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Abstract

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Abstract
A safe and effective HIV vaccine is required to significantly reduce the number of people becoming infected with HIV each year. In this study wild type Mycobacterium bovis BCG Pasteur and an attenuated pantothenate auxotroph auxotroph strain (BCGpanCD) that is safe in SCID mice, have been compared as vaccine vectors for HIV-1 subtype C Gag. Genetically stable vaccines BCG/pH5400 (BCG-Gag) and BCGpanCD/pH5400 (BCGpan-Gag) were generated using the Pasteur strain of BCG, and a pantothenate auxotroph of Pasteur respectively. Stability was achieved by the use of a codon optimised gag gene and deletion of the hsp60-lysA promoter-gene cassette from the episomal vector pCB119. In this vector expression of gag is driven by the mtrA promoter and the Gag protein is fused to the Mycobacterium tuberculosis 19 kDa signal sequence. Both BCG-Gag and BCGpan-Gag primed the immune system of BALB/c mice for a boost with a recombinant modified vaccinia virus Ankara expressing Gag (MVA-Gag). After the boost high frequencies of predominantly Gag-specific CD8+ T cells were detected when BCGpan-Gag was the prime in contrast to induction of predominantly Gag-specific CD4+ T cells when priming with BCG-Gag. The differing gag-specific T-cell phenotype elicited by the prime-boost regimens may be related to the reduced inflammation observed with the pantothenate auxotroph strain compared to the parent strain. These features make BCGpan-Gag a more desirable HIV vaccine candidate than BCG-Gag. Although no Gag-specific cells could be detected after vaccination of BALB/c mice with either recombinant BCG vaccine alone, BCGpan-Gag protected mice against a surrogate vaccinia virus infection challenge.

Introduction
Information in the 2010 UNAIDS Report on the global AIDS epidemic indicates a global decline in HIV infection, observed as a lower number of infections and deaths from AIDS. However there is a strong warning in the Report that concerted efforts to improve prevention of disease is of paramount importance as this will contribute to prevention of infection. The quest for an HIV vaccine should be an integral component of prevention strategies. The precise requirements of an HIV vaccine needed for eliciting protection against infection are as yet not known. Immune responses to human immunodeficiency virus type 1 (HIV) that participate in virus replication control, as observed in infected individuals termed long-term non progressors or elite controllers, include specific cellular responses that strongly target Gag [1,2]. Induction of such cellular HIV-specific immune responses is therefore one of the favourable and necessary requirements of a protective vaccination strategy [3]. There is abundant evidence indicating heterologous prime-boost vaccine regimens that combine the use of recombinant bacterial vaccine vectors and recombinant virus vaccine vectors expressing HIV antigens and HIV virus like particle protein vaccines induce robust HIV-specific cellular immune responses [4–6].

Mycobacterium bovis bacillus Calmette-Guérin (BCG) has been explored as an HIV vaccine vector since the early 1990s [7,8]. BCG has a record of being safe in that it has been given to billions of people worldwide with a very low incidence of serious complications and importantly it induces long lasting immunity...
were larger than those transformed with pRT106, indicating that codon-optimisation of the gag gene for expression in mycobacteria had probably reduced the metabolic load generated by expression of the viral antigen and thus improved the growth rate of the recombinant BCG. Plasmid DNA isolated from BCG[pHS300] and BCGΔpanCD[pHS300] vaccine stocks contained deletions and rearrangements. Restriction mapping and sequencing of plasmid DNA isolated from these vaccines indicated that the p7p1p6 region of the gag gene, the hup60 promoter and a portion of the lysA gene were deleted in all the rBCG analysed (Figure 1 A & B). This region of deletion varied between different vaccine stocks but, in all cases, the hup60 promoter was deleted. High levels of LysA protein were detected by SDS-PAGE analysis of the BCG lysates (data not shown). To determine whether the high levels of LysA were contributing to the instability of the rBCG, the hup60 promoter and lysA gene were deleted from the shuttle vector to generate the plasmid pH500 (expressing a codon optimised gag gene), following which BCG[pHS400] (BCG-Gag) and BCGΔpanCD[pHS400] (BCGpan-Gag) were generated. Vaccine stocks were found to be genetically stable and this stability was maintained after in vitro passage for 30 generations (Figure 1 C & D). The in vivo stability of BCG-Gag and BCGpan-Gag was also confirmed by restriction enzyme mapping of plasmid DNA isolated from rBCG recovered from mice splenocytes 6 weeks post vaccination (data not shown). Growth rates of BCG-Gag and BCGpan-Gag in broth supplemented with pantothenate were comparable to those of the respective non-recombinant BCG strains (data not shown). No growth of BCGΔpanCD was observed in the absence of pantothenate confirming its auxotrophic phenotype.

Cellular responses, bacterial burden and histopathology induced by rBCG vaccines

Vaccination of mice with the rBCG vaccines (10^7 cfu, i.p.) resulted in spleen enlargement and an associated increase in the total splenocyte yield compared to that of naive spleens (Figure 2A). At 4 weeks post vaccination with BCG-Gag or a BCG-Control (rBCG not expressing Gag), caused a 4.6-fold increase in splenocyte numbers compared to that of naive mice (p<0.01). At 12 weeks post vaccination total splenocyte numbers were always higher than that from naive spleens but the differences were not statistically significant. The splenocyte yield in response to BCGpan-Gag or BCGpan-Control (rBCGΔpanCD not expressing Gag), vaccination was 2-fold greater (p<0.05) than that from naive spleens at 4 weeks but not at 8 and 12 weeks post vaccination (Figure 2A). The proportion of CD3^+ cells in the spleens following vaccination with BCG-Gag or the control rBCG as determined by flow cytometry was consistent at 41±8% (n = 10) but not significantly different from the proportion of CD3^+ cells (39±4%, n = 10) in spleens from mice vaccinated with BCGpan-Gag or the control rBCG. These CD3^+ cells proportionally post rBCG vaccination compare with those from naive spleens which have a CD3^+ cell proportion of 43±8% (n = 10). Importantly, the CD4^+CD8^+ T cell ratio of the rBCG vaccinated mice was the same as that of naive mice, 2.4±0.3 (n = 10). Bacterial numbers in the spleen after the rBCG vaccination declined over time and there was no significant difference (p>0.05) in bacterial numbers for the vaccines at any of the observed times (Figure 2B). Histopathology analysis of liver sections 3 weeks post vaccination revealed BCG-Gag induced a significantly larger number of granulomas (p<0.05, week 2; p<0.01, week 3) that were frequently larger and contained more inflammatory cells than those isolated from mice vaccinated with BCGpan-Gag (Figure 2C & 3). For both BCG-Gag and BCGpan-Gag granulomas were well formed

Results

Development of a genetically stable rBCG vaccine

The effect of codon optimizing the gag gene for BCG usage on genetic stability of rBCG was then investigated. BCG was transformed with the plasmids pH500 and pRT106 expressing codon optimised and uncodon optimised gag genes respectively. Colonies produced by BCG transformed with plasmid pH500 were larger than those transformed with pRT106, indicating that codon-optimisation of the gag gene for expression in mycobacteria had probably reduced the metabolic load generated by expression of the viral antigen and thus improved the growth rate of the recombinant BCG. Plasmid DNA isolated from BCG[pHS300] and BCGΔpanCD[pHS300] vaccine stocks contained deletions and rearrangements. Restriction mapping and sequencing of plasmid DNA isolated from these vaccines indicated that the p7p1p6 region of the gag gene, the hup60 promoter and a portion of the lysA gene were deleted in all the rBCG analysed (Figure 1 A & B). This region of deletion varied between different vaccine stocks but, in all cases, the hup60 promoter was deleted. High levels of LysA protein were detected by SDS-PAGE analysis of the BCG lysates (data not shown). To determine whether the high levels of LysA were contributing to the instability of the rBCG, the hup60 promoter and lysA gene were deleted from the shuttle vector to generate the plasmid pH500 (expressing a codon optimised gag gene), following which BCG[pHS400] (BCG-Gag) and BCGΔpanCD[pHS400] (BCGpan-Gag) were generated. Vaccine stocks were found to be genetically stable and this stability was maintained after in vitro passage for 30 generations (Figure 1 C & D). The in vivo stability of BCG-Gag and BCGpan-Gag was also confirmed by restriction enzyme mapping of plasmid DNA isolated from rBCG recovered from mice splenocytes 6 weeks post vaccination (data not shown). Growth rates of BCG-Gag and BCGpan-Gag in broth supplemented with pantothenate were comparable to those of the respective non-recombinant BCG strains (data not shown). No growth of BCGΔpanCD was observed in the absence of pantothenate confirming its auxotrophic phenotype.

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Overall the inflammation induced by the wild type BCG was characterised by more prominent cellular infiltration and hepatocyte damage than that induced by the D<sup>panCD</sup> strain. (Figure 3). rBCG vaccines expressing Gag prime the immune system for a boost with MVA-Gag

Mice were vaccinated with the individual BCG vaccines (10<sup>5</sup> cfu or 10<sup>7</sup> cfu) and Gag-specific immune responses investigated using the IFN-γ ELISPOT assay to detect individual responses to a Gag CD<sup>8+</sup> peptide and two Gag CD<sup>4+</sup> peptides, GagCD4(13) and GagCD4(17). No Gag-specific T cell responses were detected at weekly intervals up to 12 weeks after vaccination. To detect possible priming of the immune system by these rBCG vaccines, groups of mice were primed on day 0 with the BCG vaccines then boosted at week 8 or week 12 with MVA-Gag and Gag peptide-specific immune responses were detected 12 days after the boost (Figure 4).

BCGpan-Gag at a dose of 10<sup>5</sup> cfu or 10<sup>7</sup> cfu unlike BCG-Gag, primed for a MVA-Gag boost given at week 12 after the BCG vaccine prime when the priming dose was increased to 10<sup>7</sup> cfu. Comparison of Gag peptide responses when the MVA-Gag boost was given at week 12 after a prime with either BCGpan-Gag (10<sup>5</sup> cfu) or BCG-Gag (10<sup>7</sup> cfu) is shown in Figure 4. Mean cumulative Gag-specific IFN-γ ELISPOT responses (3 separate experiments) of 412 ± 23 sfu/10<sup>6</sup> splenocytes were observed for a prime with 10<sup>5</sup> cfu BCGpan-Gag; and 835 ± 34 sfu/10<sup>6</sup> for a prime with 10<sup>7</sup> cfu BCGpan-Gag. These mean cumulative Gag peptide responses were 2.8 fold (p < 0.05) and 5.8 fold (p < 0.01) greater respectively than for a respective control BCG prime and MVA-Gag boost (Figure 4). Both Gag CD<sup>8+</sup> and CD<sup>4+</sup> peptide responses were boosted with Gag-specific CD<sup>8+</sup> cells contributing 36% and 60% to the total mean cumulative response for a dose of 10<sup>5</sup> cfu or 10<sup>7</sup> cfu BCGpan-Gag respectively. In comparison the contribution of Gag-specific CD<sup>8+</sup> cells to the total cumulative response for the control BCG prime (10<sup>5</sup> cfu or 10<sup>7</sup> cfu) and MVA-Gag boost was 17% and 22% respectively (Figure 4).

rBCG vaccines expressing Gag prime the immune system for a boost with MVA-Gag
peptide responses were boosted and the cumulative mean response (3 independent experiments) to the Gag peptides in the IFN-γ ELISPOT assay for either a prime with BCG-Gag or BCGpan-Gag were similar, 946±212 sfu/10⁶ splenocytes and 847±142 sfu/10⁶ splenocytes respectively. These cumulative Gag-peptide responses were approximately 3.4 fold higher (p<0.01) than the responses for a control BCG vaccine prime/MVA-Gag boost (Figure 4). However, when BCG-Gag was the priming vaccine, Gag-specific CD4⁺ cells contributed 82% and Gag-specific CD8⁺ cells 18% to this cumulative response, indicating MVA-Gag predominantly boosted Gag-specific CD4⁺ cells. In contrast when BCGpan-Gag was the prime Gag-specific CD4⁺ contributed 33% and Gag-specific CD8⁺ cells 67% to the cumulative Gag-specific response indicating MVA-Gag predominantly boosted Gag-specific CD8⁺ cells (Figure 4). The contribution of Gag-specific CD8⁺ cells to the cumulative Gag-specific response to a prime with both the control BCG vaccines followed by a boost with MVA-Gag was approximately 24% (Figure 4).
The rBCG prime and MVA-Gag boost vaccination regimen induced high levels of Gag-specific IFN-γ, TNF-α, and IL-6.

A cytokine bead array assay quantified the level of IFN-γ, TNF-α, and IL-6 produced by splenocytes after a prime with the BCG vaccines and a boost with MVA-Gag given at week 12 after the BCG vaccination (Figure 5). High levels of IFN-γ were released from splenocytes in response to Gag peptide stimulation (Figure 5A). When BCG-Gag was the priming vaccine 10% of the total Gag-specific IFN-γ production of 5895 ± 473 pg/10^6 splenocytes (3 individual experiments) was from CD8^+ cells, while for prime with BCGpan-Gag, CD8^+ cells contributed 63% to the total Gag-specific IFN-γ production of 6673 ± 328 pg/10^6 splenocytes (3 individual experiments). These levels of IFN-γ produced

Figure 3. Liver sections of mice vaccinated with rBCG. Histopathology examination of formalin fixed liver sections stained with haematoxylin and eosin showing the frequency of granulomas induced by the vaccines. Magnification 200 ×, and a representative view from one of 3 mice per vaccine. doi:10.1371/journal.pone.0032769.g003

Figure 4. IFN-γ ELISPOT responses induced by a prime with the rBCG vaccine and MVA-Gag boost. Groups of mice were primed with BCG-Gag (10^7 cfu) and BCG-Control (10^7 cfu), BCGpan-Gag (10^7 cfu or 10^5 cfu) or BCGpan-Control (10^7 cfu or 10^5 cfu) then boosted with MVA-Gag (10^7 pfu, i.m.) at week 8 or week 12. Spleens were harvested 12 days after the boost, pooled from 5 mice per group and used in an IFN-γ ELISPOT assay with the indicated Gag-specific CD8^+ or CD4^+ peptides. Bars indicate the mean and standard deviation of the mean IFN-γ ELISPOT responses to an individual Gag CD8^+ or Gag CD4^+ peptide for 3 separate experiments. Data are responses after subtraction of background responses which were not more than 20 sfu/10^6 splenocytes. Asterisks indicate statistical significance of the mean IFN-γ ELISPOT responses to the individual Gag peptides obtained for a rBCG vaccine and MVA-Gag boost compared to that for the respective control rBCG vaccine prime MVA-Gag boost; *<0.01; **<0.05; Student’s t-test for means of unpaired data. doi:10.1371/journal.pone.0032769.g004
by Gag peptide stimulated splenocytes were more than 6 fold greater (p<0.01) than those for a prime with the control BCG vaccines followed by a MVA-Gag boost (Figure 5A). No Gag-specific TNF-α above background responses could be detected for a BCG-Gag prime and MVA-Gag boost. This is in contrast to splenocytes from BCGpan-Gag primed and MVA-Gag boosted mice that produced a total of 1532±218 pg TNF-α/10⁶ splenocytes (3 individual experiments) when stimulated with Gag-peptides, with 31% being produced by Gag-specific CD8⁺ cells. This TNF-α production was four times that of a prime with the BCGpan-Control vaccine (Figure 5B). Splenocytes from BCG-Gag primed and MVA-Gag boosted mice produced 10771±520 pg IL-6 and all from Gag-specific CD4⁺ cells (3 individual experiments). This Gag-specific IL-6 production was 8-fold above (p<0.01) that of a prime with the BCG-Control vaccine. A prime with BCGpan-Gag elicited 4945 pg IL-6/10⁶ splenocytes with approximately equal quantities from Gag-specific CD8⁺ and CD4⁺ cells. This Gag-specific IL-6 production was 3-fold above (p<0.05) that of a prime with the BCGpan-Control vaccine (Figure 5C).

**BCGpan-Gag protects against a VV-Gag challenge**

The data from the immune assays suggest BCGpan-Gag primes predominantly Gag-specific CD8⁺ cells. Thus the ability of BCGpan-Gag to protect mice against a VV-Gag challenge was assessed. Similar titres of VV-NYCBH were measured in mice that were challenged with this control vaccinia virus after vaccination with either the BCGpan-Gag, the BCGpan-Control vaccine or the

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**Figure 5.** Gag-specific IFN-γ, TNF-α and IL-6 responses induced by a rBCG prime and MVA-Gag boost. Groups of mice were vaccinated with 10⁷ cfu BCG-Gag, BCG-Control, BCGpan-Gag or BCGpan-Control then boosted with MVA-Gag (10⁷ pfu) at week 12. Spleens were harvested 12 days after the boost and pooled from 5 mice per group and stimulated in culture with the indicated Gag-specific CD8⁺ and CD4⁺ peptides. Bars indicate the Gag peptide-specific cumulative cytokine response and blocks within each bar indicate the mean and standard deviation of the mean cytokine response for 3 individual experiments to either a Gag CD8⁺ or Gag CD4⁺ peptide. All responses are after subtraction of background responses of not more than 2 pg per 10⁶ splenocytes. Asterisks indicate statistical significance of the cumulative cytokine response for the rBCG vaccine compared to the control rBCG vaccine. *p<0.01; **p<0.05; Student’s t-test for unpaired data.
resuspension medium. This indicates that neither the rBCG vaccines nor the resuspension medium provided nonspecific vaccinia protection. In addition no non-specific protection against VV-Gag was observed in mice vaccinated with the BCGpan-Control vaccine or the BCG vaccine resuspension medium as similar VV-Gag titres were obtained after a VV-Gag challenge (Figure 6). However for mice vaccinated with BCGpan-Gag then challenged with VV-Gag, no virus was recovered from the ovaries, which translates to >7 log protection compared to that of a vaccination with the BCGpan-Control or the BCG vaccine resuspension medium followed by VV-Gag challenge.

Discussion

In this study BCG has been evaluated as an HIV vaccine vector expressing Gag. The ApaCD BCG auxotroph was compared to the wild type BCG as a vaccine vector as this strain has been shown to be less virulent than the wild type strain in mice and guinea pigs. The observation that BCG vaccination of HIV positive children has accounted for mycobacterial disease in a proportion of these immunocompromised subjects strongly indicates the need for the development of safer mycobacterial vectored vaccines [29,30]. Second generation auxotroph strains of M. tuberculosis and BCG have thus been generated for use against tuberculosis. Deletion of genes important for mycobacterial growth such as lycA, leuD, met and panCD results in attenuated growth in vivo with a consequent improved safety profile in immunocompromised animals and concomitant enhanced or comparable protection of mice and guinea pigs against M. tuberculosis challenge compared to the parent strain [28,31–34]. rBCG expressing listeriolysin or perfringolysin O have also been shown to have improved safety in SCID mice as compared to the wild type strain [13,35]. Safety issues are also of concern with the use of BCG as a HIV vaccine vector and this has led to auxotroph BCG strains being considered as alternative vectors for immunocompromised individuals [28]. These mutant strains do not show compromised immunogenicity in animal studies [20,24,36]. The ApaCD BCG auxotroph has previously been shown to require pantothenate supplementation to grow in human macrophages and is nonpathogenic in SCID mice. When used to express the M. tuberculosis 30 kDa major secretory protein in the development of a candidate TB vaccine, rBCG(panCD)30, this auxotroph was better tolerated at higher doses than the parent strain and provided protection comparable to that of BCG in guinea pigs [28]. In vitro our BCGpan-Gag vaccine grew at a rate similar to BCG-Gag in the presence of pantothenate supplementation in broth culture. Deletion of the hsp60 promoter and lycA gene from the vector resulted in genetically stable BCG and ApaCD BCG auxotroph vaccines, both in vitro and in vivo. No plasmid deletions and rearrangements were observed. This modification of the plasmid together with the use of a codon optimised gag gene was essential to prevent deletion of p7p1p6 from the C-terminus of Gag. Plasmid instability has previously been shown to be associated with the activity of the strong hsp60 promoter [37–39]. It is important to have a vaccine that includes p7p1p6 as HIV infected individuals do mount immune responses to this Gag region [40]. The BCG

![Figure 6. Protection from a Vaccinia Virus-Gag challenge by BCGpan-Gag.](doi:10.1371/journal.pone.0032769.g006)
codon optimised gag gene was used to enhance translational activity. The observed genetic stability of our vaccines may be attributed to two factors, fusion of the gag gene to the M. tuberculosis 19 kDa signal sequence, to assist with transport of the Gag protein to the surface of the mycobacterium, and control of HIV-1 gag gene expression by the M. tuberculosis melT promoter, which is up-regulated after uptake of rBCG by phagocytosing cells [6,37,41–45]. In vivo expression of HIV-1 Gag was not assessed in this study, however both BCG-Gag and BCGpan-Gag primed the immune system for a MVA-Gag boost, indicating that Gag presentation must have occurred after cellular uptake of the rBCG vaccines.

Comparison of immune responses to Gag induced by BCG-Gag and BCGpan-Gag indicated that although no Gag-specific T cells could be detected in response to the vaccines alone, Gag-specific responses were detected after the MVA-Gag boost. These responses were not due to BCG-dependent adjuvant activity as Gag-specific T cell responses induced by a MVA-Gag vaccination alone were not significantly different from that observed after a prime with the BCG-Control or BCGpan-Control vaccines and a MVA-Gag boost (data not shown). Thus both vaccines did appear to prime the immune system.

In addition BCGpan-Gag induced Gag-specific responses that protected against a surrogate vaccinia virus-Gag challenge. Greater than 7 logs of protection were seen in the vaccinia virus challenge when mice were immunised with BCGpan-Gag. Protection from challenge in this model is associated with CD8+ responses [44] and therefore this result indicated that the HIV-1 specific CD8+ T cell immune response induced with BCGpan-Gag was protective.

Data from several studies indicate mycobacterial viruses prime the immune system for a booster vaccine [5,6,17,19,20,24]. Although a pro-apoptotic M. tuberculosis AplhaAsecA2 mutant and a BCG Pasteur hsdC auxotroph have generated CD8+ cells to the HIV plasmid insert, an inability to detect responses or detection of low responses to an HIV or SIV insert delivered by BCG or BCG and M. tuberculosis single and double auxotrophs have generally been reported in murine and non-human primate vaccine studies [19,36]. Mechanisms of immune system priming by BCG are associated with in vivo replication rates and levels and duration of antigen expression in the bacteria [45]. BCG replication after vaccination is slow which favours low levels of antigen expression followed by low levels of antigen presentation [46]. Subsequently low magnitudes of induced antigen specific T cells that differentiate to the memory phenotype soon after vaccination are generated and are stimulated by the booster vaccine [47].

The responses to a MVA-Gag boost that we detect after a prime with BCG-Gag or BCGpan-Gag indicate Gag-specific T cells were probably induced by the BCG vaccines and it is possible the memory phenotype of these cells prevented their detection in the IFN-γ ELISPOT assay [47]. Gag-specific CD4+ cells were predominantly generated to the boost after a BCG-Gag prime. In contrast BCGpan-Gag primed for a boost of predominantly Gag-specific CD8+ cells. These vaccines were given at a dose of 10^5 cfu per mouse. MVA-Gag boosted primary Gag-specific CD8+ and CD4+ cells as early as 8 weeks post the BCGpan-Gag prime; but this was not achieved with a BCG-Gag prime. The overall greater inflammation generated by the wild type strain may have prevented the development of the secondary responses. These Gag-specific T cells produced IFN-γ, TNF-α as well as IL-6, cytokines which are proposed to be necessary for control of HIV infection [48–50]. Induction of a Gag-specific CD4+ response may be expected as BCG per se induces CD4+ cell responses in mice which would influence predominant insert-specific CD4+ cell development [51]. However a CD4+ response does assist CD8+ development [52,53]. Induction of Gag-specific CD4+ and CD8+ cells by the BCGpan-Gag prime and MVA-Gag boost regimen is a required favourable vaccine response and suggests both MHC class I and II presentation of the antigen occurs with generation of CD8+ cells a consequence of possible efficient cross priming by the BCG vaccine.

As inflammatory signals from BCG are expected to also influence the characteristics of the immune response to the HIV insert the reduced inflammation caused by the BCGpanCD strain may account for the immune response to the gag gene seen as both Gag-specific CD8+ and CD4+ cells [48]. This modulation of inflammation by the BCGpanCD strain vaccination was observed in this study as a reduction in early expansion of CD3+ cells as well as a reduction in granuloma size and number compared to the parent strain. Despite this lower cellular response to the BCGpanCD strain, bacterial replication was similar to that of the wild-type strain. Similar reduced pathology with constraint of bacterial growth has been observed in mice in response to a M. tuberculosis ApanCD mutant as well as the M. tuberculosis whbB2 and sigH mutants [33,54,55]. Deletion of the panC and panD genes inhibits the synthesis of pantothenate which is one of the requirements for protection of bacteria from oxidative stress. In addition there is a decrease in phospholipid and intermediate amino acid and polyketide biosynthesis through deletion of the panC and panD genes. This lack of pantothenate biosynthesis associated with decreased inflammation may play a major role in the orchestration of immune responses to Gag expressed by the BCGpan-Gag vaccine.

In conclusion this is the first study using the BCG ApanCD auxotroph as an HIV vaccine vector and shows that, when used as a vector for the gag gene, HIV-specific T cells are induced that protect against vaccinia virus surrogate challenge and can be boosted to a high level with a heterologous booster vaccine. The reduced inflammation induced by the auxotroph appears to be an important factor in the generation of this broad immune response to the HIV insert. Thus, the BCGpan-Gag vaccine appears, through its combined features of genetic stability, safety in immunocompromised mice and ability to elicit CD8+ and CD4+ T cell responses in heterologous prime boost vaccine regimes, to be a more promising HIV vaccine candidate than BCG-Gag.

**Methods**

**Construction of recombinant BCG (rBCG) expressing Gag and vaccine preparation**

Wild type M. bovis BCG Pasteur1172 P2 (BCG) (supplied by the Statens Seruminstitut, Denmark) and M. bovis BCG mc6000 (BCGpanCD), a pantothenic acid auxotroph strain derived from BCG Pasteur (constructed as per the M. tuberculosis ApanCD mutant [33]), were grown on Middlebrook 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) and 0.5% glycerol (7H10) or in Middlebrook 7H9 broth supplemented with 10% OADC, 0.2% glycerol and 0.025% tyloxapol (7H9) on rollers (4 rpm) at 37°C. Kanamycin (10 μg/ml) was included in the media for plasmid selection where required. Media was supplemented with pantothenate (48 μg/ml) and hygromycin (50 μg/ml) for the growth of BCGpanCD.

The shuttle vector pHS300 expressing HIV-1 Gag was constructed as follows: the full length HIV-1 subtype C gag gene [56] was codon optimised for use in BCG and cloned into the Apol and CiaI restriction sites of plasmid pCB119, thus fusing the gag gene to the nucleotides encoding the M. tuberculosis 19 kDa signal sequence and placing it under the control of the M. tuberculosis mttA

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promoter (Figure 1). Plasmid pH5400 was derived by deletion of the hsp60 promoter and IgsA gene from vector pH5300 (Figure 1).

The shuttle vectors pH5300, pH5400, pRT106 [6] and pCONEPI (vector not containing gag, Genbank accession DQ191753) were introduced into BCG and BCGΔpantCD by standard mycobacterial electroporation procedures to generate rBCG [57]. The rBCG were plated on 7H10 media plus kanamycin (10 μg/ml) with the appropriate supplements and incubated at 37°C for approximately 3 weeks. Vaccine stocks were prepared by selecting recombinants in 5 ml 7H9 media, then inoculating 100 ml 7H9 media in a roller bottle and growing cells until mid-logarithmic phase (∼OD600 0.5). The cultures were then harvested and the pellets resuspended in resuspension medium (0.85% NaCl; 10% glycerol; 0.025% tyloxapol) to give a final OD600 ~10 which is equivalent to a concentration of 1x10^8 cfu/ml. The vaccine stocks were stored at −80°C till required. To confirm in vivo genetic stability plasmid DNA was recovered from vaccine stocks and mapped with restriction enzymes and the HIV-1 gag gene was sequenced. Prior to vaccination the vaccines were defrosted on ice and passed through a 21 gauge syringe needle 10 times in order to disperse clumps just prior to injection.

In vitro and in vivo stability of the rBCG
To assess stability of the rBCG, cultures were passed daily for approximately 30 generations in liquid media with and without antibiotic selection. Aliquots of the culture were frozen at −80°C in 15% glycerol prior to each passage. The number of colony forming units obtained after plating suitable dilutions of the cultures on 7H10 media with and without kanamycin (10 μg/ml) were compared to determine plasmid stability. To assess the genetic stability of the rBCG, plasmid DNA was recovered after 30 generations and mapped with restriction enzymes (Figure 1). To determine in vivo plasmid stability, plasmid DNA was isolated from mycobacterial colonies and mapped with restriction enzymes 6 weeks post vaccination.

Recombinant MVA expressing Gag (MVA-Gag)
MVA-Gag expressing a matching Gag antigen was used as a booster. HIV-1 gag was inserted into the Del III region of the MVA virus genome under the transcriptional control of the modified-H5 promoter [58]. MVA-Gag was grown on the chorioallantoic membranes (CAMs) of 10–12 day old chick embryos and harvested after 72 hours. Titration was performed in BHK-21 cells, using rabbit anti-vaccinia antibody (Biogenesis, Poole, UK) and swine anti-rabbit HRP (Dako, Glostrup, Denmark) to detect MVA; and sheep anti-p24 antibody (Aalto, Dublin, Ireland) and anti-sheep HRP (Dako, Glostrup, Denmark) to detect Gag. Plaques were visualised by the reaction of peroxidase with o-dianisidine (Sigma, St Louis, USA) in the presence of H2O2 and counted to determine the virus titre. Irrespective of the antibody used to detect the plaques identical virus titres were obtained indicating MVA-Gag to be stable.

Vaccinia viruses (VV) and cells
Vaccinia virus expressing a matched HIV-1 Dut22 Gag subtype C antigen, vT369, (VV-Gag), was manufactured by Quality Biological (Gaithersburg, MD, USA) and Therion Biologics, Inc (Cambridge, USA). The New York City Board of Health (NYCBH) strain of vaccinia, Vaccinia NYCBH, was used as a control vaccinia (VV-NYCBH). Both viruses were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. These viruses were amplified on chick chorioallantoic membranes (CAMs) and titrated in CV-1 cells (Highveld Biological, Johannesburg, SA).

rBCG mouse vaccinations and MVA-Gag boost
The vaccination schedule and all the procedures using female BALB/c mice (6–12 weeks old in groups of 5) were approved by the UCT Animal Ethics Committee (reference UCTAEC 01-041) and performed by a trained animal technologist. The BCG vaccine and doses used were either 10^7 cfu or 10^7 cfu given via the intraperitoneal (i.p.) route in 200 μl resuspension medium. For mice that were primed with the rBCG vaccines and then boosted with MVA-Gag, the boost was given at week 5 or 12 after the prime, as an intramuscular (i.m.) vaccination of 10^7 pfu MVA-Gag in 100 μl PBS with 50 μl injected into each quadriceps muscle.

Bacterial burden and histopathology
Bacterial growth was determined weekly from week 2 to week 5 post vaccination (10^7 cfu, i.p.). Spleens from individual mice were collected in resuspension medium (2 ml) and homogenized. Ten-fold serial dilutions of the homogenate were plated on 7H10 agar containing the appropriate supplements in the presence or absence of kanamycin (10 μg/ml). Colonies were counted after incubation at 37°C for approximately 3 weeks.

For histopathology studies livers from individual mice were harvested at weekly intervals after vaccination (10^7 cfu, i.p.) and fixed overnight in 10% phosphate buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin. Micrographs were done with an Olympus microscope.

Preparation of splenocytes for immune assays
Spleens were harvested and pooled from 5 mice per group at weekly intervals after vaccination with the individual BCG vaccines or 12 days after the MVA-Gag boost to determine Gag-specific immune responses. A single cell suspension of splenocytes was prepared then treated with erythrocyte lysing buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA) for 1 min at room temperature before suspension in R10 culture medium (RPMI with 10% heat inactivated fetal calf serum (FCS) containing 15 mM β-mercaptoethanol, 100 U penicillin and 100 μg streptomycin/ml, [Invitrogen, Carlsbad, California, USA]). Splenocyte aliquots were stained with anti-CD3+ APC, anti-CD4+ FITC and anti-CD8+ per CP labeled antibodies (BD Biosciences, The Scientific Group, Johannesburg, SA) to determine the proportion of CD3+ cells and CD4+ and CD8+ subpopulations by flow cytometry.

IFN-γ ELISPOT assay
The Mouse IFN-γ ELISPOT set (BD Pharmingen, The Scientific Group, Johannesburg, SA) was used as per manufacturer’s instructions. Splenocytes were plated at 1x10^5/well in a 200 μl final volume of R10 alone (to determine background response) or medium containing an individual peptide (>95% HPLC pure, Bachem AG, Bubendorf Switzerland) with amino acid sequence matching BALB/c CD8+ HLA-A2 specific, at a concentration of 1μg/ml. The amino acid sequences of the peptides were AMQMLKDTI (GagCD8+ peptide) and NPPFPVGRIYKRWIILGNK (GagCD4+13 peptide) and FRDYVDRFFKTILRAEQATQE (GagCD4+17 peptide) [59–61]. The reaction was stopped after 22 hours incubation at 37°C in 5% CO2, and spots were reacted with the detection antibody then developed with Nova Red as per the kit instructions. Spots were counted and analysed using an automatic ELISPOT reader (CTL technologies, Cleveland, Ohio) and Immunospot.
individual peptides as used in the IFN-γ ELISPOT of peptide (to detect background cytokine release) or with the individual Gag peptide that arose from either a BCG-Gag or BCGpan-Gag prime and a MVA-Gag boost, was considered a positive peptide response arising from the prime-boost regimen if it was ≥1.5 fold the individual Gag peptide response for a prime with the control BCG (BCG-Control or BCGpan-Control) and a MVA-Gag boost. The sum of sfu per 10^6 splenocytes for responses to the individual Gag peptides is referred to as the cumulative Gag peptide response.

Quantification of secreted cytokines

Splenocytes at a concentration of 7.5 x 10^6 per ml R10 culture medium were cultured (48 h at 37°C in 5% CO2) in the absence of peptide (to detect background cytokine release) or with the individual peptides as used in the IFN-γ ELISPOT assay at 4 μg/ml. The cytokine content of culture supernatants was assayed using a cytokine bead array assay (BD Pharmingen, The Scientific Group, Johannesburg, SA) that detected IFN-γ, TNF-α, IL-6 and IL-10. The average of triplicate values was calculated and expressed as pg cytokine per 10^6 splenocytes. The coefficient of variation of the average value (SD of the average expressed as a percentage of the average) was not more than 7%. Cytokine values were considered positive if greater than twice background values (not more than 2 pg per 10^6 splenocytes for all assays) and are reported after background subtraction. Values below the cut off were set to zero. No IL-10 above background levels could be detected in the culture supernatant from peptide-stimulated splenocytes for any of the vaccine regimens. The sum of cytokine values obtained with the individual Gag peptide stimuli (cumulative cytokine response) for a prime with either BCG-Gag or BCGpan-Gag and a MVA-Gag boost were considered to be a positive prime-boost response if ≥1.5 fold the cumulative response for a prime with the control BCG (BCG-Control or BCGpan-Control) and a MVA-Gag boost.

Vaccinia virus challenge

Groups of 10 mice were vaccinated with BCGpan-Gag, BCGpan-Control (2 x 10^6 sfu, i.p.) or the resuspension medium used for the rBCG vaccines on day 0 and day 28. Two weeks later half the mice in each group were challenged with either VV-Gag or VV-NYCBH (1 x 10^6 pfu, i.p.). Five days after the challenge, ovaries were collected and pooled from 5 mice per group into McIlvains buffer. Pooled ovaries were finely chopped and ground in tenbrook grinders, and cell debris pelletted by low speed centrifugation. The virus in the supernatant was titrated in CV-1 cells, using serial 10-fold dilutions. Thirty six hours post infection the cells were stained with Carbol Fuschin and plaques were counted.

Statistical analysis

Results are expressed as mean and standard deviation of the mean. Data was statistically analysed using Student’s t test and p values of <0.05 were considered significant.

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Author Contributions

Conceived and designed the experiments: RC ES HS ND VS IG BR WJ AW. Performed the experiments: RC HS ND VS IG. Analyzed the data: RC ES HS ND VS IG BR WJ AW. Contributed reagents/materials/analysis tools: RC ES HS ND VS IG BR WJ AW. Wrote the paper: RC ES.


