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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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 immunization 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 and 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 and Env, resulted in a more than 10-fold increase in IFNγ ELISPOT responses in four of six animals, which were predominantly HIV-1 Pol-specific. The animals showed a balanced polyfunctional CD4 and CD8 T-cell response and high Ab titres.

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; Gómez 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 and was replicative 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 rather than 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, HIVcons, was assembled, derived from the 14 most conserved regions of the HIV-1 consensus proteomes (Létourneau et al., 2007). It was previously demonstrated that immunization with SLP covering the HIVcons sequence greatly enhanced the breadth and overall magnitude of the CD4 and CD8 T-cell response in DNA.HIVcons/human adenoavirus serotype 5 HAdV5.HIVcons/MVA.HIVcons-immunized animals, but was less effective when used for priming (Rosario et al., 2010, 2012;). However, improved adjuvantaon via simultaneous injection of pegylated type I IFN resulted in induction of high immune responses after two immunizations with SLP.SIVcons 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 HIVcons 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 developed replication-competent NYVAC vector plus type I IFN adjuvanted SLP.HIVcons.

The current study was performed in six mature captive-bred Indian-origin rhesus monkeys (R1-R6, Macaca mulatta), housed at the Biomedical Primate Research Centre, Rijswijk, The Netherlands, according to international guidelines for non-human primate care and use (The European Council Directive 86/609/EEC and Convention ETS 123, including the revised Appendix A). The animals were negative for antibodies to SIV-1, simian-type D retrovirus and simian T-cell lymphotropic virus. During the course of the study, the animals were checked twice daily for appetite and general behaviour and stools were checked for consistency. At each sedation (ketamine 10 mg kg\(^{-1}\)) for blood collection or immunization, the body weight and body temperature were measured. The Institutional Animals Care and Use Committee (DEC-BPRC) approved the study protocols developed according to strict international ethical and scientific standards and guidelines. The rhesus macaques were immunized at weeks 0, 4 and 12 by intradermal injection of NYVAC-C-KC-Gag(ZM96)-Pol-Nef(CN54) (10\(^6\) p.f.u. per immunization). The generation of replication-competent NYVAC-C-KC expressing the HIV-1 clade C genes Gag (from isolate 96ZM651, Acc.Nr. AF286224, abbreviated ‘ZM96’) and Pol-Nef (from isolate 97CN54, Acc.Nr. AX149647.1, abbreviated ‘CN54’), will be described elsewhere, and a similar vector expressing Gag-Pol-Nef from clade C CN54 was described previously (Kibler et al., 2011). The replication-competent NYVAC vector containing the cassette Gag (ZM96) and Pol-Nef (CN54) produces mainly Gag as VLPs as cell-released products and, to a lesser extent, Pol-Nef due to a ribosomal frame-shift (Perdigueru et al., 2015). The NYVAC vector was grown in primary CEF cells and purified by sedimentation through two 36% sucrose cushions. Virus titres were determined by plaque assay in monkey BSC-40 cells. Subsequently, animals were boosted at weeks 58 and 62 with SLP.HIVcons, 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 HIVcons sequence (Létourneau 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; Pol4; pp3 Pol5; pp4 Pol7,8,10 and pp5 Env regions. Synthetic peptides were dissolved in 20% DMSO, 20 mM PBS (pH 7.5) and divided into five subpools; pp1 Gag1,2,3; pp2 Pol4; pp3 Pol5; pp4 Pol7,8,10 and pp5 Env 9,14. On the day before vaccination, peptide pools were emulsified in Montanide ISA-720 (Seppic) adjuvant (DMSO/PBS/Montanide ISA-720 3:27:70, by vol.) and kept at 4 °C. Stability was checked as described previously (Miles et al., 2005). Each of the five vaccine peptide pools was injected s.c. at a separate site (right upper arm, left upper arm, right upper thigh, left upper thigh and lower back). Simultaneously, a dose of pegylated type I IFN (1 μg kg\(^{-1}\)) was given by s.c. injection. At the end of the procedure and again 48 h later, the animals received topical imiquimod containing cream (Aldara Cream 5%; 12.5 mg imiquimod/250 mg cream) at the injection sites to enhance immunogenicity (Loré 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-Teknika) and tested for antigen-specific IFNγ secretion by...
ELISpot assay as described (Koopman et al., 2008). As shown in Fig. 1(a), clearly detectable antigen-specific IFNγ ELISpot responses were already observed after one immunization. These responses were further increased after the second immunization \((P=0.049, t\text{-test})\), but could not be boosted further by a third NYVAC-C-KC immunization \((P=0.634, t\text{-test})\), probably because of the induction of high anti-vector responses \([(2850 ± 1740) \text{ spot-forming units (SFU)} \text{ per} 10^6 \text{ PBMC (not shown)}]\). Responses were modest, predominantly directed against Gag (Fig. 1b), 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., 2015). 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, two 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 also induced, besides responses against Gag, 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 (Fig. S1, available in the online Supplementary Material). 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, modest responses were nevertheless 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 seven Gag, 21 Pol and five 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 multi-parameter flow cytometry (for FACS plot analysis, see Fig. S2) 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 and TNF-α production (Fig. 2b). All six animals were included in this analysis. When comparing these results to IFNγ ELISpot and ICS responses induced by either SLP alone (SSS) or DNA prime and SLP boost (DDSS) described previously (Koopman et al., 2013, Fig. S3), it becomes clear that the

**Fig. 1.** IFNγ ELISpot responses. (a) Antigen-specific responses during NYVAC-C-KC priming (weeks 0–14), against clade C peptide pools. (b) Antigen-specific responses during SLP.HIVconsv boosting (weeks 56–70), against five conserved peptide pools. Please note the difference in scales used for the y-axes.
magnitude and diversity of both the IFN\(\gamma\) ELISPOT responses and the antigen-specific CD4 T-cell responses (ICS) were similar among the different immunization strategies (Fig. S3a, 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) (Fig. S3a, right panel, \(P=0.009\), Mann–Whitney). More importantly, the diversity of the response was highest after NNNSS immunization (Fig. S3b, 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 existed 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 (Fig. S1). However, all SLP.HIVconsv-boosted animals had strong antibody responses against SLP.HIVconsv peptides (8 weeks post-second 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 humans. 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 intra-rectal SIVmac251 challenge previously obtained in a SIVconsv DNA prime/
SLP boost strategy (Koopman et al., 2013). Unfortunately, that could not be tested in this particular study because of the HIV origin of the immunogens.

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References


