Vaccine Platform for Prevention of Tuberculosis and Mother-to-Child Transmission of HIV-1 through Breastfeeding

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Abstract

Most children in Africa receive their tuberculosis vaccine at birth. Those infants born to HIV-1-positive mothers are at high risk of acquiring HIV-1 infection through breastfeeding in the first weeks of their life. Thus, development of a vaccine, which would protect newborns against both of these major global killers is a logical, yet highly scientifically, ethically and practically challenging aim. Here, a recombinant lysine auxotroph of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), a safer BCG strain, expressing an African HIV-1 clade-derived immunogen was generated and shown to be stable and induce durable high-quality HIV-1-specific CD4+ and CD8+ T cell responses. Furthermore, when used in a heterologous prime-boost regimen, protection against surrogate virus challenge through the HIV-1-specific responses were achieved and BCG.HIVA alone protected against aerosol challenge with *M. tuberculosis*. Thus, inserting an HIV-1-derived immunogen into the scheduled BCG vaccine delivered at or soon after birth may prime HIV-1-specific responses, which can be boosted through natural exposure to HIV-1 in the breastmilk and/or by a heterologous vaccine such as recombinant MVA delivering the same immunogen, and decrease mother-to-child transmission of HIV-1 during breastfeeding.
Since the first report of AIDS in 1981, an estimated over 60 million people have become infected with HIV-1, of whom some 25 million have died. Over 60% of the global HIV-1-infected population lives in Africa and about a half of the infected adults are women of child-bearing age. Up to 40% of pregnant women attending larger urban centre clinics in Kenya are HIV-1 seropositive. Despite the fact that approximately half of mother-to-child transmission (MTCT) is due to prolonged breast-feeding (43), for many HIV-1-positive mothers bottle-feeding is not an option for social, practical and health reasons. Bottle-fed babies of infected mothers have a higher morbidity and mortality due to increased exposure and susceptibility to other infections (32). Although antiretroviral therapy can significantly reduce the risk of MTCT at parturition, it is less clear whether it is practical to use these drugs to prevent HIV-1 transmission by breast milk. The drugs are very expensive, would have to be administered at birth and be maintained throughout the whole period of breastfeeding; in addition their effectiveness might be compromised by emergence of resistant mutants. Thus, the best hope for protecting newborns in developing countries against MTCT of HIV-1 is the development of safe, effective, accessible prophylactic vaccines, which would both reduce the adult burden of infection and protect neonates against vertical HIV-1 transmission.

Development of an HIV-1 vaccine remains on a horizon. Although in principle it is possible, the induction of broadly neutralizing antibodies across HIV-1 clades has been extremely difficult to achieve by active immunization (6). While various technologies are becoming more successful at inducing HIV-1-specific T cell responses (33), a single correlate of protection against HIV-1 infection/AIDS remains elusive and possibly non-existent as protection is most likely multi-factorial. Therefore, before further progress in this area is made, the interim aim of HIV-1 vaccination is to elicit T
cell responses that are high in frequencies, multi-specific, multi-functional, capable of rapid proliferation, long-lived and of the central memory phenotype. The in vivo functionality of vaccine-induced T cells can be assessed using surrogate models of viral challenge. The ultimate goal, vaccine efficacy, can be only confirmed in adequately powered clinical trials in humans.

For a number of years, we have been developing an HIV-1 vaccine focusing on induction of protective cell-mediated responses. Our starting platform was based on a heterologous DNA prime and modified vaccinia virus Ankara (MVA) boost regimen(44) delivering a common immunogen called HIVA, which is derived from consensus HIV-1 clade A Gag protein, i.e. an immunogen derived from an HIV-1 strain prevalent in Central and Eastern Africa, and a string of CD8\(^+\) T cell epitopes(17). Extensive studies in mice, non-human primates and over four hundred healthy and HIV-1-infected humans showed that the vaccines were safe and immunogenic(18). In humans, DNA vaccine pTHr.HIVA primed weakly, but consistently HIV-1-specific mostly CD4\(^+\) T cell responses, and MVA.HIVA delivered a strong and consistent boost expanding both CD4\(^+\) and CD8\(^+\) HIV-1-specific T cells, particularly if they are well prime, e.g. by HIV-1 infection (12, 15, 37).

Infection with *Mycobacterium tuberculosis* kills about 2 million people each year(42). *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) is the only licensed vaccine and protects significantly against childhood and miliary tuberculosis(52). Globally, 80\% percent of children are vaccinated with BCG(50), the majority of them at birth. Thus, the development of a combined vaccine, which would protect neonates against tuberculosis and MTCT of HIV-1 through breastfeeding, is a logical effort in the fight against these two major global killers. One such obvious vaccine or vaccine component is a recombinant BCG (rBCG) expressing an HIV-1-derived immunogen.
BCG as a vaccine vector has a number of attractive features(10). BCG has a proven record of safety as a vaccine against tuberculosis from its use in over two billion individuals including neonates(26). BCG infects and colonizes macrophages and dendritic cells where it can survive and replicate for a long period of time. Through its persistence and potent adjuvantation by its cell wall components, it can induce long-lasting humoral and cellular immune responses. Immunity against tuberculosis following neonatal BCG vaccination lasts 10 to 15 years(20) and therefore fails to protect adults from pulmonary disease(9). Thus, it should easily cover the danger period of breastfeeding and a later vaccine boost might offer the children further protection in adolescence. BCG can be given at or any time after birth, and is not affected by maternal antibodies. Manufacturing of BCG-based vaccines is cheap as live bacteria are easy to purify. Finally, BCG is one of the most heat-stable vaccines in current use(14).

It is not known how early in life T cells can be educated to launch a protective response against intracellular microorganisms and this most likely differs from pathogen to pathogen. Qualitative and quantitative differences between responses in human newborns and adults were observed for a number of infections (1, 16, 31, 45). In HIV-1-infected infants, lower CD8 T cell responses compared to adults may play an important role in fast disease progression (7, 27, 41); children account for 4% of HIV-1 infections, yet they represent 20% of AIDS deaths (16). At the same time, mature responses to certain infections and vaccines were demonstrated during the postnatal and even foetal lives(28). This is particularly true for BCG vaccine-induced responses, which promote adult-like Th1 response in newborns (21, 29, 38, 49). Therefore, there is some evidence suggesting that protective T cell-mediated responses could be elicited by
vaccines in early life and BCG as a vaccine vector might be very well suited to prime them (30).

In this study, we have constructed a recombinant lysine auxotroph of BCG expressing the HIVA immunogen from both replicative and integrative vectors. After confirming the HIVA gene and plasmid stability and protein expression, the BCG:HIVA (episomal plasmid) alone and in a heterologous prime-boost combination was studied in a murine model for induction of HIV-1- and *M. tuberculosis*-specific immune responses. Protection was achieved against both surrogate virus expressing HIVA and aerosol *M. tuberculosis* challenge.

**MATERIALS AND METHODS**

**Construction of E. coli/mycobacterium shuttle vectors expressing HIVA.**
Parental plasmids pJH222 and pJH223 were kindly provided by Drs. W. R. Jacobs, Jr., B. R. Bloom and T Hsu. The coding sequence the HIVA gene was fused to *M. tuberculosis* 19-kD lipoprotein signal sequence in a PCR reaction and the chimeric gene was cloned into the pJH222 and pJH223 plasmids as a HindIII-HindIII fragment under the control of the *Mycobacterium ssp α*-antigen promoter using standard recombinant DNA techniques.

**Mycobacterial culture.** Lysine auxotroph of BCG, kindly provided by Drs. W. R. Jacobs, Jr., B. R. Bloom and T Hsu, was transformed by electroporation. Mycobacterial cultures were grown in Middlebrook 7H9 broth medium or on Middlebrook agar 7H10 medium supplemented with albumin-dextrose complex (ADC, Difco) and containing 0.05% Tween 80 and 25 μg/ml kanamycin. The L-lysine monohydrochloride (Sigma)
was dissolved in distilled water and used at a concentration of 40 µg/ml. For transformation, BCG cultures were grown to an O.D. of 0.9 at 600 nm, and transformed using a Biorad Gene Pulser electroporator at 2.5 kV, 25 mf, and 1000 Ω and plated on ADC-supplemented Middlebrook agar 7H10 medium containing 0.05% Tween 80 and 25 µg/ml kanamycin.

**SDS-PAGE and Western blot analysis.** Cell lysates mid-logarithmic phase of BCG transformants were prepared, separated on SDS-PAGE and electroblotted. HIVA protein was detected using anti-Pk antibodies using enhanced chemiluminiscence (Amersham International).

**Mice.** For all animal experiments, groups of five 5- to 8-week-old female BALB/c mice were used. All animal procedures and care were approved by local Ethical Committees and strictly conformed to the Spanish and U.K. Home Office Guidelines.

**Immunizations and isolation of splenocytes.** Under general anaesthesia, mice were immunized i.m. with 100 µg of endotoxin-free pTHr.HIVA DNA (Cobra Therapeutics, UK) or 10⁶ pfu of MVA.HIVA, or i.p. with 10⁸ cfu BCG.p or BCG.HIVA. For dose titration, mice received 10³-10⁷ cfu of BCG.HIVA i.p.. On the day of sacrifice, spleens were removed and pressed individually through a cell strainer (Falcon) using a 5-ml syringe rubber plunger. Following the removal of red blood cells (RBC) with RBC lysing buffer (Sigma), splenocytes were washed and resuspended in Lymphocyte medium [R10 (RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin), 20 mM HEPES and 15 mM 2-mercaptoethanol] at concentration of 2 x10⁷ cells/ml.

**In vivo stability of plasmid pJH222 BCG.HIVA.** The growth of rBCG and the stability of the extrachromosomal plasmid pJH222.HIVA were established by the recovery of BCG.HIVA colonies from spleens of mice 15 weeks after immunization.
Spleens were homogenized and plated on Middlebrook 7H10 medium supplemented with albumin-dextrose complex (ADC, Difco) and containing 0.05% Tween 80 and 25 μg/ml kanamycin. Using specific primers, the HIVA gene was detected by PCR from well-separated mycobacterial colonies.

**Peptides.** For assessing the immunogenicity of HIVA in the BALB/c mice, the following peptides were used: H-2D<sup>d</sup>-restricted epitope P18-I10 RGPGRAFVTI (48) here designated H, and subdominant H-2K<sup>d</sup>-restricted P epitope IFQSSMTKI (22). Three epitope peptides derived from p24 were pooled to investigate the MHC class II-restricted response: MHQALSPRTLNAQVKVIEEK, NPPIPVDILYKRWIILGLNK and FRDYVDRFFKTLRAEQATQE.

**In vivo killing assay.** Naïve syngeneic mouse was sacrificed, the splenocytes were prepared as above and the isolated splenocytes were incubated without or with 2 μg/ml peptides in R10 at 37 °C, 5% CO<sub>2</sub> for 90 minutes and washed 3 times. Peptide-unpulsed target cells were labeled with CMTMR (Molecular Probes, USA) only, while peptide-pulsed target cells were labeled with CFSE and either without (P peptide) or combined with CMTMR (H peptide). Briefly, H or P peptide-pulsed splenocytes resuspended in PBS at 2 x 10<sup>7</sup> cells/ml were incubated with 80 nM CFSE (Molecular Probes, USA) in dark at room temperature for 10 minutes, followed by quenching of the reaction with equal volume of FCS and three washing steps. H peptide-pulsed cells were then resuspended in R10 at 2 x 10<sup>7</sup> cells/ml, incubated with 10 μM CMTMR at 37 °C for 15 minutes and further 15 minutes in fresh R10 only. Three differentially labeled cell cultures were washed, resuspended in PBS and combined for intravenous adoptive transfer, with each animal receiving approximately 2 x 10<sup>6</sup> cells of each population. After 12 hours, animals were sacrificed, and their splenocytes were isolated and analyzed using flow cytometry. Cytotoxicity was calculated using the following
formula: Adjusted % survival = 100 x (% survival of peptide-pulsed cells / mean % survival of peptide unpulsed cells), followed by the calculation of % specific lysis = 100 - adjusted % survival(19).

**Intracellular cytokine staining.** Two million splenocytes were added to each well of a 96-well round-bottomed plate (Falcon) and pulsed with 2 (CD8 epitopes) to 5 μg/ml (CD4 epitopes) peptides or 5 μg/ml PPD tuberculin (Statens Serum Institut, Copenhagen, Denmark) together with antibodies against lysosomal-associated membrane proteins anti-CD107a-FITC/anti-CD107b-FITC (BD Biosciences) (2) and kept at 37 °C, 5% CO₂ for 90 minutes, followed by the addition of GolgiStop™ (BD Biosciences) containing monensin. After a further 5-hour incubation, reaction was terminated, the cells washed with FACS wash buffer (PBS, 2% FCS, 0.01% Azide) and blocked with anti-CD16/32 (BD Biosciences) at 4 °C for 30 minutes. All subsequent antibody stains were performed using the same conditions. Cells were then washed and stained with anti-CD8-PerCP or anti-CD4-PerCP (BD Biosciences), washed again and permeablized using the Cytofix/Cytoperm™ kit (BD Biosciences). Perm/Wash buffer (BD Biosciences) was used to wash cells before staining with anti-IL-2-FITC, anti-TNF-Δ-PE and anti-IFN-g-APC (BD Biosciences). Cells were fixed with CellFIX™ (BD) and stored at 4 °C until analysis.

**Vaccinia virus challenge/protection assay.** Groups of 4 to 5 BALB/c female mice naïve or immunized were challenged with 4 x 10⁶ pfu of vaccinia virus WR.HIVA i.p. Four days later, ovaries were collected and sonicated. Confluent Hu-TK-143B cells in 6-well plates were infected with 10-fold serial dilutions of the homogenated ovaries. Cells were stained with 0.1% crystal violet in 20% ethanol and the numbers of plaques were counted.

**FACS analysis.** All chromogen-labeled cells were analyzed by flow cytometry using
the CellQuest software (BD Biosciences).

**M. tuberculosis challenge/protection assay.** Pathogen-free 6- to 8-week-old BALB/c mice were maintained under barrier conditions in a Category III safety facility. Groups of 7 to 9 mice were vaccinated with $3 \times 10^5$ cfu of either BCG.HIVA, BCG.p or BCG wild type 1173 p2 subcutaneously into left hind foot 12 weeks prior to challenge. *M. tuberculosis* Erdman was obtained from M. Brennan (WHO), kept at -80 °C and sonicated before use. Mice were challenged by aerosol with *M. tuberculosis* using a modified Henderson apparatus (40) and exposing only the nose during challenge (ADG Developments, UK). Bacterial loads in lung and spleen were determined 4 weeks after challenge by plating 10-fold serial dilutions of organ homogenate on Middlebrook plates and counting the cfu following a 3-week incubation at 37 °C. Deposition in the lungs was estimated 24 hours after challenge and determined to be around 25 cfu/lung.

**RESULTS**

**Construction of recombinant Mycobacterium bovis BCG expressing HIV-1 clade A immunogen.** To construct a candidate HIV-1 vaccine vectored by *M. bovis* BCG, we used an immunologically well characterized protein HIVA coupled to mAb tag Pk (17). The HIVA gene was synthesized utilizing humanized GC-rich codons(5), which are similar to those used by mycobacteria(4). The HIVA open-reading frame was fused at its 5’ end to nucleotides coding for the 19-kDa lipoprotein signal sequence, which facilitates expression of foreign proteins in the mycobacterial membrane and was shown to increase the foreign protein immunogenicity(47). To facilitate the pre-clinical development of candidate vaccines, the HIVA immunogen contains an immunodominant H-2D$^d$ restricted epitope P18-I10 (48), here designated H. In addition,
it also contains at least three other subdominant H-2d epitopes recognized by CD8+ T cells including epitope P and three CD4+ T helper epitopes (24). The chimeric 19-kDa signal sequence-HIVA gene was expressed from *Escherichia coli*/mycobacterium shuttle plasmids pJH222 and pJH223 under the control of the *Mycobacteria ssp* α-antigen promoter (Fig. 1A). pJH222 is a low-copy replicative episomal vector and contains mycobacterial origin of replication (*oriM*). pJH223 is an integrative vector, which carries attachment site (*attP*) and the integrase gene (*int*) from the mycobacteriophage L5, and integrates as a single copy into mycobacterial chromosome. Both vectors also contained the kanamycin resistance gene (*aph*) as a selectable marker, *E. coli* origin of replication (*oriE*), and a wild-type lysine A-complementing gene for the vector maintenance in the BCG auxotroph. Recombinant pJH222.HIVA and pJH223.HIVA were transformed into lysine auxotroph of *M. bovis* BCG host strain Pasteur ΔlysA5::res (39). Expression of the full-size chimeric 19-kDa signal sequence-HIVA protein of *M* r65 kDa was confirmed on a Western blot of whole transformed mycobacterial cell lysates using anti-Pk mAb (Fig. 1B). This analysis also revealed that rBCG carrying episomal pJH222.HIVA expressed moderately higher levels of HIVA than rBCG with the integrated pJH223 plasmid. Growth of the transformed mycobacteria and the *in vivo* stability of episomal plasmid were established by the recovery of BCG.HIVA from the spleens of BALB/c mice 15 weeks after immunization (Fig. 1C). For further experiments, rBCG harbouring episomal vector pJH222.HIVA was used and referred to as BCG.HIVA.

**BCG.HIVA primes and enhances MVA.HIVA-elicited HIV-1-specific CD8+ T cell responses**

The ability of the candidate BCG.HIVA vaccine to induce HIV-1-specific immune responses was determined in BALB/c mice (Fig. 2A). On day 0, mice were immunized
using either BCG.HIVA with the episomal plasmid, the parental BCG (BCG.p) or left unimmunized and on day 102, half of the animals were boosted with MVA.HIVA. Because of the activation induced by BCG, detectable HIVA-specific responses peak after 12 weeks (not shown). On day 151, mice were sacrificed and the functional quality of the elicited T cells was investigated in terms of their ability to produce IFN-γ and TNF-α and degranulate (surface expression of CD107a/b) in response to peptide stimulation in a multicolour flow cytometric analysis (Fig. 2B). A number of observations were made. First, BCG.HIVA alone induced undetectable CD8+ HIV-1-specific T cell responses with the possible exception of degranulating (CD107a/b+) cells producing TNF-α (Fig. 2B, middle graph). Nevertheless, the same BCG.HIVA priming increased 5- (p ≤ 0.0004) and 14-fold (p < 0.02) the MVA.HIVA-elicited frequencies of the H- and subdominant P-specific CD8+ splenocytes producing IFN-γ and degranulating, respectively. Although some increase of the H responses was also observed after the BCG.p prime, this was not statistically significant. Thus, the BCG.HIVA enhancement of MVA.HIVA responses was not a non-specific stimulation of bystander T cells by innate anti-BCG responses, but rather a HIVA-specific response. Second, the BCG.HIVA prime-MVA.HIVA boost regimen elicited the highest proportion of HIV-1-specific bi-functional cells in all three shown analyses (Fig. 2B). Finally, no HIVA-induced CD4+ T cell responses were detected (not shown). HIV-1-specific cellular responses were also assessed in MVA.HIVA and BCG.HIVA/MVA.HIVA-vaccinated mice using an in vivo killing assay (Fig. 2C). This clearly demonstrated in vivo strong lytic activities against both the H and P epitopes primed by BCG.HIVA, which approximately doubled and tripled, respectively, responses elicited by MVA.HIVA alone.
Primining BCG.HIVA dose affects vigor and quality of CD8+ T cell response to a subdominant epitope. Next, the effect of BCG.HIVA dose on the vigor and quality of CD8+ T cell responses induced in the heterologous BCG.HIVA-MVA.HIVA regimen was investigated. Using a similar schedule as above, BALB/c mice were either unvaccinated or primed with increasing doses of BCG.HIVA ranging from 10^3 to 10^7 colony-forming units (cfu) per animal and boosted with a constant dose of MVA.HIVA (Fig. 3A). First, the frequencies of bi-functional INF-γ'CD107a/b', TNF-α'CD107a/b' and IFN-γ'TNF-α' T cells specific for the H and P epitopes were assessed. We found that for immunodominant epitope H, bi-functional responses were steadily increasing in magnitude with the dose and reached maximum of approximately 0.7% CD8+ splenocytes at 10^6 cfu of BCG.HIVA. In contrast for subdominant epitope P, 10^5 cfu of BCG.HIVA appeared to be the threshold for response induction and the P response increased up to 10^7 cfu, reaching approximately 0.2% of CD8+ splenocytes (Fig. 3B). Analysing of tri-functional cells revealed that for the immunodominant H epitope, the BCG.HIVA dose did not significantly affect the relatively high fraction of CD8+IFN-γ' splenocytes, that were also capable of producing TNF-α and degranulation. The highest group average proportion of tri-functional cells was 88% CD8+IFN-γ' splenocytes, which was detected at 10^5 cfu of BCG.HIVA (Fig. 3C). In contrast, responses to subdominant epitope P peaked at 10^7 cfu of BCG.HIVA and reached 80% of the CD8+IFN-γ' splenocytes. Similar results were obtained by the reciprocal analysis, i.e. identifying the fractions of CD8+TNF-α' cells that could both produce IFN-γ and degranulate (Fig. 3D). Thus, the priming dose of BCG.HIVA affected both the strength and quality of CD8+ T cell responses induced by a heterologous rBCG prime-rMVA boost regimen. High priming BCG.HIVA dose was particularly important for improving
the quality of responses to the subdominant P epitope, i.e. for the breadth of the vaccine-induced T cell responses.

**pTHr.HIVA DNA prime-BCG.HIVA boost protects against a surrogate virus challenge.** Relevant functionality of vaccine-induced responses against viral infection is best tested by an *in vivo* virus challenge. However, HIV-1 does not replicate in mice and there is not a relevant challenge for the HIV-1-derived immunogen HIVA in non-human primate models other than infection of chimpanzee with HIV-1, which is prohibitively costly. Thus, surrogate challenge with recombinant replication-competent vaccinia virus, strain Western Reserve, expressing the HIVA immunogen (WR.HIVA) was used as described previously (25). To avoid induction of anti-poxvirus immune responses by MVA.HIVA, a heterologous prime-boost regimen was utilized consisting of pTHr.HIVA DNA prime on day 0 and BCG.HIVA boost on day 33 together with several control groups (Fig. 4A). Mice were challenged on day 150 with WR.HIVA, sacrificed 4 days later and both the HIVA-specific immune responses and the WR.HIVA virus loads in ovaries were determined. While a 4-day infection with WR.HIVA did not elicit any HIVA-specific responses in naïve and BCG.p-vaccinated mice, H- and P-specific responses were readily detected in mice that had received BCG.HIVA, pTHr.HIVA-BCG.p and pTHr.HIVA-BCG.HIVA prior to the WR.HIVA challenge (Fig. 4B). The highest frequencies of T cells were detected in the pTHr.HIVA-BCG.HIVA-WR.HIVA group, in which the T cells mainly produced IFN-γ and degranulated. Relative to IFN-γ⁺ cells, the TNF-α⁺-producing cells were lower in frequencies, but majority of both the H- and P-specific TNF-α⁺ T cells were tri-functional suggesting again generation of a high quality T cells (Fig. 4C and D).

In this series of experiments, also CD4⁺ responses to three known HIVA epitopes were detected in all groups primed with the HIVA immunogen. Significantly
higher frequencies of bi-functional splenocytes were elicited by the pTHr.HIVA DNA-
BCG.HIVA-WR.HIVA compared to other immunization regimens (Fig. 5A, left
panels). Interestingly, for CD4+ cells, the highest quality of CD4+IFN-γ’TNF-α’TIL-2+
splenocytes was achieved equally by BCG.HIVA and BCG.p boosts of pTHr.HIVA-
primed responses (Fig. 5A, right panels), suggesting at least for the CD4’ T cell
response an augmenting role for the BCG-stimulated innate response.

Indeed, a similar synergism between the HIVA-specific and non-specific
responses was observed for the control of WR.HIVA replication in the ovaries (Fig.
5B). Compared to naïve mice, immunization with BCG.HIVA alone, but also BCG.p
alone decreased the WR.HIVA titre in ovaries by two orders of magnitude, thus
strongly implicating HIVA-non-specific protective mechanisms. While pTHr.HIVA
DNA on its own provided no protection, pTHr.HIVA priming improved the BCG.p-
generated control of the WR.HIVA replication by further two orders of magnitude
compared to BCG.p alone. Finally, a complete WR.HIVA clearance from ovaries in 5
out of 5 mice was achieved by the combination of HIVA-specific and non-specific
response following the pTHr.HIVA prime-BCG.HIVA boost regimen. Although a
surrogate virus challenge, the quality of the BCG-elicited response and complete
protection are encouraging for further development of the BCG.HIVA vaccine.

Lysine auxotroph BCG.HIVA gives similar level of protection against M.
tuberculosis challenge as the currently used BCG vaccine. We also assessed the
immunological response to purified protein derivative (PPD) following the BCG.HIVA-
dose response prime-constant MVA.HIVA boost vaccination regimen described in Fig.
3A, and found PPD-specific cells producing mainly IFN-γ and TNF-α for the higher
BCG.HIVA doses (Fig. 6A). Upon an M. tuberculosis aerosol challenge, the current
BCG vaccine (BCG wild type 1173 p2), BCG.p and BCG.HIVA provided equivalent
protection demonstrated both in lungs and spleen by a two-order-of-magnitude decrease of *M. tuberculosis* load (Fig. 6B). Thus, at least this murine experiment suggests that the safer lysine auxotroph BCG.HIVA can replace the neonatal BCG vaccine against *M. tuberculosis* without loosing the benefits of the BCG vaccination and therefore its use will not compromise or interfere with any other scheduled paediatric vaccination.

**DISCUSSION**

Here, we have engineered a novel candidate vaccine for both HIV-1 and *M. tuberculosis*, which is vectored by lysine auxotroph of *Mycobacterium bovis* bacillus Calmette–Guerin (Pasteur ΔlysA5::res) and expresses the HIV-1 clade A-derived immunogen HIVA. We demonstrated in BALB/c mice that BCG.HIVA can both prime novel and boost pre-existing HIV-1-specific cellular immune responses, which are mediated by CD4$^+$ and CD8$^+$ T cells of high quality as judged by their long-term persistence and capacity to proliferate and produce multiple cytokines upon antigenic re-exposure. Furthermore, sequential heterologous immunization involving pTHr.HIVA DNA and BCG.HIVA conferred complete protection in mice against a surrogate challenge with vaccinia virus expressing the HIVA immunogen, while BCG.HIVA alone provided protection against *M. tuberculosis* challenge comparable to that achieved by a currently used BCG vaccine strain.

Here, we constructed rBCG stably expressing immunogen HIVA from both episomal pJH222.HIVA and intergrative pJH223.HIVA plasmids. To express the HIVA protein, both plasmids used the $\alpha$-antigen promoter and 19 kDa lipoprotein signal sequence, which were found to be optimal for antigen secretion and immunogenicity(10) perhaps through acylation of the signal sequence, which was important for entering the MHC class I presentation and interaction with Toll-like
Based on higher expression levels of HIV-1, we chose to further characterize the episomal construct. The same plasmid backbones were recently compared in *M. smegmatis*, a rapidly replicating mycobacterium, expressing the HIV-1 gp120 envelope glycoprotein (8, 53). Despite higher expression levels of gp120 from the episomal over integrated plasmid, the immunogenicities in BALB/c mice were identical.

To date, vaccines vectored by BCG have been successfully constructed for a number of infectious agents such are leishmania, *Borrelia burgdorferi*, *Streptococcus pneumoniae*, *Bordetella pertussis*, malaria, cottontail rabbit papillomavirus, measles virus, and indeed human and simian immunodeficiency viruses (23). Many of these vaccines showed immunogenicity and protection in murine models, while some where also immunogenic in non-human primates (3, 51, 53). The only volunteer study conducted to date suggested that rBCG was only moderately efficient in eliciting immune responses to passenger proteins in humans, but did not use a heterologous prime-boost regimen (13). In fact, only a small fraction of the above animal experiments used rBCGs in heterologous prime-boost regimens. However, when BCG-SIVgag-induced responses were boosted by defective poxvirus-SIVgag, a partial protection against a pathogenic simian-human immunodeficiency virus challenge was obtained (3). Here, the inclusion of BCG-HIVA into heterologous vaccination protocols improved consistently frequency, quality and durability of generated HIV-1-specific responses. This was reflected in the highest detected fractions of multi-functional HIVA-specific T cells compared to other tested regimens and these responses were present over 14 weeks after rBCG administration. Also, the frequency and functionality of CD8+ T cells specific for the subdominant epitope P was significantly elevated by inclusion of rBCG suggesting that BCG may be a useful vaccine vector for induction of specificities, which might be under other circumstances overshadowed by immunodominance.
although this will need to be confirmed for other epitopes and immunogens. Detectable HIV-1-specific CD4+ T cell responses were only detected in the WR.HIVA-challenge experiment, but not following the BCG.HIVA alone or BCG.HIVA-MVA.HIVA vaccine regimens. This may simply reflect the generally lower frequencies of elicited CD4+ T cells compared to those of their CD8+ counterparts or the fact that in the former experiment, the CD4+ T cell responses were also boosted by a vigorously replicating WR.HIVA vaccinia. Nevertheless, the ability to elicit strong, multi-specific and multi-functional CD8+ T cell responses is likely to be a critical feature for the success of a candidate HIV-1 vaccine.

The BCG.HIVA vaccine candidate in this study is vectored by lysine auxotroph of BCG, which carried an E. coli-mycobacterial shuttle plasmid with lysine A complementing gene. This increases theoretically the plasmid stability in vivo, prevents genetic rearrangements of the heterologous gene and adds an additional safety feature to the vaccine. Here, we demonstrated in a mouse model, that BCG.HIVA was as efficient in protection against M. tuberculosis as the currently used BCG wild type 1173 p2 vaccine. Also, this is the first report on HIV-1 vaccine vectored by an auxotroph of BCG and the first study using rBCG prime-rMVA boost protocol as a potential anti-HIV-1 regimen, which combines the benefits of both of these promising vaccine vectors.

Protection against M. tuberculosis challenge offers the possibility to use BCG.HIVA in neonates as a substitution for the BCG vaccine in the first month of their life(35) and thus for BCG.HIVA to serve as a dual vaccine against HIV-1 and tuberculosis. The relevance of the ‘artificial’ consensus clade A HIVA immunogen to the viruses currently circulating in Nairobi has been demonstrated in HIV-1-infected or exposed uninfected infants, in whom 38-52% made T cell responses that recognized the
Gag and polyepitope domains of HIVA (46). The high frequency of exposed uninfected infants whose T cells recognize HIVA (52%) is encouraging as it suggests that exposure to virus through breast milk does not inevitably infect and can prime or boost T cell immunity. Thus the T cell responses in these vaccinated infants may exceed those seen with the same vaccines in HIV-1-unexposed adults. HIVA’s usefulness was also shown by boosting HIV-1-specific responses in patients on highly-active antiretroviral therapy, who were infected with diverse HIV-1 clades (11, 12, 37). Therefore, we propose to further develop this rBCG as a paediatric vaccine to protect children born to HIV-1-positive mothers against becoming infected with HIV-1 through breastfeeding. The regimen would consist of BCG.HIVA prime at birth followed by a boost with MVA.HIVA at one or two months of age along with the babies scheduled Extended Programme of Immunization. This boost could be combined with MVA.85A to strengthen the tuberculosis protection (34). In addition, the BCG.HIVA-primed responses can be also expanded by natural exposure to HIV-1 through breastmilk. The evaluation of safety and immunogenicity of this regimen in newborn non-human primates is the next aim. However, for a more relevant challenge with simian immunodeficiency virus (SIV), equivalent immunogen derived from SIV inner proteins will have to be constructed.

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FIG 1. Construction of BCG.HIVA. (A) A synthetic GC-rich HIVA gene was fused to the 19-kDa lipoprotein signal sequence and inserted into episomal pJH222 or integrative pJH223 E. coli mycobacterium shuttle plasmids. These both contain kanamycinr (aph) and complementing lysA genes, and E. coli origin of replication (oriE). In addition, pJH222 contains mycobacterial origin of replication (oriM) and pJH223 carries the attachment site (attP) and the integrase gene of mycobacteriophage L5 (int). BALB/c T cell and mAb Pk epitopes used in this work are shown. (B) Western blotted lysates of BCG.HIVA containing the pJH222 (lanes 1 and 2) and pJH223 (lanes 3 and 4) and parental BCG.p (lane 5) are shown. HIVA was detected using the anti-Pk mAb followed by HRP-protein A and ECL. (C) Stability of BCG.HIVA harbouring pJH222.HIVA. Following mouse injection with 10⁷ cfu BCG.HIVA i.p., rBCG was recovered from homogenized spleens 15 weeks later and plated (without kanamycin). Ten randomly picked mycobacterial colonies were tested for kanamycin resistance and two of these for the presence of the HIVA gene using HIVA-specific PCR. On the gel: Lanes 1 and 8 - molecular markers; lane 2 – BCG.p; lane 3 – no template; lane 4 – plasmid pJH222; lanes 5 and 6 – kanamycin-resistant colonies; and lane 7 – BCG.HIVA vaccine stock.

FIG 2. Induction of multifunctional HIV-1-specific CD8+ T cells by the BCG.HIVA prime-MVA.HIVA boost regimen. (A) Mice were either left unimmunized or immunized with 10⁶ cfu of parental BCG or BCG.HIVA and subsequently boosted with 10⁶ pfu of MVA.HIVA as indicated. (B) Analysis of bi-functional vaccine-elicited CD8+ T cells. The top panels provide examples of dot blots as generated for group 6 and epitope H, and the bottom panels summarize the data obtained for each vaccination group using the H (top) and P (bottom) epitopes. For the IFN-γ/CD107a/b and TNF-
α/CD107a/b analyses, the frequencies of non-degranulating (empty bars) and
degranulating (full bars) cells producing cytokine are shown. For the IFN-γ/TNF-α
analysis, group average frequencies in each quadrant I, II and III are plotted. Data are
presented as mean +/- SD (n=4 to 5). (C) A 12-hour in vivo killing of syngeneic peptide-
pulsed cells in naïve and vaccinated animals. Left is an example dot blot showing
splenocytes re-isolated from naïve mice. Right panels show H- and P-specific killing as
mean +/- SD (n=5).

FIG 3. Effect of the BCG.HIVA priming dose on the induction of HIV-1-specific CD8+ T cells. (A) Immunization groups. Mice were either left unimmunized or primed with an
increasing dose of BCG.HIVA and boosted with 10^6 pfu of MVA.HIVA using a similar
timing to Fig. 2A. (B) The top left panels provide examples of dot blots for analysis of
bi-functional vaccine-elicited CD8+ T cells as generated for group 6 and epitope H. The
bottom panels summarize the data obtained for each vaccination group using the H (top)
and P (bottom) epitopes. For the IFN-γ/CD107a/b and TNF-α/CD107a/b analyses, the
frequencies of non-degranulating (empty bars) and degranulating (full bars) cells
producing cytokine are shown. For the IFN-γ/TNF-α analysis, average frequencies in
each quadrant I, II and III are plotted. Data are presented as mean +/- SD (n=4 to 5). (C,
D) Analysis of tri-functional vaccine-elicited T cells. The two left panels indicate the
gating. The right panels give the frequencies of tri-functional cells from the upper-right
quadrants for individual mice (circles) and group means (bars) for the H (top) and P
(bottom) epitopes as a percentage of CD8+IFN-γ+ (C) and CD8+TNF-α+ (D).

FIG 4. CD8+ T cell immunogenicity of the pTHr.HIVA DNA prime-BCG.HIVA boost
regimen (A) Mice were left unimmunized, primed with 10^6 cfu of BCG.HIVA/BCG,p or
100 μg of pTHr.HIVA DNA, then groups 5 and 6 were boosted with $10^6$ cfu of BCG.HIVA/BCG.p and all groups were challenged with $4 \times 10^6$ pfu of vaccinia virus WR.HIVA. (B) The top panels provide examples of dot blots for analysis of bi-functional CD8$^+$ T cells as generated for group 6 and epitope H. The bottom panels summarize the data obtained for each vaccination group using the H (top) and P (bottom) epitopes. For the IFN-$\gamma$/CD107a/b and TNF-$\alpha$/CD107a/b analyses, the frequencies of non-degranulating (empty bars) and degranulating (full bars) cells producing cytokine are shown. For the IFN-$\gamma$/TNF-$\alpha$ analysis, average frequencies in each quadrant I, II and III are plotted. Data are presented as mean +/- SD (n=4 to 5). (C, D) Analysis of tri-functional vaccine-elicited T cells. The two left panels indicate the gating. The right panels give the frequencies of tri-functional cells from the upper-right quadrants for individual mice (circles) and group means (bars) for the H (top) and P (bottom) epitopes as a percentage of CD8$^+$IFN-$\gamma^+$ (C) and CD8$^+$TNF-$\alpha^+$ (D).

FIG 5. Induction of high quality HIV-1-specific CD4$^+$ T cells and complete protection against surrogate virus challenge. The mice and groups are the same as in Fig. 4A. (A) The left-most panels summarize the data obtained for each cytokine and vaccination group. Data are presented as mean +/- SD (n=4 to 5). The middle panels demonstrate the gating for IFN-$\gamma$-, TNF-$\alpha$- and IL-2-producing CD4$^+$ T cells as generated for group 6 and cocktail of three MHC-class II epitopes. The right-most panels give the upper-right quadrant data of tri-functional HIV-1-specific CD4$^+$ T cells for individual mice (circles) and group means (bars). Data are presented as mean +/- SD (n=4 to 5). (B) Mice were either left naïve (1), or were vaccinated with BCG.p (2), BCG.HIVA (3), pTHr.HIVA DNA (4), pTHr.HIVA DNA and BCG.p (5) or pTHr.HIVA and BCG.HIVA (6), challenged with replication-competent vaccinia virus WR.HIVA and
the WR.HIVA load in ovaries was determined 4 days later. Data are shown for individual mice (circles) and group means (bars) (n=4 to 5).

FIG 6. Immune responses and protection against *M. tuberculosis* challenge by BCG.HIVA. (A) Mice and the groups were the same as in Fig. 3 (priming with increasing dose BCG.HIVA). Left panels show examples of bi-functional analyses using PPD as an antigenic stimulus. On the right, group data are shown as mean +/- SD (n=4 to 5) for each quadrant and immunization group. (B) Mice were left naïve or immunized with the currently used BCG vaccine, parental lysine auxotroph of BCG or lysine auxotrophic BCG.HIVA into their left hind legs s.c., challenged with inhaled *M. tuberculosis* and *M. tuberculosis* loads in lung (left) and spleen (right) were determined 4 week later. Data are shown for individual mice (circles) with geometric mean indicated for each group (n=7 to 9).
Figure 1. Im et al.
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