PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	A Phase I study evaluating the safety and immunogenicity of MVA85A, a candidate TB vaccine, in HIV-infected adults
AUTHORS	Angela M Minassian, Rosalind Rowland, Natalie ER Beveridge, Ian D Poulton, Iman Satti, Stephanie Harris, Hazel Poyntz, Matthew Hamill, Kristin Griffiths, Clare R Sander, David R Ambrozak, David A Price, Brenna J Hill, Joseph P Casazza, Daniel C Douek, Richard A Koup, Mario Roederer, Alan Winston, Jonathan Ross, Jackie Sherrard, Guy Rooney, Nicola Williams, Alison M Lawrie Helen A Fletcher, Ansar A Pathan and Helen McShane

VERSION 1 - REVIEW

REVIEWER	Tim Lahey, MD MMSc Assistant Professor Dartmouth Medical School United States of America
REVIEW RETURNED	18/07/2011

THE STUDY	1. Clinical comparability of HIV-negative comparator subjects should be clarified; see main review comments for more detail. 2. ED., please note that as phrased a "Yes" answer to the last question is the red flag, not "No." See main review comments re apparent parallel recruitment of low and high dose cohorts.
RESULTS & CONCLUSIONS	None
REPORTING & ETHICS	None
GENERAL COMMENTS	Minassian et al present the results of the first study of the safety and immunogenicity of a leading TB vaccine candidate, MVA85A, in HIV-infected adults. The report is wellwritten, the complicated data clearly presented to an appropriate level of granularity, and the general findings will inform our understanding of the impact of HIV infection on TB vaccine immunogenicity. Points to consider are listed below: - The phrasing of the last sentence of the results section of the abstract is a little awkward." "Remarkably comparable, although less durable, to…" The penultimate word of the abstract is superfluous. - One of the main findings of this manuscript is that

	MVA85A is safe in HIVinfected adults. However, the safety among HIV-infected adults with lower CD4 counts is not yet established. When the authors clarify this important point, it would be reasonable to mention there is no particular reason *a priori* to expect safety of the vaccine to be altered by progression of HIV disease (but see below). - In the legend for Table 2, the authors note “there were significantly fewer systemic AEs per person, (and a lower frequency of systemic AEs overall) in the 10 HIV-infected subjects….” Could the authors clarify the whether the numerically greater incidence of local AEs among HIV-infected subjects reached statistical significance? If so, this intriguing pattern merits additional discussion. - Where AE’s graded for severity, and could these data be included, perhaps as prevalence of grade I-IV events? - Could the authors clarify what distinction is meant between “fever” and “feverish” in Table 2b? (“Measured fever” and “Subjective fever” might be more clear, if that’s the implication.) - On page 13 of the results section, the authors state that HIV transcript was detectable within CD4+ T cells from only two subjects (1004 and 1029), both post-immunization. This suggests that some proportion of subjects converted from no detectable HIV transcript to some detectable transcript after immunization, although the proportion of subjects from each group demonstrating this conversion is unclear as stated. To which dose group did these two subjects belong? How many copies were detected by qPCR? Given the known phenomenon of HIV-infection of antigen-specific CD4+ T cells, do the authors think additional evaluation of this finding is merited in subsequent or ongoing parallel studies? Would the authors like to characterize the potential implications of the finding that the two subjects with detectable HIV transcript among Ag85- specific CD4+ T cells also exhibited HIV transcript

	among CMV-specific CD4+ T cells? - It would be clearer to punch up the existing caveat in page 18 paragraph 2 to the effect that small effects of MVA85A on HIV infection of CD4+ target cells cannot be excluded given the small sample size and consequently limited study power to detect such effects. (I agree the data suggest there is no dramatic or "widespread" alteration in infection of antigen-specific CD4+ T cells, but potentially clinically significant effects could have been missed, given the fact that during chronic HIV infection only low percentages of CD4+ T cells actively harbor virus to begin with.) - In addition to the current clear characterization of the magnitude of the IFN-© ELISpot responses to peptides, the authors should provide data re responder/non-responder frequencies to allow the reader the ability to evaluate better the statement (page 14, line 20) that "the responder rate was higher in the high dose group with no observed non-responders compared with 1-2 nonresponders (depdending on antigen) in the low dose group." (The difference sounds rather subtle as stated but given the small cohort size a clear consistent 10-20% different in response rates might be relatively impressive.) - Starting on page 14 line 32, the authors compare immunogenicity of MVAAg85 among HIV-infected adults to previously-studied HIV-negative adults. To allow the reader to evaluate the validity of these comparisons, it will be important for the authors to clarify if the sex, age, continent of birth, BCG immunization, and prevalence of latent TB infection were comparable between these groups. - On page 15 line 8, the authors state that post-vaccination ELISpot responses to ESAT-6 and CFP-10 were unchanged among the four subjects with LTBI. Did the presence of LTBI impact likelihood or magnitude of Ag85-specific responses? (Figure 3 suggests not but best to be explicit.)

	- Page 17, first paragraph: The message of this paragraph would be clearer without mention of the ongoing similar studies elsewhere, although as above, should signals from this study merit further investigation then in the context of that discussion the availability of additional similar studies might be important to mention. - Page 17, line 53: These newly mentioned data regarding the single subject in each dose group that evinced a more than 0.5 log HIV viral load increase after vaccination begs the question of whether those subjects are the same as those (1004, 1029) with newly detectable HIV transcript among AG85-specific CD4+ T cells. (I'm not sure if the three digit numbers mentioned as study numbers from the same schema as the previously mentioned four digit numbers, especially given the lack of mention of four digit numbers in the key for Figure 2.) - It appears in Figure 1 that recruitment occurred separately for the low and high dose populations – please clarify. "CONSORT" should be capitalized and "diagram" might be clearer than "flowcharts." - In the legend for Figure 4, page 26 line 51 there's a space missing between the fifth and sixth words. - In Figure 5c & 5d, the qualitative difference between memory pool and AG85-specific CD4+ T cell CCR5 expression seems to hinge on whether MFI or iMFI is used. Could the authors hypothesize why? (I think this implies an effect on bystander memory T cell expression of CCR5 but I'm curious what the authors think.) I encourage the authors to devote more of the discussion section to interpretation of the findings depicted in this figure. - I am unclear what the supplemental figure adds to the manuscript.

REVIEWER	James Lewis Lecturer London School of Hygiene and Tropical Medicine UK
REVIEW RETURNED	08/07/2011

THE STUDY	None
RESULTS & CONCLUSIONS	None
REPORTING & ETHICS	None
GENERAL COMMENTS	The comparisons to HIV-uninfected subjects is clearly very important, but I would appreciate some details in this paper on who these people were and how they were selected, rather than just references to the other papers. Results para 7, starts "Ag85A-specific T cell responses..." refers to baseline responses in Table 3b (sentence 3), yet these were not in Table 3b.

REVIEWER	Andrew Nunn Associate Director, MRc Clinical Trails Unit London, UK
REVIEW RETURNED	30/06/2011

THE STUDY	There is considerable detail concerning the laboratory methods but other important details are missing. Examples of this are 1) the method of randomisation and the extent to which it was concealed, 2) the source of the HIV-negative population data and the comparability of methods used in acquiring it (both laboratory and adverse events), 3) limited information on statistical methods, e.g. page 10 line 58 No mention here of the method used to compare the areas that are calculated – footnote to Table 3b suggests Mann Whitney U test is used. It is not clear what basline refers to (page 11) in view of the variable length of time baseline data are available (page 12). what is baseline here? The description of the repeated measures analysis needs more detail, not clear to reader how to repeat this. Page 13 How has CI for the median been calculated? Bootstrap? What does a 95% CI tell us here? Are they testing whether the median number is different from zero or some other number? Page 13, line 25 Which arms are these 12 subjects from? Page 13, is it really necessary to list patient numbers? Comparisons appear to have been made within treatment arms or with the HIV negatives but not between arms.
RESULTS & CONCLUSIONS	I don'y feel fully qualified to comment on all the above. The comments relate to the presentation/interpretation of the data. There is a tendency towards over interpretation, eg Page 13 line 55 Over interpretation. Technically a (statistically) significant increase did remain but there is a clear trend back towards baseline over time which hasn't been commented on. Furthermore the authors shouldn't interpret a p-value of 0.032 so strongly considering the amount of testing that has been done. Page 13 line 60, "This response was maintained until 24 weeks ..." is wrong. The change, compared to baseline, at week 1 is 502 and

	at week 24 it is 14. Clearly the week 1 change hasn't been maintained! Page 14, line 10 Again, this is only interpreted in terms of the p-values and no consideration has been given to the point estimates! Page 14, line 13-20 Wrong interpretation. AUC analysis does not test for differences "at any time point", but rather for a difference over all time. Page 14, line 32-37 Unless those in HIV-uninfected group are followed-up at similar time points comparing AUCs should not be done. Furthermore there are 43 HIV-negatives on the low dose yet only 20 of these are used in the analysis in Table 3b. No explanation of why 23 were dropped PAge 14, line 37-41 "This difference" is confusing. It sounds like AUC analysis has been used to compare values at individual time points. Page 14, line 42-44 Not sure what they are referring to when they say "baseline response". This is not in Table 3b Page 14, line 51 Seems authprs have used AUC analysis for individual time points again. In all of the analyses in this paragraph and Table 3b they do not consider baseline values in the two groups. Page 14, line 55 Interpreted R=0.04 as a weak positive correlation, very odd. Figure 2 Difficult to read. What points in time do the "Visits" (x-axis) pre-vaccination represent? Are they the same for all patients? Not sure the figures tell us anything useful.
REPORTING & ETHICS	Not fully in line - as indicated above there is, for example, no infrmation on the randomisation procedure. MHRA and GTAC approval - not clear if ethical approval obtained also.
GENERAL COMMENTS	If this article is to be accepted it needs to undergo a major revision. My comments are limited since much of the methodology is new to me so I'm not necessarily best placed to re-review it.

VERSION 1 – AUTHOR RESPONSE

Reviewer 1: Andrew Nunn

THE STUDY
There is considerable detail concerning the laboratory methods but other important details are missing. Examples of this are
1) the method of randomisation and the extent to which it was concealed,
This study is a Phase I safety and immunogenicity study (title of trial on protocol: A Phase I study evaluating the safety and immunogenicity of a new TB vaccine, MVA85A, in healthy volunteers who are infected with HIV) and as such there was no placebo control group and no randomisation. Subjects were sequentially allocated first to the low dose group and then once safety had been

demonstrated, to the high dose group (see protocol p16). We have now added this sentence into the methods section.

2) the source of the HIV-negative population data and the comparability of methods used in acquiring it (both laboratory and adverse events),
The HIV-negative population data is data from a previous Phase I clinical trial with this vaccine in BCG vaccinated, HIV-uninfected UK healthy subjects. The low dose data used for comparison has been previously published (McShane H et al, NM 2004), and the high dose data has also been published (Beveridge N et al, Tuberculosis, 2008). We apologise for not having referenced these previous publications to make clear where the comparison data has come from and have amended the manuscript accordingly and inserted these references into the statistical methods section and the results where we refer to the comparison with these trials.
In the statistics section we say that comparisons between this trial and previous trials of MVA85A, and between low and high dose groups were conducted using the Mann-Whitney U test (Stata). We have not conducted formal statistical analysis on the adverse event data as we believe it would not be appropriate given the small numbers of subjects involved and the many different outcome measures. We have removed the sentence 'There were significantly fewer systemic AEs per person, (and a lower frequency of systemic AEs overall,) in the 10 HIV-infected subjects receiving high dose MVA85A compared with HIV-uninfected subjects receiving the same dose of vaccine (p=0.026, data not shown and Pathan et al, unpublished)' from the legend of Table 2 as although using a Mann-Whitney test there were some significant differences, we accept the numbers are very small and it is more appropriate to just show the descriptive data, as per the protocol.

3) limited information on statistical methods,
We believe we have outlined the statistical test used and the programme for all the comparisons conducted in this paper in the M&M section, but if there are specific omissions then we would be very happy to correct them.

e.g. page 10 line 58 No mention here of the method used to compare the areas that are calculated – footnote to Table 3b suggests Mann Whitney U test is used.
We have added some text in the statistics method section to clarify this.

It is not clear what basline refers to (page 11) in view of the variable length of time baseline data are available (page 12). what is baseline here?
Baseline here means pre-vaccination and we have substituted pre-vaccination for baseline here on p11 for clarity.

The description of the repeated measures analysis needs more detail, not clear to reader how to repeat this.
On review of this analysis, we agree that the numbers are too small for a meaningful statistical analysis, and have amended the text to say that there were no clinically significant effects of vaccination on CD4 count or HIV RNA load, a conclusion we feel is more clinically relevant and appropriate given the sample size in this first Phase I study.

Page 13 How has CI for the median been calculated? Bootstrap? What does a 95% CI tell us here? Are they testing whether the median number is different from zero or some other number?
Median for each group was reported with the corresponding range (i.e. min, max). However, the confidence intervals of median difference reported in this manuscript were derived using robust method suggested by Newson. We have clarified this in the Statistical Analysis section and added this reference.

Page 13, line 25 Which arms are these 12 subjects from?
Now 11 subjects (see response to reviewer 3 below). 3/11 from low dose and 8/11 from high dose. The one subject with detectable HIV transcript received high dose. We have clarified this in the text.

Page 13, is it really necessary to list patient numbers?
We agree and have removed them.

Comparisons appear to have been made within treatment arms or with the HIV negatives but not between arms.
We have compared pre and post vaccination responses within dose arms, and between dose arms. We say in the results, on p14, that 'There were no significant differences in the magnitude of the IFN-γ response between low and high dose groups at any time-point ($p=0.29$ and $p=0.68$ for summed and single peptide pools, respectively; AUC analysis, data not shown).' We have also compared with the HIV-negative data as in our response above.

RESULTS AND CONCLUSIONS
I don'y feel fully qualified to comment on all the above. The comments relate to the presentation/interpretation of the data.

There is a tendency towards over interpretation, eg Page 13 line 55 Over interpretation. Technically a (statistically) significant increase did remain but there is a clear trend back towards baseline over time which hasn't been commented on. Furthermore the authors shouldn't interpret a p-value of 0.032 so strongly considering the amount of testing that has been done.
The induction of an antigen specific cellular immune response in the trial reported here demonstrates a text-book immune response – with a peak circulating effector response early after vaccination, which contracts to a central memory response (as expected in the absence of persistent antigen). The immune response once contracted at the later time points remains significantly higher than baseline (pre-vaccination). This is exactly the same kinetic as we have reported in all our previous trials and is exactly what we expect to see (see McShane et al NM 2004, Pathan et al PLoS ONE 2005, Hawkridge et al, 2008 etc).

Page 13 line 60, "This response was maintained until 24 weeks ..." is wrong. The change, compared to baseline, at week 1 is 502 and at week 24 it is 14. Clearly the week 1 change hasn't been maintained!
See response above. A persistent immune response, above that of baseline (pre-vaccination) levels has been maintained. Immunologically, one would not expect this to remain at the peak effector response level (and indeed would not wish it to remain so high).

Page 14, line 10 Again, this is only interpreted in terms of the p-values and no consideration has been given to the point estimates!
See above response – the immune response remains significantly higher than pre-vaccination, hence the comment. We agree that it is important not to just consider the p value, but for this type of immunological analysis, a plateau immune response which remains significantly higher than baseline is considered relevant and important, as it indicates a persistent immune response which may be protective. The clinical significance of these results can only be determined in an efficacy trial (which is now ongoing in this HIV-infected population).

Page 14, line 13-20 Wrong interpretation. AUC analysis does not test for differences "at any time point", but rather for a difference over all time.
We agree with this comment and that is what we have done in this analysis, ie to perform the AUC comparison over the whole follow up period. We have then conducted a separate analysis comparing both between and within the groups at week 1 (peak) and then at week 24 (plateau). Comparisons between groups at specific time points were made using a Mann-Whitney U test and comparisons within a group at different time points made using a Wilcoxon signed rank test as indicated in the statistical methods section. We have amended the text on Page 14 to clarify this point accordingly.

Page 14, line 32-37 Unless those in HIV-uninfected group are followed-up at similar time points comparing AUCs should not be done.

The HIV-uninfected cohort are followed up at identical time points (see McShane et al, NM 2004, Beveridge et al, 2008) and we apologise for omitting those references which we have now inserted.

Furthermore there are 43 HIV-negatives on the low dose yet only 20 of these are used in the analysis in Table 3b. No explanation of why 23 were dropped
There were 12 HIV-negative subjects in the high dose comparison (see Beveridge et al, 2008) and 21 HIV-negative subjects in the low dose comparison (see McShane et al, NM 2004). In the low dose comparison there was only 24 week data for 20/21 subjects, hence only 20 in the AUC analysis and in the 24 week comparison.

PAge 14, line 37-41 "This difference" is confusing. It sounds like AUC analysis has been used to compare values at individual time points.
We believe the statistics section makes it clear that we have used the Mann-Whitney U test for all comparisons between groups at specific time points, but have added the following for clarity to this section:
Comparisons between specific time points in this trial and previous trials of MVA85A, and between low and high dose groups were conducted using the Mann-Whitney U test (Stata).

Page 14, line 42-44 Not sure what they are referring to when they say "baseline response". This is not in Table 3b
We have inserted pre-vaccination in parenthesis after the baseline for clarity.

Page 14, line 51 Seems authprs have used AUC analysis for individual time points again.
In all of the analyses in this paragraph and Table 3b they do not consider baseline values in the two groups.
See response above re statistics. There was no difference in baseline responses between the HIV-infected and HIV-uninfected groups and this is stated in the manuscript. We think it is clear that we have conducted Wilcoxon signed rank test for comparisons within groups at different time points and Mann-Whitney U for comparisons between groups at the same time point. For clarity we have added MWU analysis before each relevant p value in the text.

Page 14, line 55 Interpreted R=0.04 as a weak positive correlation, very odd.
We agree and have amended this to say 'There was no significant correlation between the CD4 count at screening and the peak summed 85A peptide pool response (R=0.04, p=0.09), nor between HIV RNA load at screening and the peak immune response (R= -0.04, p=0.08).

Figure 2 Difficult to read. What points in time do the "Visits" (x-axis) pre-vaccination represent? Are they the same for all patients? Not sure the figures tell us anything useful.
The pre-vaccination CD4 and VL data are not the same time points for all subjects but represent for each subject a series of values over the preceding few months-year leading up to vaccination. We believe this is important data to demonstrate the pre-vaccination variability in these parameters, in order to interpret the post-vaccination variability for each subject – hence including it in this figure.

REPORTING AND ETHICS
As indicated above there is, for example, no information on the randomisation procedure.
See above response. This study is a Phase I safety and immunogenicity study (title of trial on protocol: A Phase I study evaluating the safety and immunogenicity of a new TB vaccine, MVA85A, in healthy volunteers who are infected with HIV) and as such there was no placebo control group and no randomisation. Subjects were sequentially allocated first to the low dose group and then once safety had been demonstrated, to the high dose group (see protocol p16).

MHRA and GTAC approval - not clear if ethical approval obtained also.
The Gene Therapy Advisory Committee (who had until recently a remit for reviewing all clinical

trials with GMOs) are an ethics committee. So ethical approval was granted from GTAC. We have made this clearer in the text.

COMMENTS
If this article is to be accepted it needs to undergo a major revision.
My comments are limited since much of the methodology is new to me so I'm not necessarily best placed to re-review it.
We are grateful to this reviewer for his detailed review of the manuscript and believe we have addressed this reviewers concerns and queries above and in the text where appropriate.

Reviewer 2: James Lewis
The comparisons to HIV-uninfected subjects is clearly very important, but I would appreciate some details in this paper on who these people were and how they were selected, rather than just references to the other papers.
See response above. The HIV-negative population data is data from a previous Phase I clinical trial with this vaccine in BCG vaccinated, HIV-uninfected UK healthy subjects. The low dose data used for comparison has been previously published (McShane H et al, NM 2004), and the high dose data has also been published (Beveridge N et al, Tuberculosis, 2008). We apologise for not having referenced these previous publications to make clear where the comparison data has come from and have amended the manuscript accordingly and inserted these references into the results where we refer to the comparison with these trials.

Results para 7, starts "Ag85A-specific T cell responses..." refers to baseline responses in Table 3b (sentence 3), yet these were not in Table 3b.
We apologise for this error – the data is shown in Figure 3e and the reference has been amended in the revised manuscript.

Reviewer 3: Tim Lahey, MD MMSc
1. Clinical comparability of HIV-negative comparator subjects should be clarified; see main review comments for more detail. See attached file:

Minassian et al present the results of the first study of the safety and immunogenicity of a leading TB vaccine candidate, MVA85A, in HIV-infected adults. The report is wellwritten, the complicated data clearly presented to an appropriate level of granularity, and the general findings will inform our understanding of the impact of HIV infection on TB vaccine immunogenicity.
Points to consider are listed below:
- The phrasing of the last sentence of the results section of the abstract is a little awkward." "Remarkably comparable, although less durable, to…"
The wording is that the functional quality of the vaccine-induced T cell response in HIV-infected subjects was remarkably comparable (which it was) but it was also less durable – hence the phrasing.

The penultimate word of the abstract is superfluous.
We agree and have deleted therefore
- One of the main findings of this manuscript is that MVA85A is safe in HIV infected adults. However, the safety among HIV-infected adults with lower CD4 counts is not yet established. When the authors clarify this important point, it would be reasonable to mention there is no particular reason a priori to expect safety of the vaccine to be altered by progression of HIV disease (but see below).
We agree this is an important point and have inserted the following sentence into the discussion: 'Whilst we have not evaluated the safety of this vaccine in HIV-infected subjects with lower CD4 counts, we would not expect the safety profile of this vaccine to be altered by progression of HIV disease'.

- In the legend for Table 2, the authors note "there were significantly fewer systemic AEs per

person, (and a lower frequency of systemic AEs overall) in the 10 HIV-infected subjects….” Could the authors clarify the whether the numerically greater incidence of local AEs among HIV-infected subjects reached statistical significance? If so, this intriguing pattern merits additional discussion.
On review of this point after reviewer 1’s comment, we feel that although there were some statistically significant differences between HIV-infected and uninfected subjects, the numbers are very small and we have removed this comment and just left the numbers in Table 2 for interpretation.

- Where AE’s graded for severity, and could these data be included, perhaps as prevalence of grade I-IV events?
There were no severe adverse events, and only one moderate systemic AE. All other systemic AEs were mild. Local AEs were predominantly mild in keeping with previous trial experience with this vaccine. We have added 2 sentences into the text to detail this but feel that a further table with this data would not add much to the paper.

- Could the authors clarify what distinction is meant between “fever” and “feverish” in Table 2b? (“Measured fever” and “Subjective fever” might be more clear, if that’s the implication.)
Measured fever and subjective fever are exactly what we mean and have amended the text for clarity.

- On page 13 of the results section, the authors state that HIV transcript was detectable within CD4+ T cells from only two subjects (1004 and 1029), both post-immunization. This suggests that some proportion of subjects converted from no detectable HIV transcript to some detectable transcript after immunization, although the proportion of subjects from each group demonstrating this conversion is unclear as stated. To which dose group did these two subjects belong? How many copies were detected by qPCR? Given the known phenomenon of HIV-infection of antigen-specific CD4+ T cells, do the authors think additional evaluation of this finding is merited in subsequent or ongoing parallel studies? Would the authors like to characterize the potential implications of the finding that the two subjects with detectable HIV transcript among Ag85-specific CD4+ T cells also exhibited HIV transcript among CMV-specific CD4+ T cells?

We agree with the reviewer, and we choose to investigate this precisely because of the known phenomenon of HIV-infection of antigen-specific CD4+ T cells. Both subjects received high-dose MVA85A vaccination. We have amended the manuscript to make this clear. The qPCR assay used is very sensitive and we expect low copy numbers when performed with a small number of cells (due to clinical sample restrictions). The median number of cells in qPCR reactions was 73. For subject 1029 copy numbers were 0.0, 0.0, 2.3 = 0.767 mean in 173 cells at week 2 post-vaccination, and 2.4, 1.4, 0.0 = 1.27 mean in 125 cells at week 8 post-vaccination. Historically the data have been presented as gag copies per 100,000 cells, although multiplying data will multiply error rates. These data correspond to infection rates of 0.4% at week 2 and 1.0% at week 8 in subject 1029 and are within the expected range. We have withdrawn all qPCR data from subject 1004 (Ag85A and CMV), as on reviewing the data with the NIH group, it is clear that these data contain an outlying triplicate and very low cell numbers. Ideally we would like to perform additional replicates but there are no further clinical samples. We believe therefore that this is a misleading result and have removed this subject’s data from the analysis.
The reviewer is correct - the data suggest conversion from no detectable transcript to detectable transcript in two out of 12 subjects (3/32 samples). We have amended this to one out of 11 subjects (2/29 samples) (see above). Based on current understanding of the behaviour of HIV we assume the Ag85A-specific cells were infected de novo following vaccination-induced activation and proliferation. We agree with the reviewer that it would be appropriate to investigate this further. However, we are confident the clinical parameters, cytokine, chemokine and chemokine receptor expression data measured in this study do not support widespread immune activation and preferential infection by HIV.
The amended manuscript results text now reads as follows:
Of 29 Ag85A-specific CD4+ T cell samples in total (11 subjects), only one subject showed a

positive signal for HIV gag DNA by qPCR at two different post-vaccination timepoints (weeks 2 and 8 post-vaccination; data not shown). In the 11 subjects tested, resting HIV-specific and CMV-specific memory cell populations showed a positive signal in all assays (data not shown).

The amended manuscript discussion now reads as follows:

HIV preferentially infects memory CD4+ T cells 23, 24, in particular HIV-specific memory CD4+ T cells, 20 and other activated antigen-specific CD4+ T cells 25. Using a sensitive qPCR method, we detected HIV gag DNA in Ag85A-specific CD4+ T cells from only 1/11 subjects post-vaccination. This subject received high-dose MVA85A vaccination. This low positivity rate concurs with the stable CD4 count and HIV RNA load parameters in most subjects throughout the trial. Although the qPCR assay is sensitive, the low yield of Ag85A-specific CD4+ T cells entering the assay is an important limitation and provides just a snapshot of the HIV burden within the Ag85A-specific CD4+ T cell pool and warrants further future investigation. However, whilst small effects of MVA85A on HIV infection of CD4+ target cells cannot be excluded given the small sample size, these data suggest that MVA85A vaccination of healthy HIV-infected individuals does not lead to widespread preferential infection and depletion of vaccine-induced CD4+ T cell populations in the periphery. These data are supported by no change in surface expression of the HIV co-receptor CCR5 following MVA85A vaccination. In addition, the lack of effect of vaccination on chemokine and cytokine levels in unstimulated serum supports the interpretation that vaccination with MVA85A did not lead to widespread immune activation in this subject group.

Re the comment on CMV samples, all subjects exhibited detectable HIV transcript in the CMV samples – the CMV was a positive control. We are not therefore clear what the reviewer means by this comment.
- It would be clearer to punch up the existing caveat in page 18 paragraph 2 to the effect that small effects of MVA85A on HIV infection of CD4+ target cells cannot be excluded given the small sample size and consequently limited study power to detect such effects. (I agree the data suggest there is no dramatic or "widespread" alteration in infection of antigen-specific CD4+ T cells, but potentially clinically significant effects could have been missed, given the fact that during chronic HIV infection only low percentages of CD4+ T cells actively harborvirus to begin with.)
We agree and have amended the sentence so it now reads:
'However, whilst small effects of MVA85A on HIV infection of CD4+ target cells cannot be excluded given the small sample size, these data suggest that MVA85A vaccination of healthy HIV-infected individuals does not lead to widespread preferential infection and depletion of vaccine-induced CD4+ T cell populations in the periphery.'

In addition to the current clear characterization of the magnitude of the IFN-γ ELISpot responses to peptides, the authors should provide data re responder/non-responder frequencies to allow the reader the ability to evaluate better the statement (page 14, line 20) that "the responder rate was higher in the high dose group with no observed non-responders compared with 1-2 nonresponders
(depdending on antigen) in the low dose group." (The difference sounds rather subtle as stated but given the small cohort size a clear consistent 10-20% different in response rates might be relatively impressive.)
We prefer to show all the raw immunology data rather than try to define responder and non-responder statistically, given that we do not know the relationship between this immune response and protective efficacy. Non-responders here are therefore simply defined as the subjects who's responses did not increase at all after vaccination. We think this data is seen in Figure 3 (a-d), but we have added the following phrase after non-responders for clarity:
'ie those subjects with no measurable vaccine induced immune response'

- Starting on page 14 line 32, the authors compare immunogenicity of MVAAg85 among HIV-infected adults to previously-studied HIV-negative adults. To allow the reader to evaluate the validity of these comparisons, it will be important for the authors to clarify if the sex, age,

continent of birth, BCG immunization, and prevalence of latent TB infection were comparable between these groups.
See responses to reviewers 1 and 2 above. We have added in the published references for these 2 groups, where the demographic data has been reported. We have also previously published showing that immune responses between BCG vaccinated and latently infected subjects are not different (Sander et al, AJRCCM 2009), and we do not believe that the presence of LTBI in some of these subjects is responsible for the differences in their immune response durability.

- On page 15 line 8, the authors state that post-vaccination ELISpot responses to ESAT-6 and CFP-10 were unchanged among the four subjects with LTBI. Did the presence of LTBI impact likelihood or magnitude of Ag85-specific responses? (Figure 3 suggests not but best to be explicit.)
Our previously published work (Sander et al, AJRCCM 2009) has demonstrated that the presence of LTBI does not influence magnitude of Ag-85A responses post-vaccination. In this study, as there were only 2 and 3 subjects in the low and high dose groups respectively we did not consider it appropriate to do this analysis here.

- Page 17, first paragraph: The message of this paragraph would be clearer without mention of the ongoing similar studies elsewhere, although as above, should signals from this study merit further investigation then in the context of that discussion the availability of additional similar studies might be important to mention.
We think the reference to the ongoing studies in South Africa and Senegal are important and these ongoing trials have resulted directly from the data in this trial. If the editor prefers, we can remove this reference.

- Page 17, line 53: These newly mentioned data regarding the single subject in each dose group that evinced a more than 0.5 log HIV viral load increase after vaccination begs the question of whether those subjects are the same as those (1004, 1029) with newly detectable HIV transcript among AG85-specific CD4+ T cells. (I'm not sure if the three digit numbers mentioned as study numbers from the same schema as the previously mentioned four digit numbers, especially given the lack of mention of four digit numbers in the key for Figure 2.)
We agree and have taken out subject numbers as requested by reviewer 1.

- It appears in Figure 1 that recruitment occurred separately for the low and high dose populations – please clarify. "CONSORT" should be capitalized and "diagram" might be clearer than "flowcharts."
Recruitment was sequential into low and then high dose groups as specified in the protocol. Under procedures we say: 'Participants were vaccinated intradermally with either 5x107 plaque-forming units (pfu; first group of 10 subjects) or 1x108 pfu (second group of 10 subjects).' Which we believe makes this clear. We have written consort at the beginning of the results and in legend to Figure 1 in capitals and have renamed Figure 1 CONSORT diagram.

- In the legend for Figure 4, page 26 line 51 there's a space missing between the fifth and sixth words.
We cannot find this but are very happy for the editor to correct if he can.

- In Figure 5c & 5d, the qualitative difference between memory pool and AG85-specific CD4+ T cell CCR5 expression seems to hinge on whether MFI or iMFI is used. Could the authors hypothesize why? (I think this implies an effect on bystander memory T cell expression of CCR5 but I'm curious what the authors think.) I encourage the authors to devote more of the discussion section to interpretation of the findings depicted in this figure.
MFI (median fluorescence intensity) is conventionally used to display flow data and describes the 'brightness' or fluoresence intensity of the fluorochrome signal from the cells, but it gives no information about the number of cells at the brightness. Integrated MFI (iMFI) is calculated using both signal (fluorescence) intensity and the number of cells and is a function of both parameters. So, in fig 5c using MFI we see ag85a-specific cells express highest levels of CCR5, in keeping with

them being the most active antigen-specific cells. However when you take into account numbers of cells using iMFI in fig5d you see highest levels of CCR5 expression in the memory pool subset, simply because there are many more cells in that subset. Same for naïve subset.
We have added the following sentence to the discussion "These data are supported by no change in surface expression of the HIV co-receptor CCR5 following MVA85A vaccination."
I have amended the results text to "Using the integrated MFI (iMFI) function calculated using both MFI and cell frequencies, expression of CCR5 was highest in the much larger memory CD4+ T cell pool"

- I am unclear what the supplemental figure adds to the manuscript.
We think it is worth including this to show the data on CMV showing there is no bystander activation after MVA85A vaccination is important and as it is a supplemental figure do not think it is necessary to cut this, but can do so if the editor prefers.

VERSION 2 - REVIEW

REVIEWER	Andrew Nunn Senior statistician MRC Clinical Trials Unit London, UK
REVIEW RETURNED	13/09/2011

THE STUDY	None
RESULTS & CONCLUSIONS	I have made some further suggestions as to how the presentation could be improved. It is much better than before.
REPORTING & ETHICS	None
GENERAL COMMENTS	I don't need to see the manuscript again but have a few further suggestions. The reference to sequentially allocating patients to low and then high dose is at first somewhat confusing and could give the impression that the patienst received both doses. It would help to reword this to say 'subsequent patients' or a similar form of words. It would be useful if the non-HIV studies were described in more detail, perhaps in their own section in Methods. The authors say that baseline refers to pre-vaccination but how long before vaccination? They have data up to 6 years prior to vaccination. This is still unclear. We initially commented that the description of the repeated measures analysis needed more detail, since it was not clear to reader how to repeat this. On review of this analysis, the authors agreed that the numbers were too small for a meaningful statistical analysis and amended the text to say that there were no clinically significant effects of vaccination on CD4 count or HIV RNA load, a conclusion they considered more clinically relevant and appropriate given the sample size in this first Phase I study. Concluding that there are no clinically significant effects suggests that an analysis has been performed in which case it should be described. The authors say that the confidence intervals of median difference reported in this manuscript were derived using robust method suggested by Newson. Howevre, in table 3a median difference is reported with range rather than CI. In this respect tables 3a and

	3b appear to be inconsistent. Page 14 line 47 could imply that the same level of response was maintained which it was not. It is not always clear that a response is being distinguished from a level of response. As already mentioned more information on the HIV-uninfected cohort would be helpful. Table 2 which still states that there are 43 HIV –ve subjects in low dose group which is I think incorrect. The authors state that they believe it is important data to demonstrate the pre-vaccination variability in these parameters in Fig 2, in order to interpret the post-vaccination variability for each subject – hence including it in the figure. It might be easier to just calculate the variance pre and post vaccination.

REVIEWER	Tim Lahey, MD MMSc Dartmouth Medical School United States
REVIEW RETURNED	06/09/2011

THE STUDY	Please note that a "No" answer to the last question signals no problem, so you might want to rephrase the heading above.
RESULTS & CONCLUSIONS	None
REPORTING & ETHICS	None
GENERAL COMMENTS	Overall the authors' revisions are on point and adequately address the reviewers' concerns. Given the importance of the original data, and the general clarity of the manuscript, I think the manuscript is acceptable as is. To clarify a few points, however, in case they are helpful during the preparation of the final version: 1. The last sentence of results section of the abstract is still a bit awkward, although the authors' intent is clear. It would be superior as "The functional quality of the vaccine-induced T cell response in HIV-infected subjects was remarkably comparable to that observed in healthy HIV-uninfected controls, but less durable." This approach avoids the current version's embedded and ungrammatical construction 'less durable to that observed in healthy HIV-uninfected controls.' 2. The authors' discussion of the import of qPCR detection of transcript among Ag85-specific T cells is good. In my original review of the manuscript, I had the impression HIV transcript was detected in CMV-specific T cells "only" in three subjects (numbered 1004, 1029 and 1035 in the sentence now redacted near the bottom of page 14) at the pre-vaccination time point, which included the two (now one) subject with detectable HIV transcript within Ag85-specific T cells. This made me hypothesize that those two subjects had more circulating HIV available to infect any antigen-specific T cells in a general fashion that's independent of the vaccine… Now the authors state that all subjects had detectable HIV transcript among CMV-specific T cells at baseline but that this was the case in those three subjects at baseline "only," and therefore this hypothesis is less tenable. In this newly understood usage of the word "only" here, it makes me wonder if the sole subject now cited as having detectable HIV

	transcript had a negative result in what the authors call a positive control condition at the post-vaccination time point, in which case it's a judgment call whether that HIV transcript data still merits inclusion. I think it's interesting enough to include, since of course one would not expect 100% of antigen-specific cells to be infected. 3. First paragraph of Discussion, page 18. If the authors wish to include mention of the South African and Senegalese trials that resulted from this one, this is fine with me and not a deal-breaker. It might be clearer to cite the results of the current study first, and then articulate that these promising results led to the conduct of other trials. Thus, the paragraph would read "This is the first Phase I trial of a vectored TB vaccine in HIV-infected individuals. The two main findings of this study are…. As a result of these promising findings, similar studies in SA and Senegal are ongoing (refs)." But, this is a stylistic consideration I'll let the authors and editors hammer out. 4. Regarding CCR5 expression in Figures 5c and 5d, by MFI the Ag85-specific T cells appear to express more CCR5 whereas by the (more informative) iMFI measurement it's clear that AG85-specific T cells express less CCR5 compared to most other memory T cells. To clarify, this means that the bystander memory T cells and not the minority population of AG85-specific T cells are particularly juicy targets for HIV infection, which might be interesting in light of the qPCR results discussion. The authors can decide if, after that clarification, they'd like to include mention of this in their final discussion, since either way their findings are interesting and commendable.

VERSION 2 – AUTHOR RESPONSE

Reviewer: Tim Lahey, MD MMSc
Dartmouth Medical School
United States
No competing interests.

Overall the authors' revisions are on point and adequately address the reviewers' concerns. Given the importance of the original data, and the general clarity of the manuscript, I think the manuscript is acceptable as is. To clarify a few points, however, in case they are helpful during the preparation of the final version:

1. The last sentence of results section of the abstract is still a bit awkward, although the authors' intent is clear. It would be superior as "The functional quality of the vaccine-induced T cell response in HIV-infected subjects was remarkably comparable to that observed in healthy HIV-uninfected controls, but less durable." This approach avoids the current version's embedded and ungrammatical construction 'less durable to that observed in healthy HIV-uninfected controls.'

We agree and have reworded accordingly.

2. The authors' discussion of the import of qPCR detection of transcript among Ag85-specific T cells is good. In my original review of the manuscript, I had the impression HIV transcript was detected in CMV-specific T cells "only" in three subjects (numbered 1004, 1029 and 1035 in the sentence now redacted near the bottom of page 14) at the pre-vaccination time point, which included the two (now one) subject with detectable HIV transcript within Ag85-specific T cells. This made me hypothesize that those two subjects had more circulating HIV available to infect any

antigen-specific T cells in a general fashion that's independent of the vaccine… Now the authors state that all subjects had detectable HIV transcript among CMV-specific T cells at baseline but that this was the case in those three subjects at baseline "only," and therefore this hypothesis is less tenable. In this newly understood usage of the word "only" here, it makes me wonder if the sole subject now cited as having detectable HIV transcript had a negative result in what the authors call a positive control condition at the post-vaccination time point, in which case it's a judgment call whether that HIV transcript data still merits inclusion. I think it's interesting enough to include, since of course one would not expect 100% of antigen-specific cells to be infected.

We looked at 29 antigen 85A specific samples – from 11 subjects – and only 1 was positive for HIV. All CMV-positive samples examined were positive for HIV – which served as our positive control. This data supports our assertion that there is no evidence for preferential infection of antigen 85A expanded CD4 T cells and agree that the data merits inclusion and discussion.

3. First paragraph of Discussion, page 18. If the authors wish to include mention of the South African and Senegalese trials that resulted from this one, this is fine with me and not a deal-breaker. It might be clearer to cite the results of the current study first, and then articulate that these promising results led to the conduct of other trials. Thus, the paragraph would read "This is the first Phase I trial of a vectored TB vaccine in HIV-infected individuals. The two main findings of this study are…. As a result of these promising findings, similar studies in SA and Senegal are ongoing (refs)." But, this is a stylistic consideration I'll let the authors and editors hammer out.

We agree and have restructured the opening paragraph of the discussion accordingly.

4. Regarding CCR5 expression in Figures 5c and 5d, by MFI the Ag85-specific T cells appear to express more CCR5 whereas by the (more informative) iMFI measurement it's clear that AG85-specific T cells express less CCR5 compared to most other memory T cells. To clarify, this means that the bystander memory T cells and not the minority population of AG85-specific T cells are particularly juicy targets for HIV infection, which might be interesting in light of the qPCR results discussion. The authors can decide if, after that clarification, they'd like to include mention of this in their final discussion, since either way their findings are interesting and commendable.

The reviewer does raise an interesting point regarding which population is more susceptible. However we think the sample size we have here is small, both in terms of subjects and cell numbers analysed. We do not wish to over-extrapolate from the data until we have a larger dataset. There is clear evidence from others in the field that activated antigen specific cells are preferentially infected by HIV. Within the 'memory pool' population there will be activated antigen specific cells, and clearly there are many more cells in this compared to the Ag85a group so there may well be higher rates of infection on an absolute basis. However we do think this is speculating beyond what is reasonable on our data and would rather leave the interpretation as is in the discussion. We would need to analyse millions more Ag85a cells to make a direct comparison.

Reviewer: Andrew Nunn
Senior statistician
MRC Clinical Trials Unit
London, UK

I have no conflicts of interest

I don't need to see the manuscript again but have a few further suggestions.

The reference to sequentially allocating patients to low and then high dose is at first somewhat confusing and could give the impression that the patienst received both doses. It would help to reword this to say 'subsequent patients' or a similar form of words.

We have reworded the methods section to make this clearer.

It would be useful if the non-HIV studies were described in more detail, perhaps in their own section in Methods.

We think it is sufficient here to reference the published papers as all the detail on the trials is included in those references and inclusion of this information in this manuscript would make the methods section unduly long. We reference all 3 previous trials (and unpublished data, Pathan et al which is currently being prepared for submission) and feel it would be too cumbersome to include data from all 3 (some BCG vaccinated, some Mtb latently infected) here.

The authors say that baseline refers to pre-vaccination but how long before vaccination? They have data up to 6 years prior to vaccination. This is still unclear.

We have added 'prevaccination baseline on the day of screening' to the first mention of baseline in the immunological results section, to make this point clearer. In the methods section it is clear at what timepoints the immunological assays were conducted.

We initially commented that the description of the repeated measures analysis needed more detail, since it was not clear to reader how to repeat this. On review of this analysis, the authors agreed that the numbers were too small for a meaningful statistical analysis and amended the text to say that there were no clinically significant effects of vaccination on CD4 count or HIV RNA load, a conclusion they considered more clinically relevant and appropriate given the sample size in this first Phase I study. Concluding that there are no clinically significant effects suggests that an analysis has been performed in which case it should be described.

The analysis was a clinical one. No decisions re commencing ARVs were made on the basis of any fluctuations in CD4 count or HIV RNA load – hence the comment that no clinically significant effects were found. If the editor would prefer that we use the term clinically important (to remove any statistical connotations), then we are happy to do so.

The authors say that the confidence intervals of median difference reported in this manuscript were derived using robust method suggested by Newson. Howevre, in table 3a median difference is reported with range rather than CI. In this respect tables 3a and 3b appear to be inconsistent.

Table 3a (now 4a) presents the median difference between 2 paired observations, so in this case the difference between each pair is calculated, and the median value of these differences is presented along with the range. In table 3b (now 4b) it is the difference in medians which is presented and this is for unpaired data. The method used for calculating the 95% confidence interval is only valid for independent observations.

Page 14 line 47 could imply that the same level of response was maintained which it was not. It is not always clear that a response is being distinguished from a level of response.

We're not sure exactly where this comment relates to (the page and line numbers do not correspond) and think the kinetics of the immune response are clear from Figure 3 and are, as previously discussed, expected. We clearly state significantly above baseline in the text.

As already mentioned more information on the HIV-uninfected cohort would be helpful.

See our response above – we feel the inclusion of the published references is sufficient but if the editor feels strongly about this, we would be happy to add something on the previous trials. Our methodology is very similar across all the trials, apart from the trial specific details like the qPCR analysis described in this manuscript.

Table 2 which still states that there are 43 HIV –ve subjects in low dose group which is I think incorrect.

In the published references there are 21 subjects in McShane et al, NM 2004; 10 in Pathan et al, PLoS One 2007; and 12 in Sander et al, AJRCCM 2009. This makes 43 in total which is what we quote in Table 2.

The authors state that they believe it is important data to demonstrate the pre-vaccination variability in these parameters in Fig 2, in order to interpret the post-vaccination variability for each subject – hence including it in the figure. It might be easier to just calculate the variance pre and post vaccination.

This would involve calculating the variance pre and post vaccination separately for each participant (n=20) for CD4 and HIV RNA. This equates to 80 calculations of variance. We are not trying to prove a statistical difference or equality here, but are simply trying to demonstrate that the variability is similar pre and post vaccination. This is best achieved visually, through the use of figure 2.