$ T cells exhibited effector functions such as cytolysis and production of IFN-γ. The ability of recombinant, nonpathogenic rapidly growing mycobacteria to elicit antigen-specific CTL responses has never been reported previously. However, *M. tuberculosis* or recombinant *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 *M. smegmatis* was previously assessed as a vaccine in a mouse tumor model (10). Cheadle et al. showed that *M. smegmatis* was better than BCG at promoting DC maturation. However, recombinant *M. 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 *M. smegmatis* was asso-

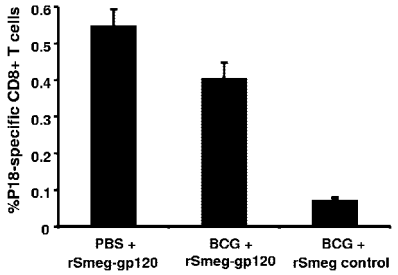

FIG. 6. Recombinant *M. smegmatis*-elicited HIV-1-specific $CD8^+$ T-cell responses in mice preimmunized with BCG. Mice were immunized with wild-type BCG (Pasteur) or PBS and 6 months later with 10^8 CFU r*M. smegmatis* expressing gp120 (integrative) (indicated as BCG + rSmeg-gp120 and PBS + rSmeg-gp120, respectively). BCG-preimmunized mice that were subsequently inoculated with rSmeg control were used as a negative control (indicated as BCG + rSmeg control). Tetramer analysis was performed on the PBMC of mice 1 week after inoculation with the r*M. smegmatis* constructs. The mean (± SEM) percent of HIV-1 HXBc2 gp120 P18-tetramer-positive $CD8^+$ T cells is shown for each group (n = 4 to 5 mice per group).

ciated with poor presentation of peptides by the nonpathogenic mycobacteria to an OVA-specific T-cell line in vitro (10). However, since 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 r*M. smegmatis*-elicited T-cell responses differed from those of T-cell responses generated using other vaccine modalities. r*M. 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). r*M. 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 r*M. smegmatis*-elicited $CD8^+$ T cells was defined using MAbs specific for CD44, CD62L, and CD127 (22). In both the peripheral blood and spleen of r*M. smegmatis*-immunized mice, the majority of the HIV-1-specific CTL generated were effector cells ($CD44^{hi}$, $CD127^-$, $CD62L^{lo}$), and very few effector memory ($CD44^{hi}$, $CD127^+$, $CD62L^{lo}$) and central memory ($CD44^{hi}$, $CD127^+$, $CD62L^{hi}$) CTL were seen at the time of peak immune responses. Interestingly, in mice receiving two immunizations with r*M. smegmatis*, we also found a small but stable population of HIV-1-specific central memory $CD8^+$ T cells. These data therefore suggest that r*M. smegmatis* is capable of generating both HIV-1-specific effector and memory cells in vivo. The ability of the r*M. 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 r*M. 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 vitro growth of multicopy r*M. 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, anti-mycobacterial immunity might diminish the immunogenicity of recombinant mycobacterial vectors such as r*M. smegmatis*. However, our data indicate that BCG immunity affects the immunogenicity of r*M. 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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