JVI Accepts, published online ahead of print on 24 August 2011
J. Virol. doi:10.1128/JVI.05165-11

The HIV/AIDS vaccine candidate MVA-B administered as a single immunogen in humans triggers robust, polyfunctional and selective effector memory T cell responses to HIV-1 antigens

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Running title: Immunogenic profile of HIV-1 vaccine MVA-B in human volunteers

Abstract

Attenuated poxvirus vectors expressing HIV-1 antigens are considered promising HIV/AIDS vaccine candidates. Here we described the nature of T cell immune responses induced in healthy volunteers participating in a phase I clinical trial in Spain after intramuscular administration of three doses of the recombinant MVA-B expressing monomeric gp120 and the fused Gag-Pol-Nef (GPN) polyprotein of clade B. The majority (92.3%) of the volunteers immunized had a positive specific T cell response at any time post-vaccination as detected by IFN-γ ICS assay. The CD4+ T cell responses were predominantly Env directed, whereas the CD8+ T cell responses were similarly distributed against Env, Gag and GPN. The proportion of responders after two doses of MVA-B was similar to that obtained after the third dose of MVA-B vaccination and the responses were sustained (84.6% at week 48). Vaccine-induced CD8+ T cells to HIV-1 antigens after one year were polyfunctional and mainly distributed within the effector memory (TEM) and terminally differentiated effector memory (TEMRA) T cell populations. Anti-vector T cell responses were mostly induced by CD8+ T cells, highly polyfunctional and of TEMRA phenotype. These findings demonstrate that the poxvirus MVA-B vaccine candidate given alone is highly immunogenic, inducing broad, polyfunctional and long-lasting CD4 and CD8 T cell responses to HIV-1 antigens, with preference for TEM. Thus, on the basis of the immune profile of MVA-B in humans, this immunogen can be considered as promising HIV/AIDS vaccine candidate.

Introduction

Since 1981, more than 25 million people have died of Acquired Immune Deficiency Syndrome (AIDS), a dramatic pandemic caused by the Human Immunodeficiency Virus (HIV). In 2009 the UNAIDS estimates that 33.4 million people now live with HIV-1 infection. Although anti-retroviral therapy (ART) can suppress viral replication increasing life expectancy among those people infected, it cannot cure the infection. Moreover, affordable ART coverage in resource-poor, HIV-1 endemic regions is a daunting global health problem. For these reasons the development of a safe and efficacious vaccine represents the best long-term solution to ending the HIV-1 epidemic.

There have been strong proponents of either antibodies or T cells alone as the most effective strategy that should be followed to prevent HIV-1 infection. However, the consensus view now is that a highly effective HIV/AIDS vaccine will need to elicit coordinated B cell, CD4+ and CD8+ T cell responses (27).

More than 30 HIV/AIDS vaccine candidates, whose prototypes have elicited varying degrees of protective responses in nonhuman primate models have advanced to human clinical trials, alone or in combinations (25, 36). These include replication-competent or incompetent viral vectors (poxvirus, adenovirus, alphavirus, adeno-associated virus) containing HIV-1 gene inserts, HIV-1 virus-like particles, HIV-1 DNA plasmids and soluble HIV-1 proteins and peptides, with or without adjuvant formulations. Among the candidate regimens that have been extended to large-scale international phase IIb or III studies only the RV144 trial, that evaluated a recombinant canarypox-HIV-1 vector prime and recombinant HIV-1 envelope gp120 subunit protein plus alum boost in Thailand, demonstrated low-level efficacy (31%) in reducing HIV-1 infection rates (35). These clinical findings provided for the first time evidence that an HIV/AIDS vaccine can prevent HIV-1 infection and highlight that poxvirus vectors should be considered as one of the future HIV/AIDS vaccine candidate vectors.

Among the poxviruses, the attenuated Modified Vaccinia Ankara (MVA) strain has received great attention in terms of vaccine development for prevention and therapeutic purposes (12). The main advantage of MVA is its safety record. Despite its limited replication in human and most mammalian cell types, MVA provides a high level of gene expression and triggers strong immune responses when delivering foreign antigens in animals and humans (12, 30, 39). In fact, in the last years several clinical trials have

been conducted using MVA-based vaccines in both healthy and HIV-1-infected human volunteers (10, 22, 24, 38, 40). These studies demonstrated that the recombinant vectors based on MVA are safe and well tolerated and are able to induce HIV-1-specific immune responses when administered alone or in combination with other vectors. However, the magnitude, response rates and durability in immunization regimens using homologous vectors were modest. These observations highlight that more efficient MVA vectors with the ability to enhance the magnitude, breadth, polyfunctionality and durability of the immune responses to HIV-1 antigens are desirable. This is particularly relevant if a single immunogen is target for mass vaccination purposes, to simplify the immunization protocol and reduce manufacture burden.

Here we have characterized the immunogenicity of the recombinant MVA-B, expressing Env, Gag, Pol and Nef HIV-1 antigens from clade B, in healthy volunteers enrolled in the RISVAC02 phase I clinical trial. The construction details and preclinical setting of this vaccine were published earlier (8, 11). We specifically addressed the breadth, phenotype, polyfunctionality and longevity of the vaccine elicited immune responses in order to provide insights into the immune protective potential of homologous MVA-B vaccine regimen in humans.

Materials and Methods

MVA-B vaccine

The generation of MVA-B vector was previously described (11). It expresses simultaneously and under the same synthetic early/late viral promoter, monomeric gp120 as a cell released product and Gag-Pol-Nef (GPN) as an intracellular polyprotein of 160 KDa. gp120 Env protein comes from the HIV-1 primary isolate BX08. Gag-Pol-Nef is a fusion protein of 1326 amino acids composed of *gag, pol* and *nef* ORFs from HIV-1 clone IIIB, that has been modified to enhance its immunogenicity and for safety by removing undesirable domains. In both cases, the codon usage was adapted to highly express human genes. The good manufacturing production (GMP) clinical lots of MVA-B were produced by IDT (Germany) and kindly provided by EuroVacc. MVA-B was genetically stable, even when grown and purified at large scale under GMP conditions as previously described (11).

Study design

The RISVAC02 study was approved by the institutional ethical review board and by the Spanish Regulatory Authorities (Government identifier: NCT00679497). The study was explained to all patients in detail, and all signed written informed consent documents. A total of 30 HIV-1 negative, vaccinia-naïve volunteers, at two clinical sites in Madrid (HGM) and Barcelona (HC) were randomly allocated to receive 3 x 1 ml injections of MVA-B (10^8 pfu/dose) (n=24) or placebo (n=6) by intramuscular route at weeks 0, 4 and 16. The duration of participant follow up was 48 weeks.

Synthetic peptides

All peptides used in this study were HPLC purified (>80% purity) and provided by EuroVacc. Overlapping peptides (15 mers with 11 amino acids overlap; n=450) covered the entire Env, Gag, Pol and Nef regions from clade B included in MVA-B. The $_{BX08}$gp120 protein (494 aa) was spanned by the Env-1 (aa: 1-251; 60 peptides) and Env-2 (aa: 241-494; 61 peptides) pools. The Gag-Pol-Nef fusion protein (1326 aa) was spanned by the following pools: Gag-1 (aa: 1-231; 55 peptides), Gag-2 (aa: 221-431; 50 peptides), GPN-1 (aa: 421-655; 56 peptides), GPN-2 (aa: 645-879; 56 peptides), GPN-3 (aa: 869-1103; 56 peptides) and GPN-4 (aa: 1093-1326; 56 peptides). For

immunological analyses we grouped the pools as follows: Env pool (Env-1+Env-2); Gag pool (Gag-1+Gag-2) and GPN pool (GPN-1+GPN-2+GPN-3+GPN-4).

Cell preparation

Whole blood samples for analyses of the immune responses were collected in cell preparation tubes (CPT Vacutainer tubes; BD) and processed within 6 hours, in accordance with manufacturer´s instructions. The yield and viability of peripheral blood mononuclear cells (PBMCs) were determined by trypan blue staining. Fresh PBMCs were used for the immunological analyses described in this study. The remaining cells were cryopreserved.

Flow cytometry analyses

Fresh PBMCs ($1–2 \times 10^6$) were stimulated during 6 hours in complete RPMI 1640 media containing 1 µl/ml Golgiplug (BD Biosciences) and 5 µg/ml of the different HIV-1 peptide pools. When the anti-vaccinia response was assayed, the PBMCs were stimulated during 6 hours in complete media containing 1 µl/ml Golgiplug (BD Biosciences) and autologous cells infected with MVA at 2 pfu/cell in a ratio 10:1. For functional analyses the following fluorochromes-conjugated antibodies were used: CD3-AmCyan; CD4-Alexa 700; CD8-PerCPCy5.5; IFN-γ-V450 or -PECy7; IL-2-APC; TNF-α-PECy7 and MIP1β-PE. In addition, for phenotypic analyses the following antibodies were used: CCR7-PE and CD45RA-FITC. All antibodies were from BD Biosciences. At the end of the stimulation period, cells were stained for the surface markers, permeabilized (Cytofix/Cytoperm kit; BD Biosciences) and stained intracellularly using the appropriate fluorochromes. Cells were collected on an LSR II flow cytometer (BD Immunocytometry Systems). Analyses of the data were performed using the FlowJo software version 8.5.3 (Tree Star, Ashland, OR). The number of lymphocyte-gated events ranged between 10^5 and 10^6. After gating, Boolean combinations of single functional gates were then created using FlowJo software to determine the frequency of each response based on all possible combinations of cytokine expression or all possible combinations of differentiation marker expression. Background responses detected in negative control tubes were substracted from those detected in stimulated samples for every specific functional combination.

Data analysis and statistics

To correct measurements of the medium response (RPMI) we used a novel statistical approach previously described (8, 29). An ICS was considered positive if the percentages of cytokine+ cells in the stimulated samples were 3 times over the values obtained in the unstimulated controls and if the background-substracted magnitudes were higher than 0.02%. The background for the different cytokines in the unstimulated controls never exceeded 0.015%. Each participant was classified as a responder if there was at least one positive IFN-γ ICS response against any of the HIV-1 peptide pools at weeks 6, 18 or 48 and as a nonresponder if responses at these weeks were all negative.

The magnitude of the ICS responses and other continuous variables were compared between groups using the nonparametric tests Wilcoxon rank sum test and Mann-Whitney U test. The differences among cumulative proportions have been tested by comparing two binomial distributions as described in (41) (implemented by the R function prop. test). For correlation analysis between variables the Pearson´s correlation coefficient test was used.

The data analysis program, Simplified Presentation of Incredibly Complex Evaluations (SPICE, version 4.1.5, Mario Roederer, Vaccine Research Center, NIAID, NIH), was used to analyze and generate graphical representations of T cell responses detected by polychromatic flow cytometry. All values used for analyzing proportionate representation of responses are background-substracted.

Results

Study design

The main objective of this study was to characterize the magnitude, breadth, phenotype, function and durability of the T cell responses induced by the single recombinant MVA-B administered in three doses in human healthy volunteers enrolled in the RISVAC02 phase I clinical trial in Spain.The MVA-B vaccine is a non-replicating viral vector in human cells that expresses simultaneously the gp120 Env protein from the BX08 HIV-1 isolate as a cell released product and Gag-Pol-Nef (GPN) from the IIIB HIV-1 isolate as an intracellular polyprotein (11). GPN has been engineered by the removal of immunosuppressed sequences and to prevent virus-like particles (VLP) formation. A total of 30 healthy, HIV-1 negative volunteers, naïve for smallpox vaccine, were enrolled. The study was randomized and double-blinded with respect to active vaccine or placebo. The participants received 3 x 1ml injections of MVA-B (10^8 pfu/dose) intramuscularly in the deltoid at weeks 0, 4 and 16. The immune responses were evaluated at weeks 6, 18 and 48 by polychromatic intracellular cytokine staining (ICS) (Figure 1A). This assay was done in 16 volunteers due to rapid availability of freshly isolated peripheral blood mononuclear cells (PBMCs) to ensure no loss of functional activity of T cells.

Analyses of the demographics of the trial population and of the safety of the vaccine will be described elsewhere (García, F et al., submitted). No related serious adverse events occurred during the study indicating that MVA-B was safe and well tolerated.

Vaccine-induced T cell responses

Vaccine-induced T cell responses were assessed in 16 volunteers by ICS assay after the stimulation of freshly isolated PBMCs with a panel of 450 HIV-1 peptides (15 mers overlapping by 11 amino acids) grouped in three pools: Env (121 peptides), Gag (105 peptides) and GPN (224 peptides). The peptides encompassed the Env, Gag, Pol, and Nef proteins of HIV-1 and were designed based on the sequence of the immunogens expressed by MVA-B.

The response rates at weeks 6, 18 and 48 were determined for each T cell population based on the percentage of antigen-specific IFN-γ+ cells. Cumulative analysis of the data demonstrated that MVA-B induced HIV-1-specific T cell responses that were balanced and significantly different to those determined in the placebo group (p=0.04)

(Table 1). CD4+ and CD8+ T cell responses to any HIV-1 peptide pool at any time post-vaccination were detected in 69.2% (9/13) and 92.3% (12/13) of the vaccines, respectively. The CD4+ T cell responses were predominantly Env directed (Env: 69.2% vs Gag: 15.4% and GPN: 7.7%) whereas the CD8+ T cell responses were similarly distributed against the three peptide pools (Env: 61.5%, Gag: 69,2% and GPN: 69.2%).

The assessment of vaccine-induced T cell responses at different time points, determined as the rate of CD4+ and/or CD8+ responses to any HIV-1 antigen, indicated that the proportion of responders after 2 doses of MVA-B (W6) was similar to that obtained after the third dose of MVA-B vaccination (W18) (75% vs 69.2%) and was sustained by 32 weeks after the last immunization (84.6% at W48) (Figure 1B). The mean values for the total HIV-1 responses (Env+Gag+GPN) in each T cell population are shown in Figure 1C. For CD4+ T cells both the magnitude and response rates peaked after 2 MVA-B doses, declining with time. The response rates to any antigen decreased from 58.3% at W6 to 23.1% at W18 and to 38.5% at W48 (Table 1). For CD8+ T cells both magnitude and response rates were higher than for CD4+ T cells, specially at weeks 18 and 48. The magnitude of the responses remained similar during the study as well as the response rates to any antigen (50% at W6; 61.5% at W18 and 69.2% at W48) (Table 1). There was no significant correlation between the magnitude of the response for CD4+ and CD8+ T cells in individuals.

The CD4+ T cell response was essentially directed against 1 HIV-1 peptide pool (Env) at all time points assayed, with occasional recognition of 2 antigens (Env and Gag), whereas the CD8+ T cell response was broad and evenly distributed to 1, 2 or 3 HIV-1 peptide pools (Figure 1D).

The cross-sectional responsiveness per antigen showed that Env response was mediated by both CD4 and CD8 T cell subsets whereas the Gag and GPN responses were mainly mediated by the CD8 T cell population (Figure 1E).

Functional profile of vaccine-induced CD4 and CD8 T cell responses

The profile of vaccine-induced CD4 and CD8 T cell responses was analysed in those volunteers with IFN-γ+ ICS responses. The polychromatic ICS assays were performed on fresh PBMCs at 2 weeks after both the second (W6) and third dose (W18) of MVA-B vaccine. The panel of T cell functions analyzed included IL-2, TNF-α, MIP1β and IFN-γ secretion. For each subpopulation the background, as detected in the unstimulated

control sample, was substracted. Only responses exceeding a predefined threshold level after background substraction were considered.

The mean values for the total responses (Env+Gag+GPN) in each T cell population considering the frequencies of all the cytokines are represented in Figure 2A. The magnitudes of the total HIV-1-specific responses were similar for both populations at the two time points. Among the cytokine producing CD8+ T cells, IFN-γ and MIP1β predominate at both weeks 6 and 18 whereas no single cytokine prevail in the CD4+ T cells at any time assayed (Figure 2B). Representative functional profiles of vaccine-induced CD4 and CD8 T cell responses were shown for one of the responders at week 18 (Figure S1).

The quality of a T cell response can be characterized in part by the pattern of cytokine production. On the basis of the analysis of IL-2, TNF-α, MIP1β and IFN-γ secretion, 15 distinct HIV-1-specific CD4+ and CD8+ T cell populations were identified (Figure 3). Vaccine-induced CD4+ T cell responses at weeks 6 and 18 were mainly represented by cells expressing 1 function, although about 25% of CD4+ T cells exhibit two or three functions. In contrast to CD4+ T cells, vaccine-induced CD8+ T cells were more polyfunctional, with about 45% of vaccine-induced HIV-1-specific CD8+ T cells exhibiting more than one function (Figure 3). In both subsets there were no changes in the polyfunctional profile after the third dose of MVA-B.

To define if polyfunctionality is a feature of an individual or of responses to particular antigens, we performed a two-way ANOVA (response as a function of the patient and the antigen) of the responses for CD8+ and CD4+ T cells after 2 (W6) and 3 doses (W18). We found that all patients responded similarly (except one individual who is particularly polyfunctional responsive for CD8+ after 2 doses). At week 6 we found significant differences ($p < 0.05$) between the polyfunctional response of CD8+ T cells to Env, Gag and GPN versus the polyfunctional response to MVA. Similarly, the polyfunctional response of CD4+ T cells to Env was significantly larger than that to GPN. The rest of the responses were not significantly different. At week 18 we did not find any difference between the polyfunctional responses of individuals and antigens at the level of CD8+ T cells. Moreover, when we determined if the magnitude or breadth of the response correlate with the polyfunctionality we only found a positive correlation (0.78) between breadth and CD8+ polyfunctionality after 2 doses (W6). Otherwise, these variables are not correlated (with Pearson's correlation coefficient test).

Phenotypic profile of long-lived memory HIV-1-specific T cell responses

Phenotypic analysis of long-lived memory vaccine-induced T cell responses was carried out at 32 weeks after the last MVA-B immunization (W48) by polychromatic ICS assay. Fresh PBMCs were stimulated with the HIV-1 peptide pools Env, Gag and GPN for 6 hours and stained with specific antibodies to identify T cell lineage (CD3, CD4 and CD8), responding cells (IL-2 and IFN-γ) as well as memory stages (CD45RA and CCR7).

At this time point the HIV-1-specific response was mainly mediated by CD8+ T cells, although in 3 out of 11 responders (27.3%) was mediated by both CD4+ and CD8+ T cells. 55.6% of the responders at W48 had specific-CD8+ T cells against 2 or 3 pools, correlating with the individuals that secrete more IFN-γ ($p<0.05$).

Since previous studies have shown that CD45RA and CCR7 define functionally distinct populations of memory antigen-specific T cells (4, 19, 37), we characterized the differentiation stages of the responding CD4 and CD8 T cells into central memory (TCM: $CD45RA^{-}CCR7^{+}$), effector memory (TEM: $CD45RA^{-}CCR7^{-}$) or terminally differentiated effector memory (TEMRA: $CD45RA^{+}CCR7^{-}$) populations. For each vaccinee we summed the totality (single IL-2 plus dual IL-2/IFN-γ plus single IFN-γ) of Env+Gag+GPN specific T cell responses and determined for CD4 and CD8 T cell subsets the percentages of the specific responses with phenotype TCM, TEM or TEMRA (Figure 4). The HIV-1-specific CD4+ T cell responses were mainly distributed within the TCM and TEM cell populations whereas the CD8+ T cell responses were mainly distributed within the TEM and TEMRA cell populations (Figure 4A). In both CD4 and CD8 T cell subsets the higher numbers of cytokine secreting cells were found within the TEM cell population. Figure 4B shows representative phenotypic profiles of long-lived memory HIV-1-specific T cells in one of the volunteers.

Anti-vector T cell responses

Vaccine-induced anti-vector T cell responses were assessed by ICS assay after the stimulation of freshly isolated PBMCs with autologous cells infected with MVA. The response rates at weeks 6, 18 and 48 were determined for each T cell population based on the percentages of MVA-specific IFN-γ+ cells following the same criteria described above. The analysis of anti-vector T cells responses at different time points, determined as the rate of CD4 and/or CD8+ responses to MVA-infected cells, indicated that the proportion of responders after 2 doses of MVA-B (W6) was similar to the obtained after

the third MVA-B vaccination (W18) (83.3% vs 84.6%) and remained unchanged over time (91.7% at W48) (Figure S2A). There was not correlation between vector and HIV-1 antigen responses. None of the placebo recipients had a positive response against the vector. The responses were mostly induced by the CD8 T cells (Figure 5A) and were highly polyfunctional, with about 70% of MVA-specific CD8+ T cells displaying more than one function (Figure 5B). The magnitude and polyfunctionality of anti-vector CD8 T cell responses were maintained after the third dose of MVA-B. Representative functional profiles of the anti-MVA responses in one of the volunteers at week 18 are shown in Figure S2B. Although anti-vector CD8+ T cell responses appeared to be more polyfunctional than responses to the HIV-1 antigens, we have to take into consideration that the different assay system used (one stimulated with peptide pools, the other with virus-infected cells) might influence the result and do not allow the direct comparison between the polyfunctional degree against the vector and against the HIV-1 antigens.

To define if strong responses to the vector at earlier times reduce the benefit of boosting for the HIV-1 antigens, we analyzed using Pearson's correlation coefficient test if the anti-vector response at week 6 affect the anti-HIV-1 response at week 18, and we found that strong responses to the vector at earlier times do not reduce the benefit of boosting for the HIV-1 antigens (0.348).

At week 48, the totality (single IL-2 plus dual IL-2/IFN-γ plus single IFN-γ) of MVA specific CD4+ T responses were mainly distributed within the TEM cell population whereas the CD8+ T cell responses were mainly distributed within the TEMRA cell population (Figure 5C). Representative phenotypic profiles of long-lived memory MVA-specific T cells are shown in one of the volunteers (Figure S2C).

Discussion

At present it remains unclear which elements of the immune system need to be stimulated to provide protection against HIV-1 infection and to improve viral control in already HIV-1 infected individuals. For this reason, HIV/AIDS vaccine development is currently directed towards the quantitative and qualitative improvements of vaccine induced immune responses through the use of novel vectors administered either alone or in prime-boost heterologous combination. The modest efficacy and low-level immune responses of the RV144 Thai phase III trial based on the poxvirus vector ALVAC in combination with the protein gp120 (35), suggest that improved poxvirus vectors may be effective components of a realistic strategy for vaccination against HIV-1 infection.

We have previously described the generation and characterization of the MVA-B vaccine candidate against HIV/AIDS (11). MVA-B used alone, or in combination with DNA vectors expressing the same HIV-1 antigens, was able to induce in mice robust, polyfunctional and durable T cell HIV-1-specific responses (8, 11). In macaques, a similar MVA construct expressing Env (gp120 from $SHIV_{89.6P}$) and Gag-Pol-Nef (from SIV_{mac239}) induced strong specific CD4+ and CD8+ T cell immune responses with a bias for CD8+, and high protection after challenge with $SHIV_{89.6P}$ (28). Furthermore, expression of HIV-1 antigens from MVA-B selectively induced in human monocyte-derived dendritic cells (moDCs) the expression of different cellular genes that might act as regulators of immune responses to HIV-1 antigens (14), and MVA-B-infected moDCs co-cultured with autologous T lymphocytes induced a highly functional HIV-1-specific CD8+ T cell response including proliferation, secretion of IFN-γ, IL-2, TNF-α, MIP1β, MIP1α, RANTES and IL-6, and strong cytotoxic activity against autologous HIV-1-infected $CD4^+$ T lymphocytes (2). Based on these previous results, MVA-B was approved in Spain for a phase I clinical trial in healthy volunteers (RISVAC02).

The primary aim of this study was to characterize in detail the magnitude, breadth phenotype, function and type of memory T cell responses induced by the recombinant MVA-B in participants enrolled in the RISVAC02 clinical trial. The availability of fresh PBMCs from 16 volunteers obtained at different times post-immunization made it possible to analyze directly the T cell profile in all of these samples, thus assuring minimal loss of T cell functions. The analysis of the vaccine-induced T cell responses was performed by polychromatic ICS assay from PBMCs stimulated with a panel of peptide pools encompassing Env, Gag, Pol, and Nef HIV-1 antigens from clade B

included in the MVA-B vector. Although the IFN-γ ELISPOT is the best standardized assay used internationally for measuring HIV-1 vaccine induced immune responses (1, 5, 13), it provided limited information on a spectrum of cytokine/chemokine profiles. To overcome the limitation of evaluating a single cytokine, novel techniques, as the polychromatic ICS assay, are becoming increasingly more stringent in assessing HIV-1-specific immune responses in different clinical settings (3, 10, 16, 22). This assay provides simultaneous information on multiple markers measured at the single cell level allowing a detailed characterization of the vaccine specific T cell responses.

Here we demonstrate that the vaccination regimen based on 3 doses of 10^8 pfu of MVA-B given intramuscularly is highly immunogenic, induces high frequency of HIV-1-specific CD4+ and CD8+ T cells which are polyfunctional and with broad IFN-γ ICS reactivity, and more importantly, this vaccine regimen induces long-lasting T cell immunity activating specific subset of memory T cell populations. The majority (12 out of 13, 92.3%) of the volunteers immunized with MVA-B had a positive HIV-1-specific T cell response at any time post-vaccination detected by IFN-γ ICS assay. While direct comparison of overall response rates between MVA recombinants tested in clinical trials has to be taken with caution due to differences in the HIV-1 expressing cassette of the vectors, simple comparison with other stand-alone MVA-based HIV-1 vaccine products revealed that MVA-B appears to be as good or even better immunogen than MVA-CMDR (84.6%) (3), more immunogenic than MVA62 (43%) (10) and substantially more immunogenic than MVA.HIVA (0%) (21, 32). Other studies using the same immunization regimen, but with higher doses of MVA products, had reported similar or even lower response rates than those reported here. The use of ADMVA (40) and TBC-M4 (34) at 2.5 fold higher doses than MVA-B gave response rates of 62% and 100%, respectively. Furthermore, after a third dose of 10^9 pfu of MVA-HIV a response rate of 41.4% was reported (22). MVA-B was also more immunogenic than the related attenuated poxvirus vector NYVAC-C used in homologous combination (16, 26). Overall, the response rates assigned to MVA-B in comparison with other MVA-HIV related vaccines provided strong support for the potential benefit of this vector as an HIV/AIDS vaccine candidate.

Considering the consensus that for an HIV/AIDS vaccine to be effective it should aim to trigger specific T cell immune responses with an immunogenic profile of high frequency of CD4+ and CD8+ T cells, polyfunctional and durable, the immunogenic characteristics of MVA-B described in this work fulfil these criteria. The HIV-1-

specific T cell responses induced by MVA-B vaccine were balanced, with CD4+ and CD8+ T cell responses detected in 69.2% and 92.3% of the vaccinees, respectively. The CD4+ T cell response peaked and then decline after the second dose of MVA-B and was directed almost entirely to Env, whereas the CD8+ T cell response slightly increases over time and was more evenly distributed between Env, Gag and GPN antigens. These results were in line with the preclinical evaluation of MVA-B in mice (8, 11) and also with the results obtained in macaques using an analogous MVA expressing gp120 from $SHIV_{89.6P}$ and Gag-Pol-Nef from SIV_{mac239} (28), but differed from studies by others that suggest that MVA-vectored constructs expressing multigenic products induced primarily a CD4+ T cell response (3, 10, 22). Using flow cytometry-based assays, Currier et al. reported that Env antigen was consistently the predominant target of the cellular immune response, and CD4+ T cells were the most frequently detected responder cell type when using 10^8 pfu of MVA-CMDR (3). Using the MVA62 in homologous regimen it was reported a 2.4-fold excess of CD4+ over CD8+ T cells responses with strong bias towards Gag (10). More recently it was reported that after 3 doses of 10^9 pfu of MVA-HIV there was 3-fold excess of CD4+ over CD8+ responses, being the CD4+ T cell response more frequently directed at Gag than Env, and the CD8+ T cell response directed entirely at Env (22). The divergences observed between the studies described above and our study must be attributed to the delivery format and the nature of the HIV-1 antigens expressed by the different vaccine candidates. The MVA-CMDR and MVA62 share similar features. In both recombinants the truncated gp160 env gene was inserted into the deletion II, whereas the modified gag-pol gene was inserted into the deletion III. In addition, both viruses expressed the Env protein on the surface of the infected cells while Gag and Pol antigens are produced as noninfectious virus-like particles (VLPs) (6, 10). On the other hand, MVA-HIV represents a mix of two different MVA recombinants, one expressing the structural env and gag genes and the other expressing the regulatory tat, rev, nef and reverse transcriptase (RT) genes, all at different locus (22). Our MVA-B vaccine has inserted in the single viral TK locus the env and gag-pol-nef genes, and both are expressed in infected cells simultaneously, with the monomeric gp120 Env protein as a cell released product, and Gag-Pol-Nef (GPN) as an intracellular polyprotein. The better stimulation of CD4 T cells in the previous studies might be related with the preferential activation of the exogenous pathway of antigen presentation by secreted products as VLPs or Env protein. In fact, in our study almost all the vaccine-induced CD4+ T cell response was

449 directed against Env. The MVA-B-induced T cell responses against Gag and GPN
450 antigens were mainly mediated by CD8+ T cells and this might be related with the
451 activation of the intrinsic pathway of antigen presentation by the Gag-Pol-Nef
452 intracellular polyprotein. As we have reported, both the expression of gp120 and GPN
453 by MVA-B on moDCs had an effect on host cellular functions. In fact, expression of
454 HIV-1 proteins from moDCs infected with MVA-B induced the expression of
455 cytokines, cytokine receptors, chemokine receptors, and molecules involved in antigen
456 uptake and processing, including major histocompatibility complex (MHC) genes,
457 whose products might act as regulators of immune responses to HIV-1 antigens (14).
458 Moreover, MVA-B infection of moDCs stimulate strong HIV-1 immune response,
459 mainly induced by CD8+ T cell proliferation together with high secretion of CD8+-
460 polyfunctional-related cytokines (2). Thus, the preferential induction of CD8+ T cells
461 by MVA-B might be related to the intrinsic innate vector-effects on target cells.
462 In addition to T cell responses, MVA-B also elicited strong and durable Env-specific
463 humoral response. Binding antibodies against HIV-1 LAVgp160 were detected in
464 45.8% of the volunteers after the second MVA-B dose, while nearly all recipients
465 (95.8%) tested positive by ELISA after the third MVA-B dose. At 32 weeks after the
466 last immunization 72.7% of the vaccinees had detectable levels of anti-Env antibodies
467 (García, F et al., submitted). These results are comparable to the previous studies
468 reported by Currier et al. (3) and Goepfert et al. (10) in which the anti-Env antibody
469 responses peaked after the third dose of MVA-CMDR (90%) and MVA62 (86%)
470 respectively.
471 In our vaccination scheme with MVA-B, the last boost was needed to enhance humoral
472 HIV-1-specific responses in vaccinees, and might also be important for increasing and
473 maintaining the anti-Gag and anti-GPN CD8+ T cell responses. The ICS data correlated
474 with the immune responses detected by IFN-γ ELISPOT in all volunteers included in
475 the RISVAC02 study, where at early times the higher responses were detected against
476 Env and after the third dose of MVA-B these responses were against Gag and GPN
477 (Garcia,F et al., submitted). Similar remarks were reported in the MVA62 study
478 although the specific responses were lost 6 months after the last dose (10). The
479 induction of Gag responses in vaccinees could be favourable for a vaccine since in the
480 natural HIV-1 infection it has been reported that Gag-specific CD8+ T cell responses
481 are associated with better control of HIV/AIDS disease in individuals with chronic
482 HIV-1 infection (7, 9, 23). The HIV-1-specific CD4+ and CD8+T cell responses

induced by MVA-B vaccine were polyfunctional and both T cells subsets maintained similar functional profiles after 2 or 3 doses of the MVA-B vaccine. In this regard several studies performed in the setting of HIV-1 infection have shown that polyfunctional T cell responses are associated with better clinical outcome and protection from disease progression (20, 31, 33).

A critical component of the effectiveness of vaccines is their ability to induce long-lasting immunity. Here we observed that 84.6% of volunteers have HIV-1-specific T cell responses at week 48. This response rate is higher than the reported in other studies using multigenic vaccines such as MVA-CMDR (about 60%) (3), MVA62 (8%) (10) or TBC-M4, that although reported 100% of responders after the third dose, point out that only few vaccinated individuals exhibited long lasting responses (34). The T cell responses at week 48 were balanced and do not differ with the response obtained 2 weeks after the third booster. In our volunteers the vaccine-induced CD4+ T cell populations had mainly TCM ($CD45RA^{-}CCR7^{+}$) or TEM ($CD45RA^{-}CCR7^{-}$) phenotypes which correspond to cells with effector functions but also with the ability to secrete IL-2 and endowed with proliferation capacity (4, 17, 19, 37). In the case of CD8+ T cells the memory phenotypes were either TEM ($CD45RA^{-}CCR7^{-}$) or TEMRA ($CD45RA^{+}CCR7^{-}$). The presence of both memory populations at 8 months after the last vaccination is an important consideration since they have been implicated in the control of different virus infections. The presence of $CD45RA^{+}CCR7^{-}$ CD8 T cells has been found in controlled chronic virus infections such as CMV and EBV (4, 18, 42), and a correlation between the percentage of this cell population and virus control has also been shown in HIV-1 infection (31). Moreover, it was recently described the relevance of the effector memory T cells on the early control of highly pathogenic SIV (15).

As others (33), we observed that MVA-B vaccine also induced specific anti-vector immune responses mainly mediated by the CD8 T cells. The responses were highly polyfunctional, with about 70% of MVA-specific CD8+ T cells displaying more than one function. Significantly, the magnitude and polyfunctionality of anti-vector CD8+ T cell responses were maintained after the third dose of MVA-B and were durable, with a phenotype related with advanced stages of differentiation. The anti-vector memory responses were predominantly of TEM phenotype for CD4+ T cells and of TEMRA for CD8+ T cells.

In conclusion, this study revealed a number of significant findings on the immune profile of the MVA-B vector as an HIV/AIDS vaccine based on ICS data from human

PBMCs. First, the vector MVA-B given alone is highly immunogenic as over 90% recipients responded to the vaccine; second, MVA-B induces broad HIV-1-specific T cell responses, comprising of both CD4 and CD8 T cells, which were balanced after the third dose; third, the HIV-1-specific immune responses triggered by MVA-B were polyfunctional; fourth, MVA-B responses were maintained at least for one year in 85% of vaccinees, with HIV-1-specific memory T cells being of TEM and TEMRA phenotypes for CD8+ T cells; fifth, the anti-vector responses were largely polyfunctional with predominance of memory CD8+ T cells of TEMRA phenotype. This immune profile fulfils immune requirements as a promissing HIV/AIDS vaccine candidate, and support to move forward the MVA-B product into larger clinical trial, alone or combined with other HIV-1 immunogens, like DNA or proteins. Undoubtedly, the immune value of MVA-B vaccine to impact the outcome of HIV-1 infection can only be tested in an efficacy trial.

Acknowledgments

This investigation was supported by grants from FIPSE-360731/09, the Ministry of Science and Innovation (SAF2008-02036) and Foundation Botín of Spain (to ME). We are grateful to EuroVacc Foundation for peptide pools of HIV-1 antigens from clade B. We thank Dr. Alexandre Harari for evaluation of the ICS data and critical advice.

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728 **Figure Legends**

729 **Figure 1:** MVA-B-induced HIV-1-specific T cell responses across the study. (A)
730 Chronological diagram showing the vaccination schedule followed in the RISVAC02
731 study and the immunogenicity endpoints. (B) Percentage of responders at the different
732 time points. The percentage of responders was calculated on the basis of volunteers with
733 a positive IFN-γ ICS. (C) Magnitude of vaccine specific CD4+ and CD8+ T cells at the
734 different time points. The mean values for the total responses (Env+Gag+GPN) in each
735 T cell population are shown. The box plots showed the distribution of responses in
736 positive responders only. The box indicated the median (solid line), mean (dash line),
737 and interquartile range (IQR). p values for significant differences were determined using
738 Mann-Whitney U test and are represented. (D) Breadth of CD4+ and CD8+ T cell
739 responses at the different time points. Percentage of responders that recognized 1, 2 or 3
740 HIV-1 peptide pools in both T cell subsets are shown. (E) Percentage of CD4+ and
741 CD8+ T cells producing IFN-γ in response to Env, Gag or GPN peptide pools as
742 measured by ICS at the different time points. The box plots showed the distribution of
743 responses in positive responders only. The box indicated the median (solid line), mean
744 (dash line), and interquartile range (IQR). p values for significant differences were
745 determined using Wilcoxon rank sum test with continuity correction and are
746 represented. All data are background substracted.
747
748 **Figure 2:** Vaccine-induced T cell responses at primary immunogenicity endpoints
749 (weeks 6 and 18). (A) Magnitude of HIV-1-specific CD4+ and CD8+ T cells after two
750 and three doses of MVA-B. The mean values for the total responses (Env+Gag+GPN)
751 in each T cell population are shown. The box plots showed the distribution of responses
752 in positive responders only. The box indicated the median (solid line), mean (dash line),
753 and interquartile range (IQR). (B) Percentages of HIV-1-specific T cells secreting
754 cytokines in the CD4 and CD8 T subsets. The box plots showed the distribution of
755 responses in positive responders only. The box indicated the median (solid line), mean
756 (dash line), and interquartile range (IQR). Data points represent the sum of the
757 frequencies obtained against Env+Gag+GPN peptide pools. All data are background
758 substracted. ** represents p values<0.005 determined using Wilcoxon rank sum test
759 with continuity correction comparing at the same time points the secretion of the
760 different cytokines.
761

762 **Figure 3:** Functional profile of vaccine-induced CD4 and CD8 T cells. The results
763 shown are generated from the determinations in responders at weeks 6 and 18. All the
764 possible combinations of the responses are shown on the x axis, whereas the percentage
765 of the functionally distinct cell populations within the total CD4 and CD8 T cell
766 populations are shown on the y axis. Responses are grouped and colour-coded on the
767 basis of the number of functions. The bars correspond to the individual data point and
768 interquartile range (IQR) after 2 (W6) or 3 (W18) doses of MVA-B. The pie charts
769 showed the average proportion of the CD4 or CD8 vaccine-specific T cell responses
770 according to the functions.
771
772 **Figure 4:** Phenotype of long-lived memory vaccine-induced T cell responses. (A)
773 Distribution of HIV-1 antigen-specific T cells at week 48 based on CCR7 expression in
774 combination with CD45RA. The bars correspond to the individual data point and
775 interquartile range (IQR) of the CD4+ and CD8+ T cell responses against
776 Env+Gag+GPN with phenotype central memory (TCM: CD45RA−CCR7+), effector
777 memory (TEM: CD45RA−CCR7−) or terminally differentiated effector memory
778 (TEMRA: CD45RA+CCR7-). The pie charts showed the average proportion of the
779 CD4+ or CD8+ vaccine-specific T cell responses according to the memory phenotype. *
780 represent distributions that are different from the CD4 T cell subset at $p<0.05$ (Student
781 T test). All data are background substracted. (B) Representative phenotypic profiles of
782 long-lived memory HIV-1-specific CD4 and CD8 T cells. Fresh PBMCs obtained from
783 the responder volunteers at week 48 were stimulated with Env, Gag or GPN peptide
784 pools. The red dots indicate antigen-specific (IL-2 plus IFN-γ) vaccine-induced CD4+ T
785 cells and blue dots indicate antigen-specific (IL-2 plus IFN-γ) vaccine-induced CD8+ T
786 cells, both overlaid on the total T cell subsets (grey). Neg, background values in
787 unstimulated cells.
788
789 **Figure 5:** Anti-vector-induced T cell responses across the study. (A) Percentage of
790 CD4+ and CD8+ T cells producing IFN-γ against MVA-infected cells as measured by
791 ICS at the different time points. The box plots showed the distribution of responses in
792 positive responders at weeks 6 and 18. The box indicated the median (solid line), mean
793 (dash line), and interquartile range (IQR). All data are background substracted. p values
794 for significant differences were determined using Wilcoxon rank sum test with
795 continuity correction and are represented. (B) Functional profile of MVA-specific CD8

T cells. The results shown are generated from the determinations in all the responders. All the possible combinations of the responses are shown on the x axis, whereas the percentages of the functionally distinct cell populations within the total CD8 T cell populations are shown on the y axis. Responses are grouped and colour-coded on the basis of the number of functions. The bars correspond to the individual data point and interquartile range (IQR) after 2 (W6) or 3 (W18) doses of MVA-B. The pie charts showed the average proportion of the MVA-specific CD8+ T cell responses according to the functions. (C) Phenotype of long-lived memory MVA-specific T cell responses. Distribution of MVA-specific T cells at week 48 based on CCR7 expression in combination with CD45RA. The bars correspond to the individual data point and interquartile range (IQR) of the CD4+ and CD8+ T cell responses against MVA-infected cells with phenotype central memory (TCM: CD45RA−CCR7+), effector memory (TEM: CD45RA−CCR7−) or terminal effector memory (TEMRA: CD45RA+CCR7-). The pie charts showed the average proportion of the CD4+ or CD8+ MVA-specific T cell responses according to the memory phenotype. * represent distributions that are different from the CD4 T cell subset at $p<0.05$ (Student T test). All data are background substracted.

TABLE 1: Vaccine responsiveness based on IFN-γ+ ICS assay across the RISVAC02 study

Vaccination group	**Antigen**	**Week 6** (2 Wks post 2nd)		**Week 18** (2 Wks post 3rd)		**Week 48** (30 Wks post 3rd)		**Cumulative** (Any post vacc)	
		CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
MVA-B (10^8 PFU)	Env	7/12 (58.3%)	4/12 (33.3%)	3/13 (23.1%)	5/13 (38.5%)	5/13 (38.5%)	5/13 (38.5%)	**9/13 (69.2%)**	**8/13 (61.5%)**
	Gag	1/12 (8.3%)	3/12 (25.0%)	0/13 (0%)	5/13 (38.5%)	1/13 (7.7%)	6/13 (46.1%)	**2/13 (15.4%)**	**9/13 (69.2%)**
	GPN	0/12 (0%)	3/12 (25.0%)	0/13 (0%)	4/13 (30.8%)	1/13 (7.7%)	5/13 (38.5%)	**1/13 (7.7%)**	**9/13 (69.2%)**
	Any	**7/12 (58.3%)**	**6/12 (50.0%)**	**3/13 (23.1%)**	**8/13 (61.5%)**	**5/13 (38.5%)**	**9/13 (69.2%)**	**9/13 (69.2%)**	**12/13 (92.3%)***
Placebo	Env	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	**0/3 (0%)**	**0/3 (0%)**
	Gag	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	**0/3 (0%)**	**0/3 (0%)**
	GPN	1/3++ (33.3%)	1/3++ (33.3%)	0/3 (0%)	0/3 (0%)	1/3++ (33.3%)	0/3 (0%)	1/3++ (33.3%)	1/3++ (33.3%)
	Any	**0/3 (0%)**	**0/3 (0%)**	**0/3 (0%)**	**0/3 (0%)**	**0/3 (0%)**	**0/3 (0%)**	**0/3 (0%)**	**0/3 (0%)**

An ICS was considered positive if the percentages of IFN-γ+ cells in the stimulated samples were 3 times over the values obtained in the unstimulated controls and if the background-substracted magnitudes were higher than 0.02%. One volunteer at week 6 did not have data. Cumulative analysis represents a positive response at any time point post-vaccination.

++One placebo recipient was excluded for the cumulative analysis due to the reactivity against GPN pool at baseline and at subsequent time points. For this reason, the GPN pool was excluded in the comparison of the cumulative responses between vaccinees and placebo groups.

* The differences among cumulative proportions between vaccinees and placebo groups have been tested by comparing two binomial distributions (implemented by the R function prop. test).

Figure 1

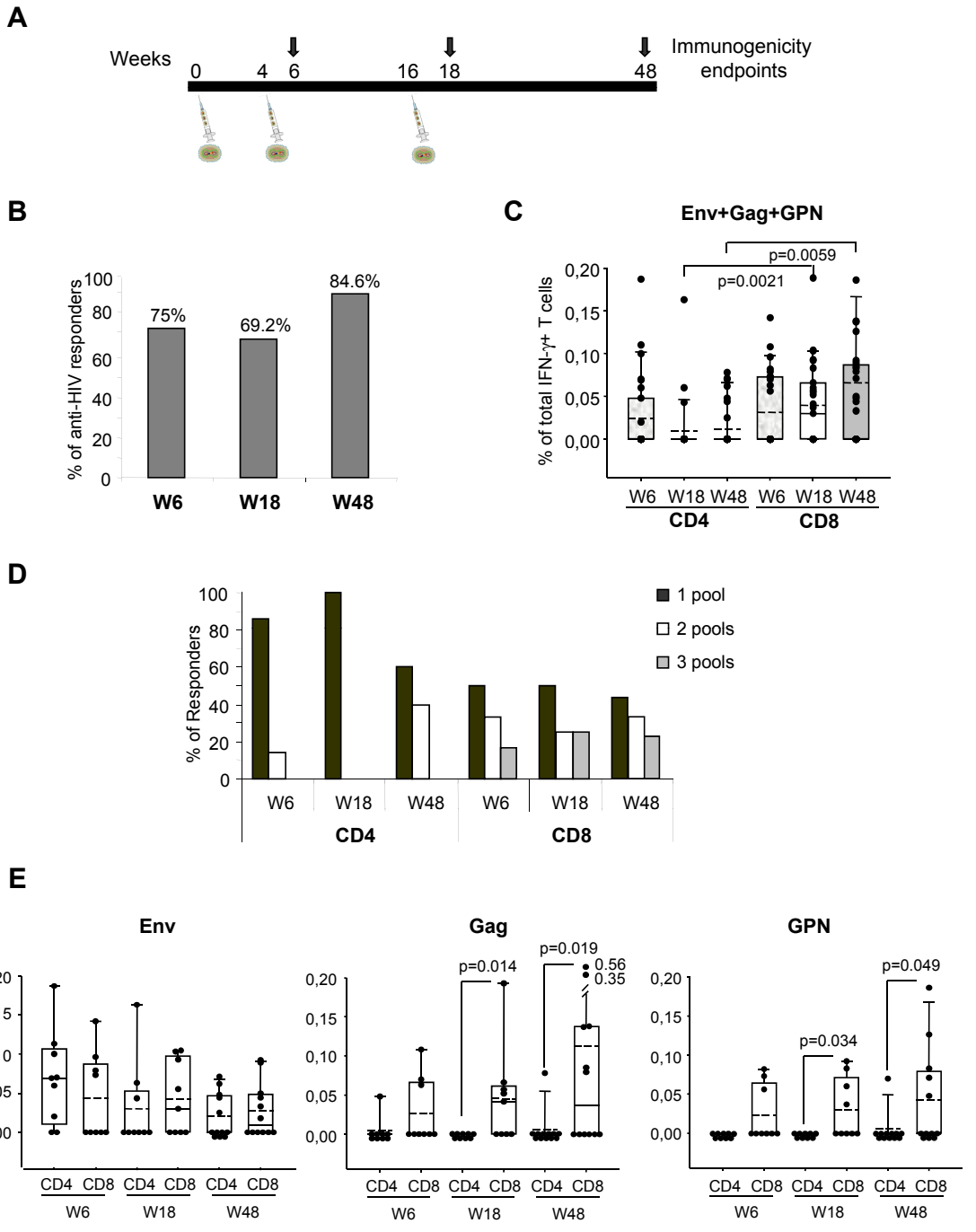

Figure 2

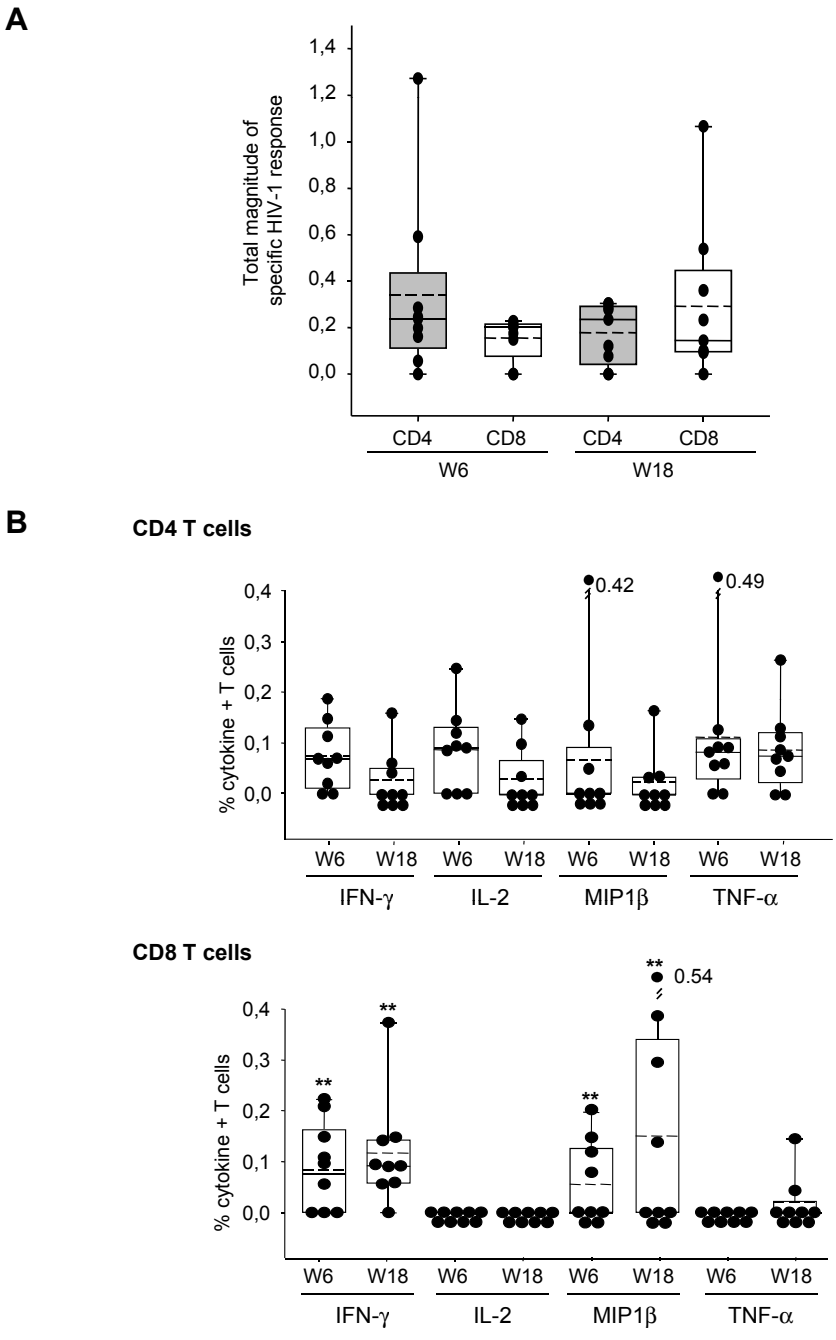

Figure 3

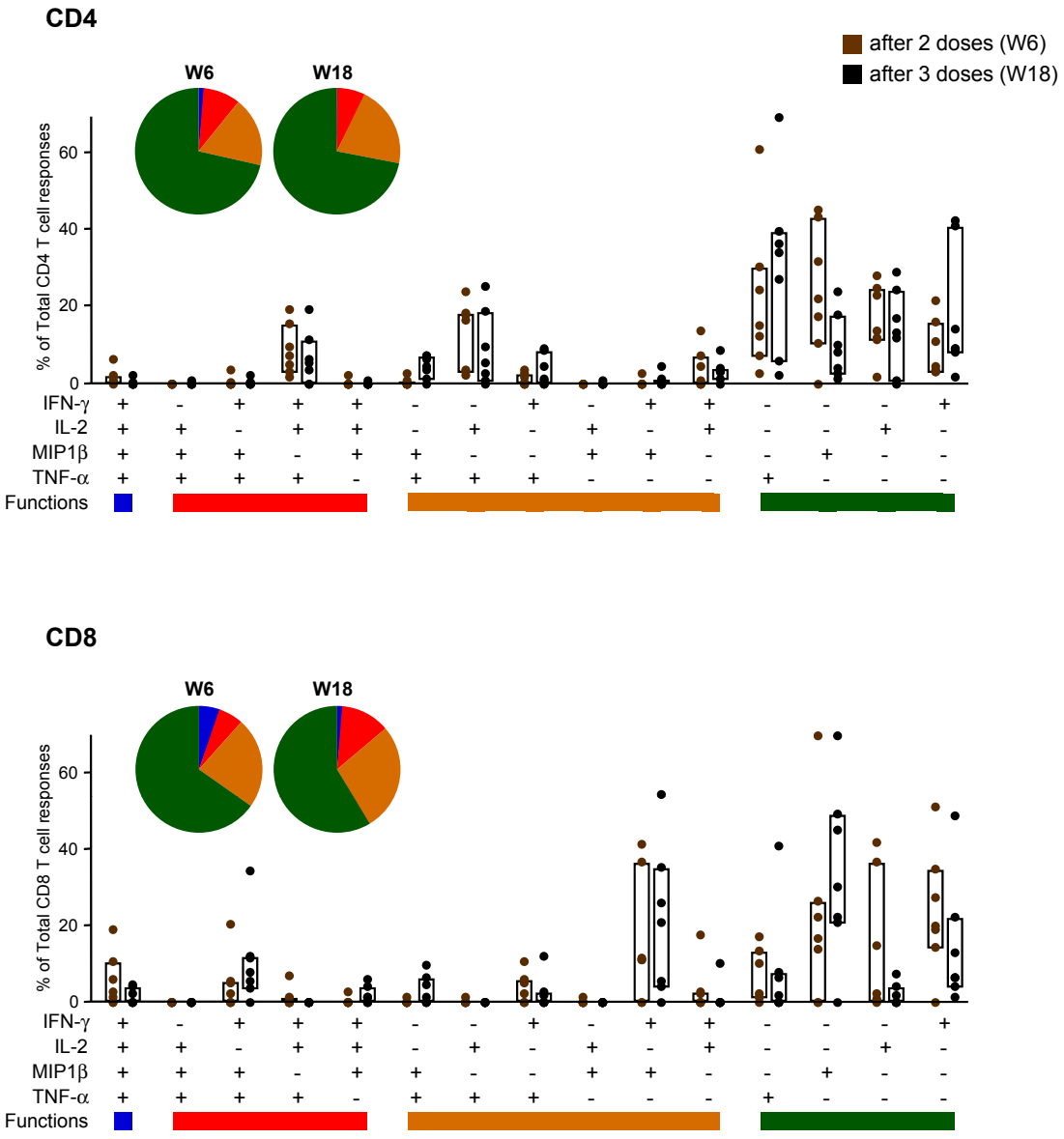

Figure 4

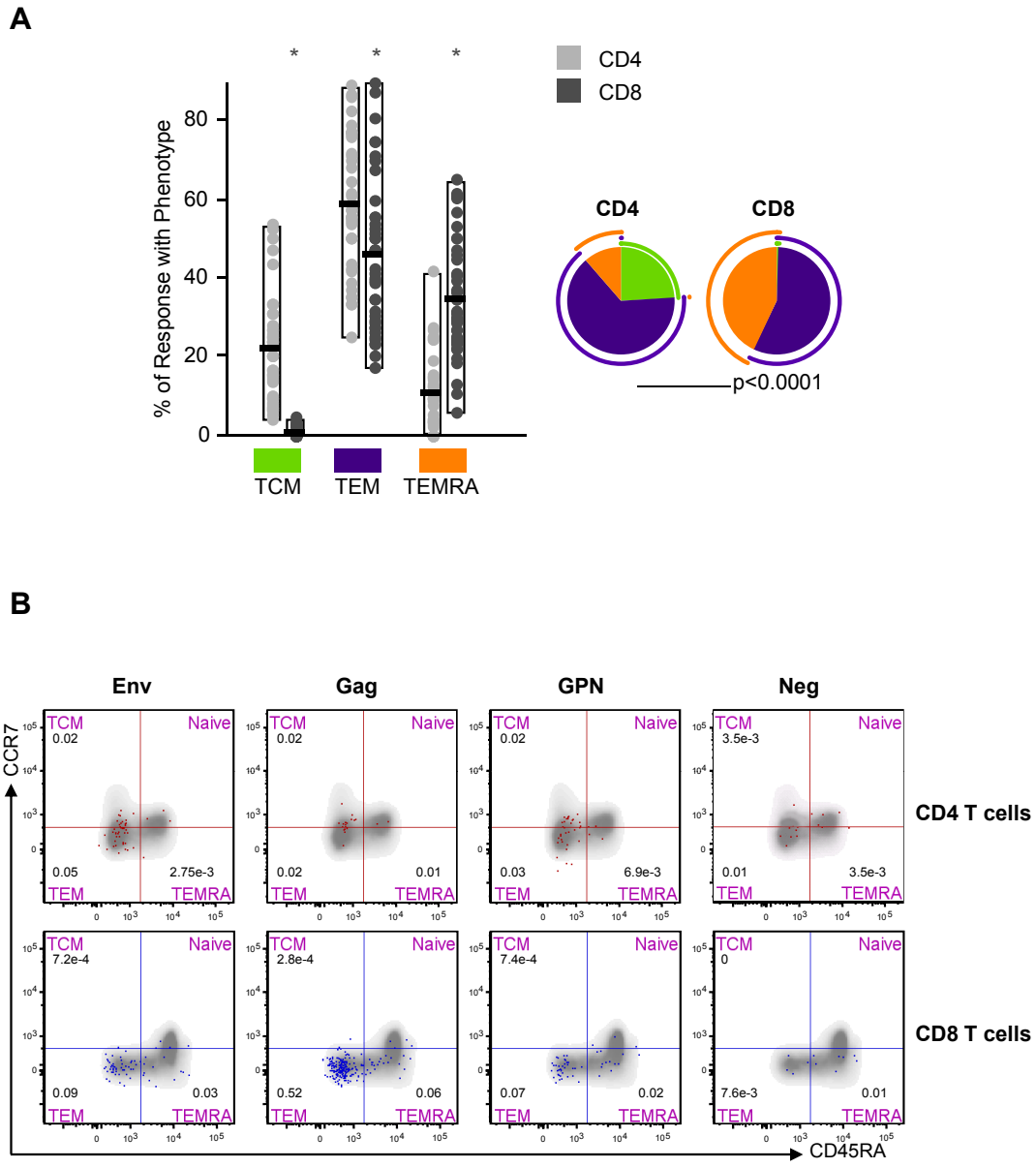

Figure 5