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HIVIS-DNA or HIVISopt-DNA priming followed by CMDR vaccinia-based boosts induce both humoral and cellular murine immune responses to HIV

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Abstract

Background: In order to develop a more effective prophylactic HIV-1 vaccine it is important optimize the components, improve Envelope glycoprotein immunogenicity as well as to explore prime-boost immunization schedules. It is also valuable to include several HIV-1 subtype antigens representing the world-wide epidemic.

Methods: HIVIS-DNA plasmids which include Env genes of subtypes A, B and C together with Gag subtypes A and B and RTmut/Rev of subtype B were modified

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as follows: the Envelope sequences were shortened, codon optimized, provided with an FT4 sequence and an immunodominant region mutated. The reverse transcriptase (RT) gene was shortened to contain the most immunogenic N-terminal fragment and fused with an inactivated viral protease vPR gene. HIVISopt-DNA thus contains fewer plasmids but additional PR epitopes compared to the native HIVIS-DNA. DNA components were delivered intradermally to young Balb/c mice once, using a needle-free Biojector® immediately followed by dermal electroporation. Vaccinia-based MVA-CMDR boosts including Env gene E and Gag-RT genes A were delivered intramuscularly by needle, once or twice.

Results: Both HIVIS-DNA and HIVISopt-DNA primed humoral and cell mediated responses well. When boosted with heterologous MVA-CMDR (subtypes A and E) virus inhibitory neutralizing antibodies were obtained to HIV-1 subtypes A, B, C and AE. Both plasmid compositions boosted with MVA-CMDR generated HIV-1 specific cellular responses directed against HIV-1 Env, Gag and Pol, as measured by IFNγ ELISpot. It was shown that DNA priming augmented the vector MVA immunological boosting effects, the HIVISopt-DNA with a trend to improved (Env) neutralization, the HIVIS-DNA with a trend to better (Gag) cell mediated immune reponses.

Conclusions: HIVIS-DNA was modified to obtain HIVISopt-DNA that had fewer plasmids, and additional epitopes. Even with one DNA prime followed by two MVA-CMDR boosts, humoral and cell-mediated immune responses were readily induced by priming with either DNA construct composition. Priming by HIV-DNA augmented neutralizing antibody responses revealed by boosting with the vaccinia-based heterologous sequences. Cellular and antibody responses covered selected strains representing HIV-1 subtypes A, B, C and CRF01_AE. We assume this is related to the inclusion of heterologous full genes in the vaccine schedule.

Keywords: Immunology, Infectious disease, Vaccines, Virology

1. Introduction

The best and most impressive preventive measure against infectious diseases has been vaccination. Over twenty different infectious diseases are preventable today by immunization. However, several serious viral infections, such as human immunodeficiency viruses, most of the herpes viruses and the hepatitis C virus lack efficient vaccines. One common property of these viruses is that they rapidly become chronic or persistent before protective immunity is established. Attempts to develop prophylactic or therapeutic vaccines or a cure against HIV have been going on for more than three decades, with lessons learned from several trials [1, 2, 3, 4, 5]. The Immune space presents variables such as immunoprofiles that can be used to assess and select future vaccine components [6].

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Genetic/DNA vaccines have shown promise for priming of immune responses, both for antibody production and for cell-mediated immunity. In non-human models the induction of protective immunity to infectious diseases and tumors has been impressive [7] [8] [9] [10]. In humans and larger animals, difficulties were encountered with delivery systems and with expression of correct conformational proteins/peptides. A few protective or therapeutic schedules obtained by genetic vaccination were revealed [8, 11, 12, 13].

To obtain broad antibody and cell mediated immunities, the concept of prime-boost has proven effective. Heterologous vaccine strategies incorporating DNA, MVA and or HIV protein have been shown to augment and improve the quality of the immune response in mice [14] [15]. Both these approaches have met with modest success, and at best one of the prophylactic HIV-1 vaccine trials has obtained 60% early and 31,2% endpoint protective effects [16, 17]. These findings emphasize the importance to induce high quality long-term memory, recently acquired by a prime-boost schedule containing heterogeneous HIV-1 components [18, 19, 20] [21].

An HIV-1 vaccine for prophylactic use should be capable of eliciting potent antibody-mediated protection but also cell-mediated immunity against several HIV-1 subtypes, while a therapeutic vaccine is expected to work mainly by cell-mediated immunity and antibody dependent cellular immunities (ADCC) [22, 23].

HIVIS-DNA represents HIV-1 subtypes A, B and C [21, 24, 25]. The HIVIS-DNA has been the priming part followed by a vaccinia-based boost (MVA-CMDR) in several clinical trials that have induced broad and strong and long-lasting humoral and cellular immune reponses [19] [20] [26]. The priming schedule is however complex since several plasmids are combined. We have reduced the complexity in clinical trials by showing that intradermal needle-free delivery gives better immune responses than intramuscular [27], that higher doses in smaller volumes can improve responses [28] and that, in difference to animal studies, a combination of all plasmids results in as good responses as the separation of Gag from Env plasmids [29]. In order to reduce the complexity of the HIVISDNA and retain the reduced DNA amount to be given intradermally and still represent the various HIV antigens in separate plasmids, we optimized Env plasmids further by codon optimization, reduction in length and introduced an FT4 trimerization unit, thus eliminating the Rev plasmid that is not highly immunogenic. The RT plasmid was shortened to include the most prevalent epitopes and expressed together with an enzymatically destroyed PR which harbors several potent epitopes for cell mediated immunity. We thus obtained HIVISopt-DNA which was compared in an experimental system to reveal better immune responses or non-inferiority to HIVIS-DNA, finally for clinical purposes.

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The murine model may be used to assess applicability of several variables that would benefit a human vaccine schedule. Generally we could establish a non-inferiority of the new HIV-DNA priming composition, and since these comparisons were done in parallel we might assume that the optimized HIVISopt-DNA can act to induce broad humoral and cellular immune responses in humans, similar to or better than the HIVIS-DNA, followed by the MVA-CMDR.

Finally, trials in humans need further preparation, such as selection and composition of plasmids in relation to one another, dosing of primes and boosts, and decisions on rapid or protracted immunization schedules to obtain long-term memory. The present aim was to refine immunization components and schedules using a preclinical model.

2. Materials and methods

2.1. Immunogens

Fig. 1 HIVIS-DNA represents HIV-1 subtypes A, B and C as follows, Table 1: Gag p37 A and B, Env gp160 A, B, C, reverse transcriptase (RT) and Rev B [21] [24] [30] [31]. HIVISopt-DNA plasmids consist of gp150 subtypes A, B and C modified to contain FT4C-terminal ends to improve trimerization by T4 fibritin [32] together with p37 Gag A and B plus PRidelRT (aa mut, 413 aa) [33]. In summary, the original immunogen composition of HIVIS-DNA contains a Rev-expressing plasmid, which was exchanged for shortening of the Env plasmids at

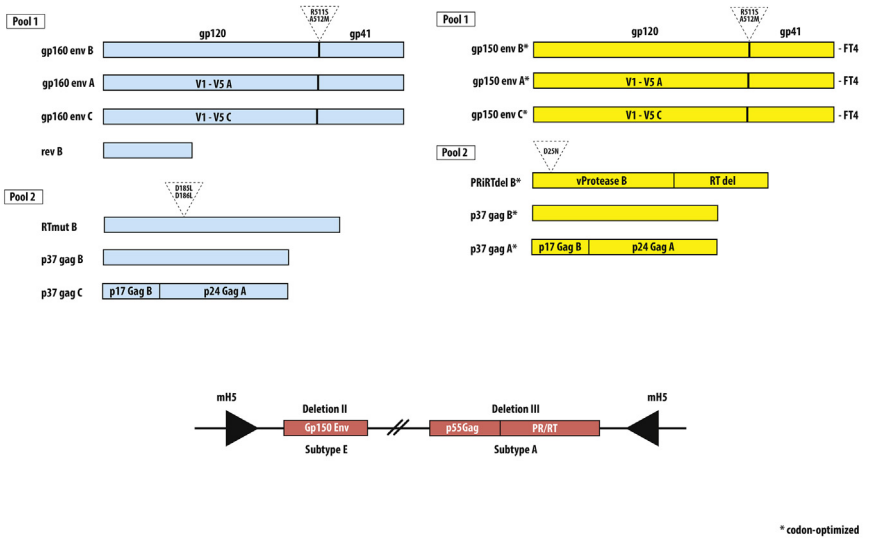

Fig. 1. Overview of HIVIS-DNA and HIVISopt-DNA compounds. Blue color indicates components of HIVIS-DNA, yellow color indicates components of HIVISopt-DNA and red color indicates components of MVA-CMDR.

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Table 1. Immunization schedule of HIVIS-DNA, HIVISopt-DNA and HIV-MVA-CMDR.

Groups of immunized animals	Day 0	Days 21, 42
1 A. 1 x HIVIS-DNA; 1x MVA	DNA id/EP	1 x MVA im
Content	Env A, B, C/Rev	Env E
Content	Gag p37A B, RT B	Gag Pol A
1 B. 1 x HIVIS-DNA; 2 x MVA	DNA id/EP	2 x MVA im
Content	Env A, B, C/Rev	Env E
Content	Gag p37A B, RT B	Gag Pol A
2 A. 1 x HIVIS-DNAopt; 1 x MVA	DNAopt id/EP	2 x MVA im
Content	Env A, B, C	Env E
Content	Gag p37A B, RTPR B	Gag Pol A
2 B. 1 x HIVIS-DNAopt; 2 x MVA	DNAopt id/EP	2 x MVA im
Content	Env A, B, C	Env E
	Gag p37A B, RTPR B	Gag Pol A
3 A. 1 x MVA	MVA im	
Content	Env E	
Content	Gag A, Pol A	
3 B. 2 x MVA	MVA im	1 x MVA im
Content	Env E	Env E
Content	Gag A, Pol A	Gag A, Pol A
4. Naive controls	none	none

Five Balb/c mice were immunized per group. HIV-DNA doses: 200 μg DNA Gag and RT/PR, 200 μg Env A-C, Rev/mouse/immunization; id Bioject and id EP. 107 pfu of MVA-CMDR/mouse was divided equally between the right and left hind legs, 50 μl/side. Mice were sacrificed at day 30 (groups 1A, 2A, 3 A), or days 63-70 (groups 1B, 2B, 3B).

the C-terminal site, providing them with FT4 endings and a change of several nucleotide triplets to improve expression. The immunodominant gp41 region was mutated in all constructs. The RT plasmid was shortened to include the most prevalent epitopes inducing cell mediated immunity (Los Alamos) and expressed together with an enzymatically destroyed PR which harbors several potent epitopes for cell mediated immunity. Gag plasmids were not revised. Fig. 1 shows a schematic picture of vaccine compounds.

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The recombinant vector virus, MVA-CDMR was designed and produced by the National Institutes of Health and Walter Reed Army Institute of Research (MD, USA) to express the following HIV-1 antigens, Gag p55 subtype A, protease and RT of subtype A and Env gp150 subtype E derived from CRF01_AE [34, 35, 36, 37].

In the prime-boost schedule genes for Env covered HIV-1 subtypes A, B, C and E, genes encoding Gag, Rev and RT/PR represented HIV-1 subtypes A and B (Tables 1, 2, 3, 4, 5 and http://www.hiv.lanl.gov/ contain sequence information).

2.2. Animals and immunizations

Female BALB/c mice (6–8 weeks old, H-2D^{d}) were housed at the Astrid Fagraeus animal facility at Karolinska Institutet. HIV-1 DNA-plasmids were dissolved in saline and given intradermally (ID) at two separate injection sites on the shaved flanks of the mice. Plasmids encoding Gag and RT were mixed and given intradermally on the shaved right flank (100 μl volumes, 200 μg, at a DNA concentration of 2 mg/ml). Plasmids encoding Env subtypes A, B and C with (HIVIS-DNA) or without (HIVISopt-DNA) Rev subtype B were mixed and given intradermally on the shaved left flank. Both immunizations were given by Biojector®, immediately followed by electroporation (EP) [15, 38, 39, 40, 41, 42]. EP was performed over the immunization site (DermaVax, Cellectis, Romainville, France; pulse frequency and amplitudes according to Roos et al. [38]). MVA-CMDR was given intramuscularly (IM) twice. Mice immunized with recombinant vaccinia vector were given 10^{7} plaque forming units (PFU) of MVA-CDMR intramuscularly in the hind legs in 50 μl of phosphate buffered saline (PBS) [34]. Sera were sampled before and at 10–20 days after boost immunizations. Spleens were sampled at the end of each study (Table 1). In these, as well as in previous experimental studies, general health and significant and similar growth curves of individual mice participating in the prime-boost studies were noted. Ethical permission was obtained from the Northern Stockholm Board of Animal Ethics dnr N66/13.

2.3. Enzyme-linked immunosorbent assay

ELISA was performed with recombinant HIV-1 antigens: Recombinant proteins (1 μg/ml) Gag p55 (Protein Sciences, CT), Gag p17/p24 (ARP6010, Centre for AIDS Reagents, NIBSC) and CN54 gp140C (Polymun, AU), baculovirus recombinant protein subtype B gp160/LAI (Protein Sciences) and synthetic peptides (10 μg/ml) representing the HIV-1 gp120 subtype B V3-epitope (aa 308–323) or gp41 subtypes A, B, C). Gp41 peptides were synthesized as homologs of the antiretroviral compound T20 (Enfuvirtide, Roche, NL) represent the ELDKWAS epitope of broadly neutralizing human monoclonal antibody 2F5 from subtypes A

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Table 2. HIV-1 neutralizing serum titers (medians and ranges) from 1 x HIVIS-DNA or HIVISopt-DNA prime followed by 2 x MVA-CMDR.

Group		HIV–1 A 92UG29 R5		HIV-1 B 6920 R5		HIV-1C J10687 R5		HIV-1 AE 1525		HIV-1 III B LAI X4
		50% NT	80% NT	50% NT	80% NT	50% NT	80% NT	50% NT	80% NT	50% NT
1B	Median	140	60	360	210	85	60	160	120	760
	Range	(60–320)	(<20–185)	(110- > 540)	(40–300)	(<20–210)	(<20–100)	(110–450)	(75–230)	
	Response	5/5	4/5	5/5	5/5	4/5	2/5	5/5	5/5	5/5
2B	Median	100	20	625	240	220	140	160	75	700
	Range	(30–220)	(<20–100)	(260–880)	(60–540)	(70–300)	(75–170)	(40- > 540)	(<20–540)	
	Response	5/5	2/5	5/5	5/5	5/5	5/5	5/5	3/5	5/5
3B	Median	160	55	80	40	40	<20	60	<20	110
	Range	(25–180)	(<20–140)	(30–170)	(<20–100)	(<20–55)	(<20)	(<20–320)	(<20–20)	
	Response	5/5	2/5	5/5	1/5	2/5	0/5	4/5	0/5	5/5
4	Median	<20	<20	<20	<20	<20	<20	<20	<20	<20
	Range	<20	<20	<20	<20	<20	<20	<20	<20	<20
	Response	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

HIV-1 isolates were used at 25–30 TCID50 concentration. 50% and 80% neutralization titers are show, as well as titer ranges between individuals. No. of responding individuals are shown for 50% neutralization of each strain.

Group 1B received Env components of subtype A (DNA and MVA), B (DNA), C (DNA), E (MVA).

Group 2B received Env components of subtype A (DNA and MVA), B (DNA), C (DNA), E (MVA).

Group 3B received Env components of subtype E (MVA).

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Table 3. ELISpot IFNγ cellular responses against peptide pools representing HIV-1 RT and PR of subtype B.

	Responder frequencies with HIV RT and PR peptides		
	RT I (aa 38–109)	**RT II (aa 110–179)**	**PR (aa 12–38)**
Groups of immunized animals			
Group 1A			
1 x HIVIS; 1 x MVA	3/5	5/5	2/5
Median IFNγ SFC/106	40	45	144
Range	(30–73)	(35–95)	(55–152)
Group 2A			
1 x HIVISopt; 1 x MVA	2/5	3/5	3/5
Median IFNγ SFC/106	33	105	196
Range	(29–112)	(35–95)	(61–1744)
Group 3A			
2 x MVA	1/5	2/5	2/5
Median IFNγ SFC/106	96	167	108
Range	96	(40–294)	(98–118)

Abbreviations: RT I = N-terminal peptides of reverse transcriptase protein subtype B, RT II = C-terminal peptides of reverse transcriptase protein subtype B, PR = peptides representing the protease protein of subtype B.
Group 1A received RT components of subtype A (MVA), B (DNA); no PR.
Group 2A received RT components of subtype A (MVA), B (DNA); PR of subtype B (DNA).
Group 3A received RT components of subtype A (MVA); PR of subtype A (MVA).

(aa 652–661), B (aa 661–676) and C (aa 551–566). The CCR5 peptide represents the cellular HIV-1 co-receptor (Los Alamos database, http://www.hiv.lanl.gov/and Thermohybaid, DE). Microplates (Nunc Maxisorb, DK) were coated with 100 µl/well of each antigen and sera assayed as described [33, 43]. For peptide ELISAs, absorbance values are given for serum dilutions of 1:20.

2.4. Neutralization assay

Viral isolates used for neutralization derived from subtype A 92UG29/WHO strain, subtype B laboratory strain IIIB LAI, HIV-1 B/6920 R5, HIV-1 B/6794 X4, HIV-1C J10687 and HIV-1CRF01_AE 1525 (http://www.hiv.lanl.gov/, Table 2), selected from strains close to the DNA plasmid constructs [37] [31]. The III B LAI is the comparator sequence for subtype B (http://www.hiv.lanl.gov). Sera were studied individually or pooled and inactivated at 56 °C for 1 h to prevent

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Table 4. HIVIS-DNA and HIV-MVA subtypes in prime-boost-boost schedules.

HIV-1 protein expressed	HIV-1 subtype	Name, accession nos.*	Vector	Ref.
HIVIS-DNA prime				
Env gp160	A1	A1.UG92031 KY4773268	pkCMV	Ljungberg et al 2002
Env gp160	B	B.FR.HXB2, KY4773267	pkCMV	Ljungberg et al 2002
Env gp160	C	C.BR.92BR025, KY4773269	pkCMV	Ljungberg et al 2002
Gag p37	A1/B	A1.UG92031 B.FR.HXB2 KY4773265	pkCMV	Bråve et al 2005
Gag p37	B	B.FR.HXB2 KY4773266	pkCMV	Bråve et al 2005
Rev	B	B.FR.HXB2 KY4773270	pkCMV	Kjerrström et al 2001
RTmut	B	B.FR.HXB2 KY4773271	pkCMV	Isaguliants et al 2000
HIV-MVA boost				
Env gp150	E	CRF01_AE CM235	MVA**	Earl et al 2009
Gag p55	A	CRF01_AE CM240	MVA	Earl et al 2009
Pol (RTmut, PRmut)	A	CRF01_AE CM240	MVA	Earl et al 2009

* HIV database: http://www.hiv.lanl.gov and Genbank gb-admin@ncbi.nml.nih.gov.**Modified Vaccinia Ankara MVAp579.

complement-mediated neutralization. Sera were diluted in RPMI 1640 supplemented with 5% FCS and antibiotics (Invitrogen Life Technologies, SE) in 96-well tissue culture plates (Nunc Microwell plates, Pierce Thermo Scientific, Walthman, MA). Sera were assayed individually or pooled from mice with the highest serum IgG titers against the envelope proteins, and a second pool with sera from the animals with the lowest anti-Env IgG titers (Fig. 4). Each dilution was mixed with virus (25–80 $TCID_{50}$) and incubated at 37 °C for 1 h followed by the addition of 10^5 human PBMCs activated by phytohemagglutinin (PHA) and rIL-2 (PeproTech, Rocky Hill, NJ) or CCR5 receptor-rich C8166 rR5 cells. The cells were incubated at 37 °C in 5% CO_2 in air over night, washed twice with RPMI 1640 and received new medium. After 6 further days of culture, the presence of HIV-1 p24 antigen in the culture medium was measured by ELISA for HIV-1 subtypes A and B [43] or the CavidiTech Lenti-RT assay (Uppsala, Sweden) for HIV-1 subtypes C and CRF01_AE. The background of the p24 ELISA was determined for each plate and subtracted from all wells before the percentage neutralization was determined as [1-(mean p24 OD in the presence of test serum/mean p24 OD in the absence of test serum)] × 100. CavidiTech results were calculated as pg of RT/ml.

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Table 5. HIVISopt-DNA and MVA-CMDR subtypes in prime-boost schedules.

HIV-1 proteins expressed	HIV-1 subtype	Name, accession nos.*	Vector	Ref.
HIVISopt-DNA prime				
Env gp150	A1opt	A1.UG92031 KY773272	pkCMV	Ljungberg et al 2002, Hallengärd 2014
Env gp150	Bopt	B.FR.HXB2, KY773273	pkCMV	Ljungberg et al 2002, Hallengärd 2014
Env gp150	Copt	C.BR.92BR025 KY773274	pkCMV	Ljungberg et al 2002, Hallengärd 2014
Gag p37	A1/B	A1.UG92031 B.FR.HXB2 KY773265	pkCMV	Bråve et al 2005
Gag p37	B	B.FR.HXB2 KY773266	pkCMV	Bråve et al 2005
PRidelRT	B	B.FR.HXB2 KY773275	pkCMV	Hallengärd et al 2014
HIV-MVA boost				
Env gp150	E	CRF01_AE CM235	MVA**	Earl et al 2009
Gag p55	A	CRF01_AE CM240	MVA	Earl et al 2009
Pol (RTmut, PRmut)	A	CRF01_AE CM240	MVA	Earl et al 2009

* HIV database: http://www.hiv.lanl.gov and Genbank gb-admin@ncbi.nml.nih.gov.

** Modified Vaccinia Ankara MVAp579.

2.5. ELISpot assays

Cell-mediated immune responses were analyzed by interferon gamma (IFNγ) ELISpot assays (MabTech, Nacka, SWEDEN). Anti-IFNγ pre-coated ELISpot plates were used, and another monoclonal antibody (Mab) AN18 for IFNγ detection. A total of 2×10^5 cells were plated per well and stimulated for 24 h with pools of overlapping peptides of HIV-1 representing MVA-CMDR peptides representing Env (subtype E), Gag (subtype A) and Pol (subtype A) of CRF01_AE (2.5 μg/ml) (Nilsson Wahren 2015) (a kind gift from Dr. J. Cox, WRAIR, MD). Concanavalin A (Con A, 5 μg/ml, Sigma-Aldrich, S:t Louis, MA) was used as a positive control, peptide CCR5 as a background control and culture medium (MDEM with 2 mM L-glutamine, 1% Penicillin-Streptomycin and 5% bovine calf serum) as negative control. ELISpots were developed with biotinylated detection Mab R4-6A2 (1 μg/ml) followed by streptavidine alkaline phosphatase (ALP) and 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium (BCIP/NBT) substrate. The number of spot-forming cells (SFCs) was determined using an ELISpot reader (BioSys, BioReader Autoplate 5000, Karben, Germany). The HIV-1CRF01_AE peptides contain subtype A for Gag and Pol (RT and PR), subtype E for Env (Table 3 and Tables 4 and 5).

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2.6. Statistical analysis

Statistical analyses were performed using Prism 5 GraphPad Software, CA. Statistical methods used were the non-parametric Mann-Whitney U test and Kruskal- Wallis non-parametric analysis with Dunńs correction.

3. Results

3.1. Binding antibodies

An overview of antigens used is given in Fig. 1. Groups of Balb/c mice were primed once by HIV-1HIVIS-DNA (Groups 1 A and B) or HIVISopt-DNA (Groups 2 A and B), followed by one or two MVA-CMDR boosts, or given only MVA-CMDR once or twice (Groups 3 A and B) (Table 1).

Anti-Env gp140C and anti-Env gp160 B (not shown) binding antibodies were primed by both HIVIS-DNA and HIVISopt-DNA and were considerably boosted by the second MVA-CMDR boost (Fig. 2A, $p < 0.01$, $p < 0.05$ and $p < 0.05$). The group primed by HIVISopt-DNA following the first MVA-CMDR boost obtained slightly higher (ns) median titers than the group primed by the native HIVIS-DNA. Following the second MVA-CMDR boost, both DNA primed groups obtained high and similar titers ($p = 0.29$, median titers 10 000–50 000). Without DNA priming, the MVA-CMDR second immunization resulted in significant boosting of anti-Env ($p < 0.05$) with an endpoint median titer of around 5 000. The highest median endpoint titer of around 50 000 against Env was obtained in the HIVISopt-DNA

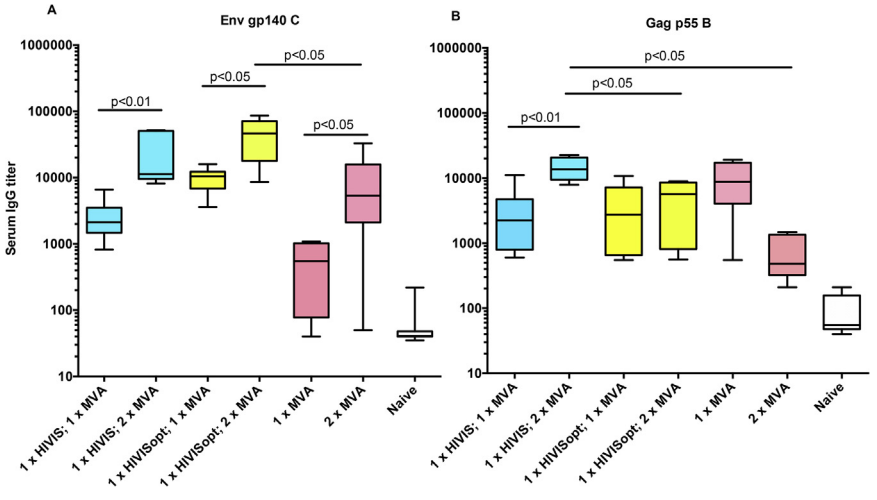

Fig. 2. Antibody titers. A.Serum IgG ELISA titers against HIV-1 Env gp140C. B. Serum IgG ELISA titers against HIV-1 Gag p55 B were compared after one and two MVA-CMDR boost immunizations. Median titers and ranges are shown as box plots. Significant differences after one and two boosts are shown, as analyzed by the non-parametric Mann-Whitney U test. Blue color indicates components of HIVIS-DNA, yellow color indicates components of HIVISopt-DNA and red color indicates components of MVA-CMDR.

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primed group. It is notable that the group primed with HIVISopt-DNA (both primes contain a plasmid expressing Env C) primed group gave higher reactivity to Env gp140C antigen than the non-primed group after two MVA-CMDR immunizations (MVA-CMDR does not contain Env C) (Fig. 2A, $p < 0.05$).

Antibody measurements to Gag p55 B (Fig. 2B) shows that HIVIS-DNA and HIVISopt-DNA induce similar levels of antibodies after the first boost with MVA-CMDR. After the second boost with MVA-CMDR, the HIVIS-DNA had primed antibody titers to Gag to higher levels than HIVISopt-DNA ($p < 0.05$). There is no difference in Gag plasmid composition between HIVIS and HIVISopt immunizations (Table 1). The highest median binding endpoint titer against Gag was obtained in the HIVIS-DNA primed group, around 15 000. A substantial reduction in antibody titers to Gag was observed after the second injection of MVA-CMDR.

Sera from five animals were pooled within groups in an attempt to identify anti-peptide serological immune responses. The gp120 V3 peptide was recognized by all groups (Fig. 3A-C). The Gp41 peptides were selected from sites of HIV-1 subtypes A, B and C strains assumed to induce broad neutralization (see Methods). Here, the gp41 subtype B peptides were clearly recognized by all immunized groups, while the subtype A gp41 peptide was weakly recognized only by HIV-DNA primed animals. Control peptides representing the human HIV co-receptor CCR5 of cellular origin and not present in the vaccines were used to estimate background antibody levels. Pre-immune sera had very low responses to any peptide (Fig. 3D).

3.2. Neutralizing antibodies

Neutralizing titers were assayed against viruses of HIV-1 subtypes A, B, C and CRF01_AE strains (Table 2). Neutralization titers were assessed after HIVIS-DNA or HIVISopt-DNA priming and twice boosted by MVA-CMDR. There was no major difference between the neutralization titers between HIVIS-DNA and HIVISopt-DNA primed mice after 2 MVA-CMDR boosts. Two immunizations with MVA-CMDR also resulted in neutralizing titers to all strains, with generally lower median titers. The HIV-1CRF01_AE strain was neutralized by all groups at titers of 60–160.

DNA priming generally increased median neutralizing titers to subtypes B and C. Prime-boosted groups had the highest median neutralization titers of around 700–760 against the consensus HIV-1 III B strain. Sera from non-immunized controls (and pre-immunization sera, not shown) were all negative (Table 2).

Fig. 4A shows a detailed picture of virus subtype CRF01_AE neutralization, indicating that pooled sera from mice with high binding titers from the HIVISopt-DNA group had IC_{50} titers of 220 while pooled sera with high binding titer from

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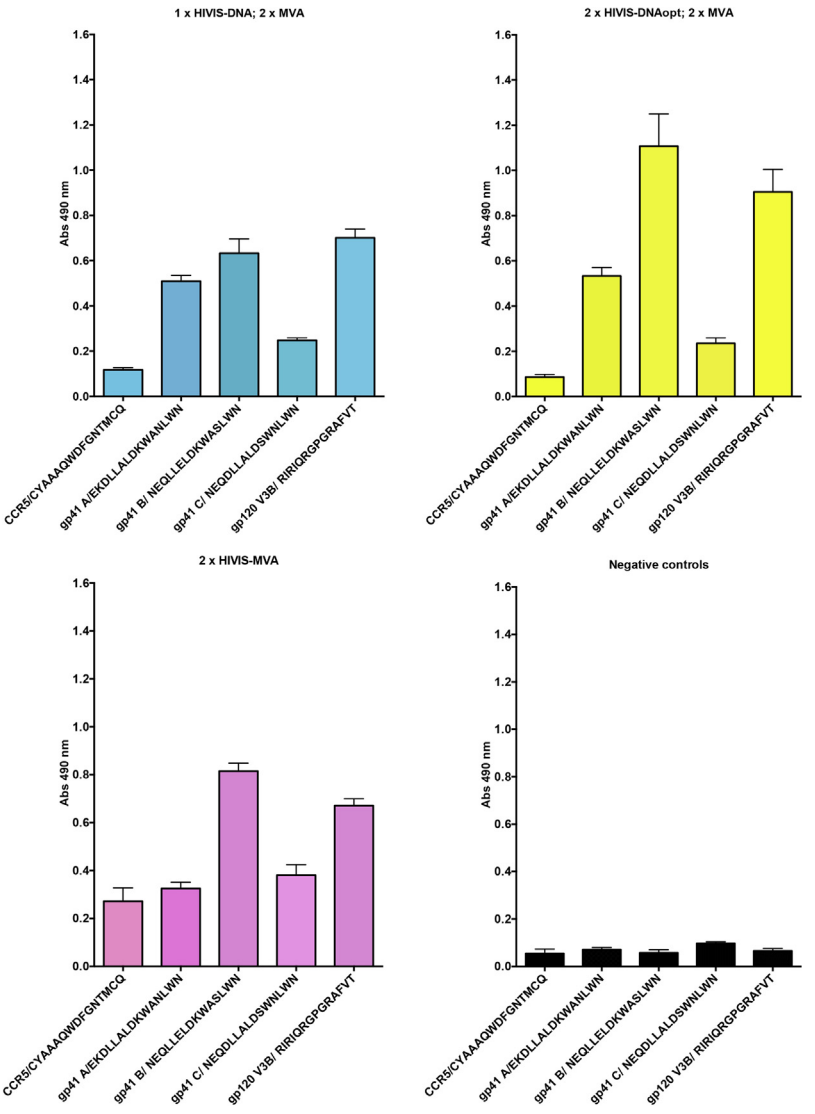

Fig. 3. Anti-peptide gp41 and anti-V3 titers. A. Serum IgG ELISA reactivity of HIVIS-DNA against HIV-1 envelope peptides representing HIV-1 subtypes A, B and C epitopes tested after HIVIS-DNA and two MVA-CMDR boost immunizations. B. Serum IgG ELISA reactivity of HIVISopt-DNA against HIV-1 envelope peptides representing HIV-1 subtypes A, B and C epitopes tested after HIVISopt-DNA and two MVA-CMDR boost immunizations. C. Serum IgG ELISA reactivity of MVA-CMDR against HIV-1 envelope peptides representing HIV-1 subtypes A, B and C epitopes was tested after two MVA-CMDR boost immunizations. D. Serum IgG ELISA reactivity in non-immunized mice against HIV-1 envelope peptides representing HIV-1 subtypes A, B and C epitopes. Absorbance values are given for serum dilutions of 120. + Blue color indicates components of HIVIS-DNA, yellow color indicates components of HIVISopt-DNA and red color indicates components of MVA-CMDR.

the HIVIS-DNA group had titers of around 200, indicating that two MVA-CMDR (AE) boosts induce similar titers to AE strains irrespective of priming. Fig. 4B shows an overview of subtype C neutralization with individual sera from animals receiving a single HIVISopt-DNA prime followed by two MVA-CMDR boosts

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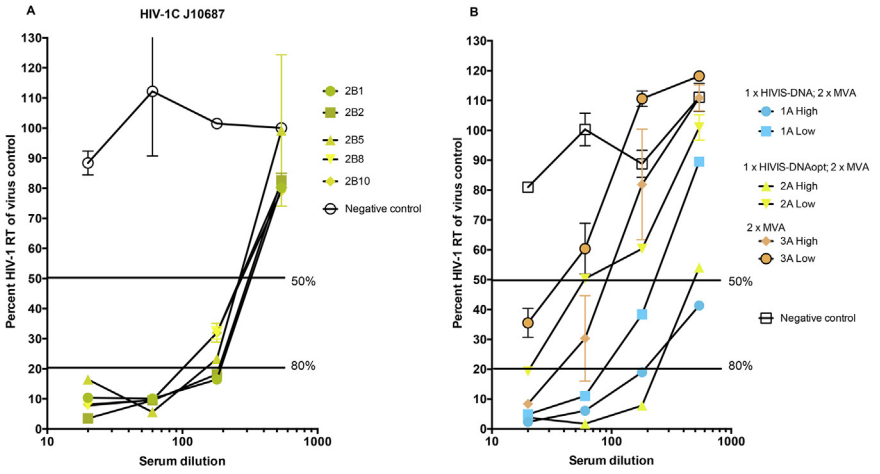

Fig. 4. Virus neutralization results with sera from mice with high or low ELISA binding titers. A. Sera pooled from HIVIS-DNA, HIVISopt-DNA or MVA groups and naive mice were tested at dilutions 20, 60, 180 and 540 against HIV-1CRF01_AE strain 1525. B. Individual sera from HIVISopt-DNA boosted twice by MVA-CMDR were tested at dilutions 20, 60, 180 and 540 against HIV-1 subtype C strain J10687. Numbers 2B1-2B10 indicate single mouse identifications. RT measurements define viral replication, lower concentrations reflect a better neutralization. Blue color indicates components of HIVIS-DNA, yellow color indicates components of HIVISopt-DNA and red color indicates components of MVA-CMDR.

(group 2 B see Table 1). All five animal sera neutralized HIV-1 subtype C, with a median neutralization titer of 220.

3.3. Cell mediated immune responses

After one HIV-DNA priming followed by a first and second MVA-CMDR boost we noted cell mediated responses to peptides derived from Env (subtype E), Gag (subtype A) and Pol (subtype A) (Fig. 5A and B).

Fig. 5A compares the Env-specific cell mediated responses by spot forming spleen cells (SFC) secreting IFNγ following HIVIS-DNA or HIVISopt-DNA boosted with MVA-CMDR once or twice. HIVIS-DNA primed mice had lower reactivity against Env CMDR after the 1st MVA-CMDR boost than HIVISopt-DNA primed group. The second MVA-CMDR boost increased reactivity considerably in the HIVIS-DNA primed group ($p < 0.01$). After HIVISopt-DNA priming and MVA-CMDR boosting there was no further increase.

Gag reactivity after HIVIS-DNA or HIVISopt-DNA priming was boosted by one MVA-CMDR immunization (Fig. 5B). Two MVA-CMDR boosts raised IFNγ spots significantly for the HIVIS-DNA primed group ($p < 0.01$). The cell mediated responses were lower when the group that received MVA-CMDR only, had not been primed ($p < 0.01$).

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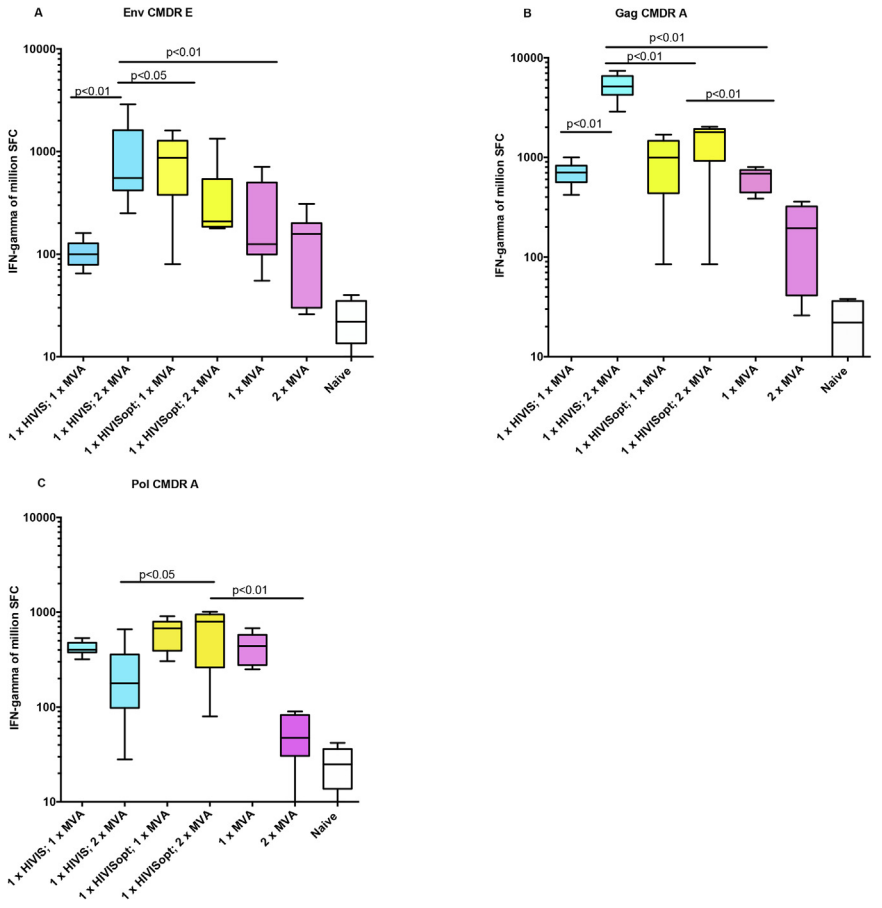

Fig. 5. Cell mediated immune responses. A. Median and ranges of IFNγ ELISpot reactivity against HIV-1 peptide pools representing Env E. B. Median IFNγ ELISpot reactivity against HIV-1 peptide pools representing Gag A. C. Median IFNγ ELISpot reactivity against HIV-1 peptide pools representing Pol A. The figures show responses obtained after DNA prime and one or two MVA-CMDR boost immunizations. Blue color indicates components of HIVIS-DNA, yellow color indicates components of HIVISopt-DNA and red color indicates components of MVA-CMDR.

Pol peptides of the CMDR subtype A origin were used for a similar experiment, Fig. 5C. Here all three animal groups showed 400–800 SFC/million spleen cells after one MVA-CMDR boost. Two MVA-CMDR immunizations did not increase the anti-Pol cellular immunity (Fig. 5C), similar to findings with the Env or Gag peptide antigens (Fig. 5A and B). It is likely that addition of PR sequences to HIVISopt-DNA (Table 3) contributed to the higher ($p < 0.05$) cellular reactivity following HIVISopt-DNA priming and MVA boosting.

The Con A activation of spleen cells from immunized mice showed a median of around 8 000–10 000 SFC/10^6 cells, the medium control 40–70 SFC/10^6 cells (not shown). Control non-immunized mice had a median of around 2 000 SFC/10^6 cells after Con A activation, and 30 SFC/10^6 cells with Env, Gag and Pol peptides.

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Peptide pools representing segments of the HIV-1 subtype B Pol region were used in an attempt to map cellular responses (Table 3). The addition of PRidelRT plasmid to HIVISopt-DNA gave rise to cell mediated responses to both RT and PR peptides. In total, these responses were similar after priming with HIVIS-DNA or HIVISopt-DNA, however the variation of reactivity to these peptide pools was considerable. The highest median spot level was obtained for PR peptides after HIVISopt-DNA priming and one MVA-CMDR boost (196, range 61–1744 spots).

Thus, for cell mediated immunity, MVA-CMDR appears to be a strong boosting agent for both types of HIV-DNA priming whether HIVIS-DNA or HIVISopt-DNA (Fig. 5A and B), as measured by stimulation with CMDR-analogous peptides. The highest IFNγ spot forming units for Gag A peptides were discovered after one HIVIS-DNA prime and two HIV-MVA boosts. Gag A sequences are present in both the HIV-DNA plasmid compositions and in MVA-CMDR (Table 1).

4. Discussion

Recent clinical HIV-1 vaccine trials or studies in primates have used prime-boost schedules, often with combinations of DNA plasmids with viral vectors such as Pox- or Adenovirus–based vectors [1, 20, 44, 45, 46, 47, 48]. It is also anticipated that the novel idea of using a latency-forming vector, based on replicating Cytomegalovirus, may be helped by HLA-E/KIR in inducing novel CD8+ cellular responses [49, 50].

Broadly neutralizing humoral antibodies have been described following multi-clade HIV-DNA plasmids [51, 52] and recombinant protein booster immunizations in primates [47], rabbits [53], and rodents [21, 52, 54, 55]. In clinical studies with HIV-DNA prime, the virus-reducing activities of antibodies have been characterized as neutralization [56] or prominent ADCC reactivity [23], for a review see [22].

In a series of clinical studies we documented broad and durable immune responses to an HIV-DNA prime and modified Vaccina Ankara-based MVA-CMDR boost schedule, which warrants further exploration. DNA-MVA based HIV-1 vaccines induce both binding, neutralizing and high frequencies of ADCC antibodies to relevant conserved and variable sites of the HIV-1 subtypes A, B, C and AE [23] [57]. Likewise, cell mediated immune responses of long duration have been induced to several subtypes as represented by peptides overlapping the HIV-1 Env and Gag sequences [19, 20]. The studies have demonstrated dose sparing and simplification of DNA administration and priming in human trials [27, 28].

For larger clinical studies it is important to reduce the number of vaccine components while still not compromising the excellent broad cell mediated

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immunities and functional antibody responses of long duration. For this purpose we modified the HIV-DNA, MVA-based immunization schedule. Our rational was to retain representation of long Env and Gag proteins from multiple HIV subtypes, while reducing the number of plasmids and still add immunogenic epitopes. An overview of the subtype-specific antigens represented in the prime and boost immunizations is given in Table 1.

The relative immunological responses to two prime-boost schedules were evaluated in a murine model. HIVIS-DNA prime resulted in serum IgG binding titers against Env gp140 C, Env gp160 B, Gag p55 B, RT and PR B antigens. Mice receiving the HIVISopt-DNA prime followed by one MVA-CDMR boost responded with similar or higher IgG titers compared to those obtained by HIVIS-DNA and MVA-CDMR boost. Binding antibodies were seen to different subtypes of HIV-1, although it is difficult to determine whether such antibody binding cross-reactivities would extend to inhibit primary HIV-1 infection in humans.

Virus-neutralizing antibodies were found to several HIV-1 subtypes following both HIVIS-DNA and HIVISopt-DNA priming. Both DNA primed groups, which received B and C Env components in addition to the E Env component, acquired good neutralizing titers to the selected B and C HIV-1 strains. Thus, neutralizing activity to HIV-1 subtypes A, B, C and CRF01_AE was induced, indicating a broad response, similar to that found in clinical studies [2] [19]. We ascribe these results to the heterogenous composition of the vaccine, including full sequences from strains of subtypes A, B and C in the prime and E (AE) in the boost.

This was a first attempt to compare neutralizations between the two immunization schedules. We have therefore started to assay against strains of several subtypes with which we are familiar and also represent the subtypes included in the vaccine schedules. Human sera derived from clinical studies with HIVIS-DNA priming have been assayed with the TZM.bl as well as PBMC assays (Joachim et al. PlosOne 2016).

By measuring cell-mediated immune responses, multi-subtype reactivities were found related to the peptides of both A and E subtypes used for the stimulation of IFNγ secretion. Our previous studies have shown cellular reactivities by ELISpot and lymphoproliferation to peptide and proteins of subtypes A, B, C, D and E reagents of mice and humans [20, 58] (and unpublished). Both HIVIS-DNA and HIVISopt-DNA primed anti-Gag cellular responses as measured by ELISpot.

The HIVISopt-DNA primed a larger number of epitope responses to Pol, also anticipated since the antigen PR was added and RT modified in these plasmids. Expression of RT has [57] been shown to be significantly higher in the PRidRT construct of HIVISopt than that of RT from the RTmut construct of HIVIS-DNA

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[33]. In that study, PR related cell mediated immune responses occurred of around 2 000 SFC/10^6 cells, a reactivity that was similar or higher when PRidRT was combined with HIVIS-DNA plasmids [33].

The reasons for the MVA-CMDR not continuing to boost cell mediated immunity when given the second time as seen in the present study, cannot be easily explained. Similar phenomena occur with relative antigen overload or when timing between two strong antigens is too short. Two immunizations with MVA-CMDR as given here may require a longer time interval than 3 weeks between these potent boost immunizations to obtain the best contraction of primarily induced B- and T-cell immune responses. The MVA-CMDR humoral Env responses were boosted by the second HIV-MVA immunization, but Gag antibody and Gag cellular responses were not boosted. It is known that kinetics for humoral and cell mediated responses as well as for different antigens differ, and we may not have been able to access the optimal time point for both [59]. Here, this was evident for antibody induction to Gag and cell mediated reactivities to Gag and Pol. This work, as well as other studies, highlight that DNA vaccines potently prime potent immunogenicity in mice although this may not translate in nonhuman primates and human studies. In fact, the DNA prime and MVA boost model has shown variable levels of immunogenicity in humans [60] [20] [19] [1].The reasons for the variability between studies are multiple. Except for the species selected, the age and sex play roles [19]. The expression of plasmids and vectors may vary related to tissue in which they are delivered, facts that might be modulated with new formulations of DNA to better enhance immunogenicity.

Correct delivery and dosing of DNA are key questions for good expression of plasmid DNA. Both needle-less Biojector® and electroporation have been proven efficient means to increase numbers of transfected cells expressing antigens [2, 40, 41, 61, 62]. Delivery of DNA to mice appears to be optimal by Biojector® or electroporation targeted in the skin, which is resident to large numbers of dendritic cells [15]. In humans, the needle-less Biojector® works well after delivery of DNA plasmids to the skin, comparable to vaccine delivery by electroporation [2].

It is well known that mice respond well to DNA vaccines in contrast to many larger animals. The limitations of the presented results generated in Balb/c mice do not take into account dosing or delivery systems for humans, which we have shown to be important for the HIVIS-DNA priming in clinical trials.However, we feel that the small animal system can be used for comparisons between vaccine schedules related to certain criteria such as general trends for indicating whether B-cell or T-cell responses would be improved or similar.

We intended to show non-inferiority of the reduction and compression of antigens in the HIVIS-DNA vaccination schedule, which would be useful for clinical trials.

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This was successful and allows continuation with either the 6 or 7 component DNA vaccine.

Late boosts are essential to retain good immunological memory. Extended identification of remaining B- and T-cell memories have been revealed after DNA prime, MVA boosts in the clinic [20] [26]. The clinical study RV144 experienced a higher rate for protection early, which waned until the pre-decided endpoint [17]. It was notable in the present study, that priming once with DNA followed by one MVA boost induced higher immune responses than one MVA alone and at times even two MVA boosts. In a coming clinical trial it might therefore be optimal to start immunizations by one high dose composite DNA prime and one high dose MVA boost, followed at 2–3 yearly intervals with the same schedule. This would provide good initial priming and very long-term memory responses.

Declarations

Author contribution statement

Jorma Hinkula, Stefan Petkov: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Karl Ljungberg: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

David Hallengärd, Sumit Sharma: Performed the experiments; Wrote the paper.

Andreas Bråve, Merlin Robb, Bernard Moss: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Maria Isaguliants, Charlotta Nilsson: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tina Falkeborn: Performed the experiments; Wrote the paper.

Valentina Liakina: Performed the experiments.

Mike Eller: Analyzed and interpreted the data; Wrote the paper.

Gunnel Biberfeld, Britta Wahren: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Eric Sandström: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Kerstin Markland, Pontus Blomberg: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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