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Synthetic long peptide booster immunization in rhesus macaques primed with replication competent NYVAC-C-KC induces a balanced CD4/CD8 T-cell and antibody response against the conserved regions of HIV-1

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Abstract:	The Thai trial (RV144) indicates that a prime/boost vaccine combination that induces both T-cell and antibody responses may be desirable for an effective HIV vaccine. We have previously shown that immunisation with synthetic long peptides (SLP), covering the conserved parts of SIV, induced strong CD4 T-cell and antibody responses, but only modest CD8 T-cell responses. To generate a more balanced CD4/CD8 T-cell and antibody response, this study evaluated a pox-vector prime/SLP boost strategy in rhesus macaques. Priming with a replication competent NYVAC, encoding HIV-1 clade C gag, pol, nef, induced modest IFNγ T-cell immune responses, predominantly directed against HIV-1 gag. Booster immunization with SLP, covering the conserved parts of HIV-1 gag, pol, env, resulted in a more than 10 fold increase in IFNγ ELISpot responses in 4 of 6 animals, which were predominantly HIV-1 Pol-specific. The animals showed a balanced polyfunctional CD4 and CD8 T-cell response and high Ab titers.

Synthetic long peptide booster immunization in rhesus macaques primed with replication competent NYVAC-C-KC induces a balanced CD4/CD8 T-cell and antibody response against the conserved regions of HIV-1.

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Summary

The Thai trial (RV144) indicates that a prime/boost vaccine combination that induces both T-cell and antibody responses may be desirable for an effective HIV vaccine. We have previously shown that immunisation with synthetic long peptides (SLP), covering the conserved parts of SIV, induced strong CD4 T-cell and antibody responses, but only modest CD8 T-cell responses. To generate a more balanced CD4/CD8 T-cell and antibody response, this study evaluated a pox-vector prime/SLP boost strategy in rhesus macaques. Priming with a replication competent NYVAC, encoding HIV-1 clade C *gag, pol, nef,* induced modest IFNγ T-cell immune responses, predominantly directed against HIV-1 gag. Booster immunization with SLP, covering the conserved parts of HIV-1 gag, pol, env, resulted in a more than 10 fold increase in IFNγ ELISpot responses in 4 of 6 animals, which were predominantly HIV-1 Pol-specific. The animals showed a balanced polyfunctional CD4 and CD8 T-cell response and high Ab titers.

Keywords:

HIV vaccine; conserved regions; synthetic long peptide; NYVAC-C-KC; replication competent pox-vector; non-human primate

Ideally a vaccine against human immunodeficiency virus type 1 (HIV-1) should be capable of inducing broadly neutralizing antibodies as well as effective T-cell responses (Walker & Burton, 2008). Although these goals have not yet been achieved, results from the phase III Thai trial (RV144) indicate that with a recombinant canarypox (ALVAC-HIV, vCP1521) prime – gp120 (AIDSVAX B/E) protein boost immunization strategy, that induces both CD4 T-cell as well as antibody responses, the risk of acquiring HIV-1 infection is decreased (Haynes *et al.*, 2012; Rerks-Ngarm *et al.*, 2009).

Three of the best characterized highly attenuated pox vectors are ALVAC, Modified Vaccinia virus Ankara (MVA) and NYVAC (Drexler *et al.*, 2004; Franchini *et al.*, 2004; Gomez *et al.*, 2011; Paoletti *et al.*, 1994). NYVAC was derived from the parental Copenhagen strain by deletion of 18 specific open reading frames, including the host range genes K1L and C7L. Reinsertion of these two genes resulted in an improved vaccine vector, designated NYVAC-KC, which yielded higher levels of antigen expression in infected cells, was replication competent in human keratinocytes and dermal fibroblasts, but maintained a highly attenuated phenotype (Kibler *et al.*, 2011). In addition, NYVAC-KC showed enhanced capacity to stimulate dendritic cell maturation, antigen processing and presentation and stimulation of CD8 T-cell responses through cross presentation (Quakkelaar *et al.*, 2011).

Synthetic long peptides (SLP) are a relatively novel vaccine modality designed as approximately 30-mer peptides overlapping by 10 to 15 amino acids. The peptide length strongly favours processing by ‘professional’ antigen-presenting cells instead of direct binding to major histocompatibility complex class I molecules on the cell surface and this provides a parallel stimulation of both CD4 T-helper and CD8

cytotoxic T-cells (Melief & van der Burg, 2008; Zhang *et al.*, 2009). Using this approach for human papilloma virus 16 we have previously demonstrated a clinical benefit in patients with high-risk human papilloma virus type 16-induced premalignant vulvar lesions (Kenter *et al.*, 2009; Welters *et al.*, 2010).

Both broad neutralizing antibody and T-cell inducing vaccines face the problem of the extreme variability of the HIV-1 genome. To address HIV-1 variability and escape, a novel pan-clade immunogen HIVconsv was assembled, derived from the 14 most conserved regions of the HIV-1 consensus proteomes (Letourneau *et al.*, 2007). It was previously demonstrated that immunization with SLP, covering the HIVconsv sequence greatly enhanced the breadth and overall magnitude of the CD4 and CD8 T-cell response in DNA.HIVconsv/human adenovirus serotype 5 HAdV5.HIVconsv/ MVA.HIVconsv immunized animals, but was less effective when used for priming (Rosario *et al.*, 2012; Rosario *et al.*, 2010). However, improved adjuvantation via simultaneous injection of pegylated type I IFN resulted in induction of high immune responses after two immunizations with SLP.SIVconsv only (Koopman *et al*, 2013). In addition, increased expression of TRAIL on NK cells and CD80 on plasmacytoid dendritic cells was noted 2 days following SLP immunization in the presence of type I IFN, suggesting enhanced activation of the innate immune system. In contrast to the HIVconsv prime/boost strategies described by Rosario et al, the type I IFN adjuvanted SLP was found to induce predominantly CD4 T-cell responses of central memory phenotype, while only modest CD8 T-cell responses with limited breadth were generated (Koopman *et al.*, 2013).

Here, we explored the possibility of obtaining a more balanced CD4/CD8 T-cell response by using a pox-vector prime/SLP boost strategy, employing the recently

95 developed replication competent NYVAC vector plus type I IFN adjuvanted
96 SLP.HIVconsv.

97 The current study was performed in six mature captive-bred Indian origin
98 rhesus monkeys (R1-R6, *Macaca mulatta*), housed at the Biomedical Primate
99 Research Centre, Rijswijk, The Netherlands, according to international guidelines for
100 non-human primate care and use (The European Council Directive 86/609/EEC, and
101 Convention ETS 123, including the revised Appendix A). The animals were negative
102 for antibodies to SIV-1, simian type D retrovirus and simian T-cell lymphotropic
103 virus. During the course of the study, the animals were checked twice daily for
104 appetite and general behaviour and stools were checked for consistency. At each
105 sedation (ketamine 10mg/kg) for blood collection or immunization the body weight
106 and body temperature were measured. The Institutional Animals Care and Use
107 Committee (DEC-BPRC) approved the study protocols developed according to strict
108 international ethical and scientific standards and guidelines. The rhesus macaques
109 were immunized at week 0, 4 and 12 by intradermal injection of NYVAC-C-KC-
110 Gag(ZM96)-Pol-Nef(CN54) (10^8 pfu per immunization). The generation of
111 replication competent NYVAC-C-KC expressing the HIV-1 Clade C genes Gag (from
112 isolate 96ZM651 (Acc.Nr. AF286224), abbreviated "ZM96") and PolNef (from
113 isolate 97CN54 (Acc.Nr. AX149647.1), abbreviated "CN54"), will be described
114 elsewhere, and a similar vector expressing Gag-Pol-Nef from clade C CN54 was
115 described previously (Kibler *et al.*, 2011). The replication competent NYVAC vector
116 containing the cassette Gag (ZM96) and Pol-Nef (CN54) produces mainly Gag as
117 VLPs as cell-released products and to a lesser extent Pol-Nef due to a ribosomal
118 frame-shift (Perdiguero *et al.*, 2014). The NYVAC vector was grown in primary CEF
119 cells and purified by sedimentation through two 36% sucrose cushions. Virus titers

were determined by plaque assay in monkey BSC-40 cells. Subsequently, animals were boosted twice at week 58 and 62 with SLP.HIVconsv, given in a decreasing dose range of 100 and 30 μg of each peptide. SLP used in this study were based on the previously described HIVconsv sequence (Letourneau *et al.*, 2007) and comprised a set of 33 peptides ranging in length from 26 to 27 amino acids (aa) and covering the Gag1,2,3, Pol 4,5,7,8,10 and Env 9,14 regions. Synthetic peptides were dissolved in 20% dimethyl sulfoxide (DMSO), 20 mM PBS (pH 7.5) and divided into five sub-pools; pp1 Gag1,2,3; pp2 Pol4; pp3 Pol5; pp4 Pol7,8,10; pp5 Env 9,14. On the day before vaccination, peptide pools were emulsified in Montanide ISA-720 (Seppic, Paris, France) adjuvant (DMSO/PBS/Montanide ISA-720 3:27:70, v/v/v) and kept at 4 °C. Stability was checked as described before (Miles *et al.*, 2005). Each of the 5 vaccine peptide pools was injected s.c. at a separate site (right upper arm, left upper arm, right upper thigh, left upper thigh, lower back). Simultaneously, a dose of pegylated type I IFN (1 μg/kg) was given by s.c. injection. At the end of the procedure and again 48 hours later, the animals received on the injection sites topical imiquimod containing cream (Aldara Cream 5%, 12.5 mg imiquimod/250 mg cream) to enhance immunogenicity (Lore *et al.*, 2003; Othoro *et al.*, 2009).

In order to evaluate the immune potency of the prime with replication competent NYVAC-C-KC (Quakkelaar *et al.*, 2011), PBMC from six immunized animals were isolated using LSM density gradient centrifugation (Organon-Teknica) and tested for antigen-specific IFNγ secretion by ELISPOT assay as described (Koopman *et al.*, 2008). As shown in Fig. 1A, clearly detectable antigen-specific IFNγ ELISpot responses were observed already after one immunization. These responses were further increased after the second immunization ($p = 0.049$, t-test), but could not be boosted anymore by a third NYVAC-C-KC immunization ($p = 0.634$, t-test) probably

because of the induction of high anti-vector responses (2850 ± 1740 spot forming units (SFU) per 10^6 PBMC (not shown). Responses were modest, predominantly directed against gag (Fig. 1A), most probably because of a higher production of Gag VLPs than of Pol-Nef, due to the nature of the NYVAC vector that makes mainly extracellular VLPs (Perdiguero *et al.*, 2014). Responses were too low to further characterize multifunctionality by ICS.

At week 56, 44 weeks after the last NYVAC-C-KC immunization, memory T-cell responses measured against HIV-1 Gag (ZM96, 2 pools), the most dominant antigen after priming, were found to be negative in all animals (not shown). Likewise no IFNγ ELISpot responses were seen when animals were tested against the five conserved peptide pools (Fig. 1B). In contrast to the Gag dominated responses seen after priming, the SLP booster immunization induced besides responses against Gag also strong responses to Pol peptide pools. With two SLP booster immunizations, responses were amplified to above 2000 SFU/10^6 PBMC, but only in four out of six animals, the other two animals (R3 and R4) generating about 500 SFU/10^6 PBMC. In contrast, previously reported SLP booster immunizations in DNA.HIVconsv/ HAdV5.HIVconsv/MVA.HIVconsv primed animals or DNA.SIVconsv primed animals gave a more uniform induction of high responses in all animals over time (Koopman *et al.*, 2013; Raab *et al.*, 2010). Possibly, the application of different antigenic inserts, used for NYVAC priming and SLP booster immunization, may have contributed to less effective triggering of memory responses in some animals in this study, despite the considerable sequence overlap between the antigens (supplementary figure 1). Genetic differences, for instance in MHC or KIR expression pattern (not tested) may have resulted in less efficient peptide presentation or innate immune stimulation in animals R3 and R4, but this remains speculative. Importantly, even

though the responses against Pol were very low after priming, the SLP booster immunization resulted in high Pol specific responses in three animals, indicating that HIVconsv specific cross reactive memory responses can be triggered. Although Env was only included during boosting, still modest responses were induced in two animals (Fig. 1B). The preferential amplification of Pol over Gag specific responses may be related to the composition of the SLP.HIVconsv immunogen, which contained 7 Gag, 21 Pol and 5 Env peptides.

Further functional characterization of vaccine-induced cellular immune responses for detection of IFN-γ, IL-2, and TNF-α (cytokine production, within CD4 and CD8 T-cell subsets was performed by multiparameter flow cytometry (for FACS plot analysis, see supplementary figure 2) at the end of the study when animals were sacrificed and sufficient PBMC could be obtained for this extensive analysis, as described (Koopman *et al.*, 2013). In the four animals with a high IFNγ ELISpot response at week 70, strong antigen-specific CD4 and CD8 T-cell responses were observed (Fig. 2A). Both CD4 and CD8 T-cell responses were polyfunctional with 10-15% triple IFN-γ, IL-2, TNF-α production (Fig. 2B). All six animals were included in this analysis. When comparing these results with IFNγ ELISpot and ICS responses induced by either SLP alone (SSS) and DNA prime and SLP boost (DDSS) described before (Koopman *et al.*, 2013, supplementary figure 3), it becomes clear that the magnitude and diversity of the IFNγ ELISpot responses as well as the antigen specific CD4 T-cell responses (ICS) were similar between the different immunization strategies (supplementary figure 3A and B, left and middle panels). However, NYVAC priming followed by SLP boosting (NNNSS) induced higher CD8 T-cell responses than DNA priming/SLP boosting (DDSS) (supplementary figure 3A, right panel, $p = 0.009$, Mann-Whitney). More importantly, the diversity of the response was

highest after NNNSS immunization (B, right panel, NNNSS versus SSS: $p = 0.009$ and NNNSS vs DDSS: $p = 0.003$, Mann-Whitney), indicating that NYVAC priming followed by SLP boosting induced the most balanced CD4/CD8 T-cell response (both CD4 and CD8 T-cell responses with highest CD8 T-cell diversity).

Antibody responses to SLP.HIVconsv peptides measured by standard ELISA techniques (Koopman *et al.*, 2013) were not induced by NYVAC-C-KC immunization (measured at 4 and 44 weeks post third immunization, not shown). Although it cannot be excluded that at these time points some antibody reactivity exists against the whole Gag and Pol proteins, this is unlikely as these responses should have been detected with the SLP.HIVconsv peptides due to the considerable sequence overlap between the Gag and Pol antigens (supplementary figure 1). However, all SLP.HIVconsv boosted animals had strong antibody responses against SLP.HIVconsv peptides (8 weeks post 2^{nd} boost, Fig. 3).

The increase in HIVconsv specific responses in animals primed with a divergent immunogen suggests that this strategy might also be useful for therapeutic vaccination in HIV-1 infected people. However, lack of pre-existing memory responses against the HIVconsv immunogen might be an issue, resulting in either poor enhancement or induction of responses with limited breadth, a phenomenon also observed in two of our SLP boosted animals. This NYVAC-C-KC prime/SLP.HIVconsv booster vaccination strategy demonstrated proof-of-concept induction of balanced CD4/CD8 T-cell responses and antibody responses, albeit not in all animals immunized. Potentially, this strategy could increase the level of protection against intrarectal SIVmac251 challenge that was obtained in a SIVconsv DNA prime/SLP boost strategy (Koopman *et al.*, 2013). Unfortunately, this could not be tested in this particular study because of the HIV origin of the immunogens.

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Conflict of interest:

C.J.M. Melief has a 2% stock appreciation share in ISA Pharmaceuticals and is CSO of this biotech company. ISA Pharmaceuticals has licensed from LUMC the technology for application of synthetic peptide vaccine against high-risk HPV and several other targets.

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336 **Figure legends**

337

338 **Fig. 1.** IFNγ ELISpot responses. (a) Antigen specific responses during NYVAC-C-
339 KC priming (week 0-14), against clade C peptide pools. (b) Antigen specific
340 responses during SLP.HIVconsv boosting (week 56-70), against five conserved
341 peptide pools. Please note the difference in scales used for the Y-axis.

342

343 **Fig. 2.** Antigen specific cytokine responses measured by ICS. (a) Magnitude of
344 combined IFNγ, IL-2, TNFα cytokine response measured after the second
345 SLP.HIVconsv booster immunization (week 70). Expressed is the percentage of
346 positive CD4 and CD8 T-cells, specified for each of the five peptide pools. (b)
347 Cytokine expression pattern of total antigen specific response (mean responses of all 5
348 peptide pools combined of all six animals) in CD4 and CD8 T-cells. Pies indicate the
349 relative number of cells expressing one (dark), two (dark grey) or three (light grey)
350 cytokines. Arcs indicate production of IFN-γ, IL-2 and TNF-α.

351

352 **Fig. 3.** Antibody responses. Antibody responses in serum against the HIVconsv long
353 peptides, measured at week 70. Shown is dilution titre of positive response. Lowest
354 dilution tested =1:100.

Figure
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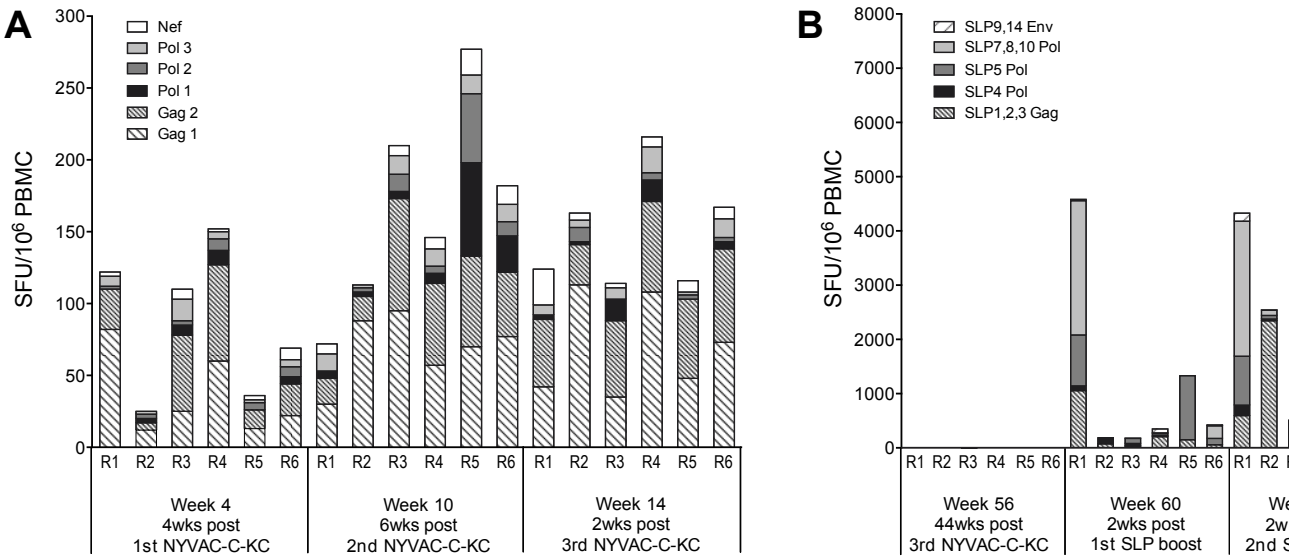

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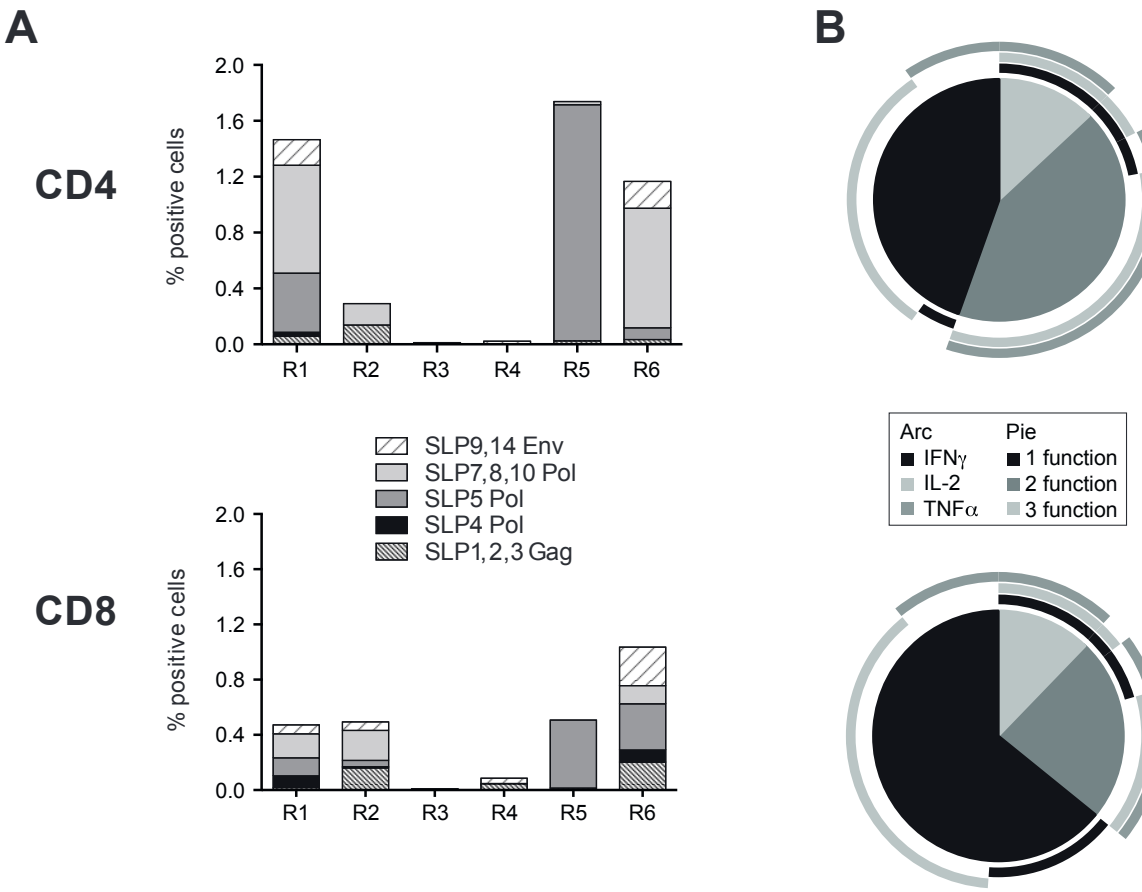

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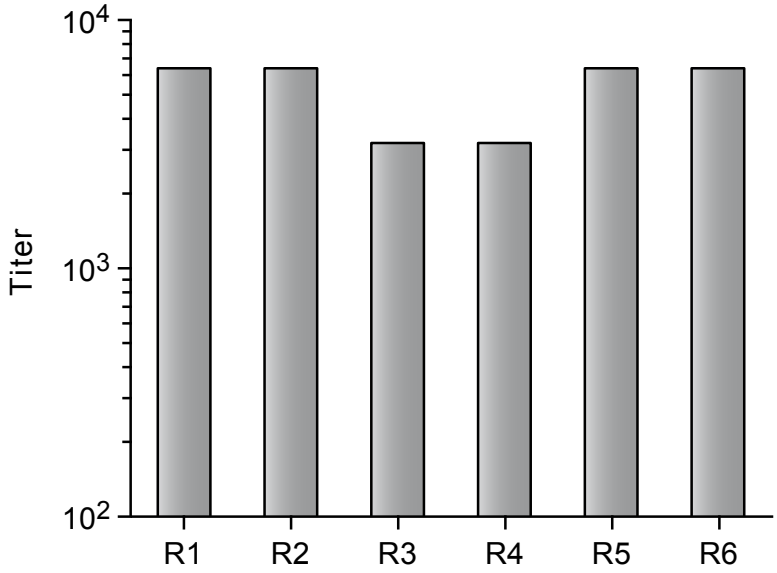

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