Vaccine 34 (2016) 1744–1751

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The improved antibody response against HIV-1 after a vaccination based on intrastructural help is complemented by functional CD8+ T cell responses

Michael Storcksdieck genannt Bonsmann[a], Thomas Niezold[a], Drew Hannaman[b], Klaus Überla[a,c], Matthias Tenbusch[a,*]

[a] Department of Molecular and Medical Virology, Ruhr-University Bochum, Germany
[b] Ichor Medical Systems, Inc., San Diego, CA, USA
[c] Universitätsklinikum Erlangen, Institute of Clinical and Molecular Virology, Friedrich-Alexander University Erlangen-Nürnberg, Erlangen, Germany

ARTICLE INFO

Article history:
Received 12 October 2015
Received in revised form 3 February 2016
Accepted 24 February 2016
Available online 3 March 2016

Keywords:
HIV-1
AIDS
Vaccine
DNA
VLPs
CTLs
Antibodies

ABSTRACT

Despite more than three decades of intense research, a prophylactic HIV-1 vaccine remains elusive. Four vaccine modalities have been evaluated in clinical efficacy studies, but only one demonstrated at least modest efficacy, which correlated with polyfunctional antibody responses to the HIV surface protein Env. To be most effective, a HIV-1 vaccine probably has to induce both, functional antibody and CD8+ T cell responses. We therefore analyzed DNA/DNA and DNA/virus-like particle (VLP) regimens for their ability to induce humoral and cellular immune responses. Here, DNA vaccination of mice induced strong CD8+ responses against Env and Gag. However, the humoral response to Env was dominated by IgG1, a subclass known for its low functionality. In contrast, priming only with the Gag-encoding plasmid followed by a boost with VLPs consisting of Gag and Env improved the quality of the anti-Env antibody response via intrastructural help (ISH) provided by Gag-specific T cells to Env-specific B cells. Furthermore, the Gag-specific CD8+ T cells induced by the DNA prime immunization could still protect from a lethal infection with recombinant vaccinia virus encoding HIV Gag. Therefore, this immunization regimen represents a promising approach to combine functional antibody responses toward HIV Env with strong CD8+ responses controlling early viral replication.

1. Introduction

Due to the steadily increasing number of HIV-1-infected people, a prophylactic vaccine represents a global health priority. While most licensed vaccines are thought to protect by provoking sufficiently strong neutralizing antibody responses [1,2], induction of these antibodies by vaccination against HIV-1 has proven extremely complicated [3–6]. However, antibodies can also mediate a plethora of Fc-dependent effector functions, like antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), or NK cell activation. Indeed, comparative analyses of clinical HIV-1 vaccine trials indicate that polyfunctional antibodies able to mediate several of the aforementioned functions could provide protection against infection as long as less functional subclasses do not interfere [7–9]. Furthermore, even broadly neutralizing monoclonal antibodies against HIV-1 demonstrated superior efficacy in a murine model if provided as a polyfunctional subclass, namely IgG2a, as compared to the less functional IgG1 [10]. Therefore, the functional profile seems to be an important determinant for the protective efficacy of a humoral immune response induced by vaccination.

In addition to functional antibodies, cytotoxic T cells (CTLs) may be beneficial or even necessary for an HIV vaccine to be efficacious [11]. SIV-specific CD8+ T cells were shown to reduce the viral loads after challenge in the macaque model [12–14]. Furthermore, SIV-specific CTLs were also able to preserve CD4+ central memory T cells and increase the survival of macaques [15]. However, a clinical HIV-1 vaccine trial based solely on the induction of HIV-specific T cells by adenoviral vectors failed to demonstrate efficacy [16]. While this study did not include an Env component, the follow-up study using DNA and adenoviral vector vaccines encoding Env, Gag, Pol, and Nef

* Corresponding author at: Department of Molecular and Medical Virology, Ruhr-University Bochum, Universitaetsstrasse 150, 44780 Bochum, Germany. Tel.: +49 234 32 23189; fax: +49 234 32 14352.
E-mail address: Matthias.tenbusch@rub.de (M. Tenbusch).

http://dx.doi.org/10.1016/j.vaccine.2016.02.059
0264-410X/

also failed to provide any protection or alteration of viral kinetics following infection, despite the induction of T cell and antibody responses [6]. Collectively, the clinical studies suggest that qualitative properties of humoral and cellular immune responses are likely to be critical for the efficacy of an HIV-1 vaccine.

Therefore, our aim was to develop a vaccination strategy which combines functional antibody responses against Env, as seen in the RV144 trial, with substantial T cell responses able to control early viral replication, which were undetectable in RV144. To this end, we studied DNA and VLP vaccine candidates in different prime-boost regimens in the mouse model (Fig. S1). We found that priming with a DNA vaccine encoding GagPol followed by a VLP boost containing Env and Gag could improve the functional profile of Env-specific antibodies via intrastructural help [17], while still inducing $CD8^+$ T cell responses capable of providing protection. Therefore, the GagPol-DNA prime VLP booster immunization regimen represents a promising strategy for the development a prophylactic HIV-1 vaccine.

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.02.059.

2. Results

2.1. Immunization against HIV-1 Env and GagPol by DNA electroporation induces protective $CD8^+$ T cell responses

Due to the intracellular expression of their respective antigens, genetic vaccines like nucleic acids or viral vectors are known as good inducers of CTL responses. Therefore, we first immunized mice with escalating doses of plasmids encoding HIV-1 Env and GagPol by intramuscular electroporation twice in a three week interval (Fig. S1A). Five weeks after the second immunization, the animals were challenged intranasally with a lethal dose of recombinant vaccinia virus expressing HIV-1 Gag (rVV-Gag).

Compared to naïve mice, all immunized animals demonstrated significantly reduced weight loss ($p < 0.01$) in a dose dependent manner (Fig. 1A). Quantitative PCR (QPCR) analysis of viral loads in lung homogenates on day six after challenge revealed a similar dose-dependency (Fig. 1B). In contrast, naïve animals showed high viral loads, indicative of an uncontrolled replication. Finally, flow-cytometric analysis of Gag-specific $CD8^+$ T cells in the spleens revealed substantial anamnestic responses after challenge in immunized animals (Fig. 1C). Surprisingly, IFN-γ-producing Gag-specific T cells were lowest in animals receiving the intermediate antigen dose. This indicates that the responses were affected by both, the antigen dose and the extent of viral replication. Almost all IFN-γ-producing cells also demonstrated CD107 surface expression. While IFN-γ is well recognized for its antiviral potential, surface expression of CD107 is considered a surrogate marker for the cytotoxic potential of T cells. Furthermore, a substantial fraction of Gag-specific $CD8^+$ T cells was also able to express IL-2. Thus, DNA immunization against GagPol leads to polyfunctional $CD8^+$ T cell responses with the capacity for protection against a viral challenge.

Although the induced Gag-specific T cell responses were encouraging, we found a strong bias in the antibody response to the Env protein toward IgG1 (Fig. 1D). This subclass was shown to be a weak

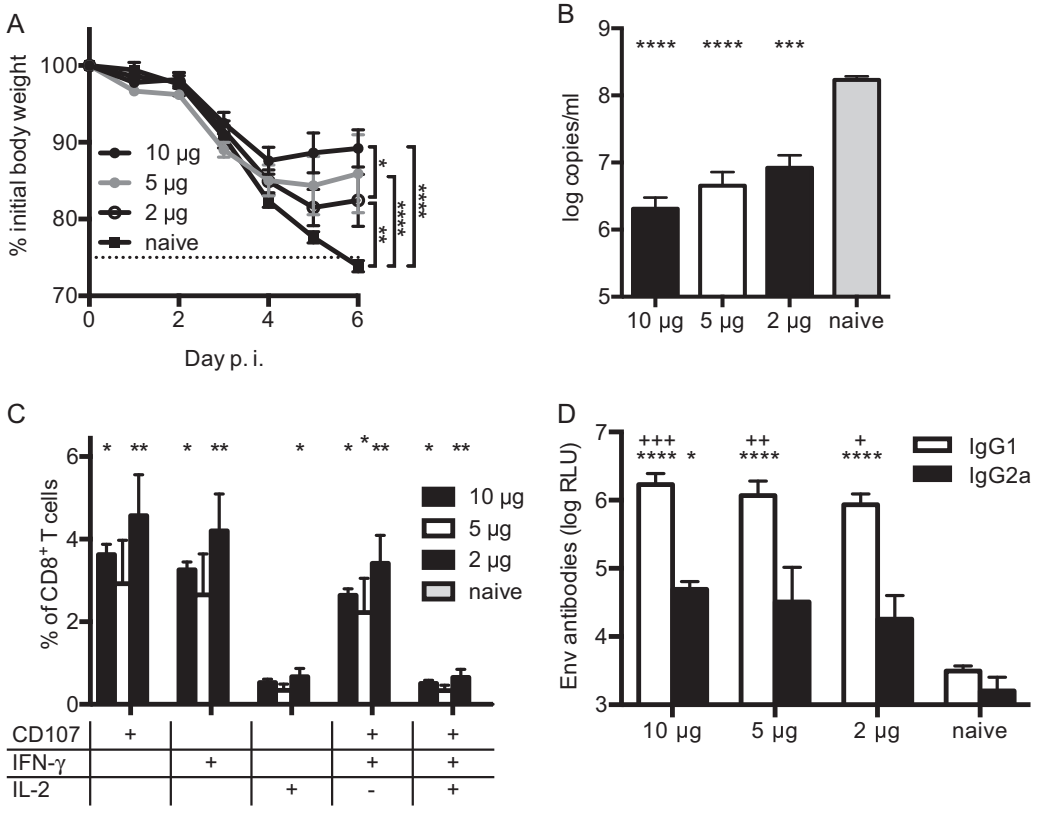

Fig. 1. Titration of a bivalent HIV-1 DNA vaccine. Groups of five mice were immunized twice with different doses of a combined HIV-1 Env and Gag DNA vaccine by intramuscular electroporation. Two weeks after the boost immunization Env-specific antibody responses were determined and three weeks later the animals were intranasally challenged with 5×10^4 PFU of a recombinant vaccinia virus expressing HIV-1 Gag. (A) Body weight curves for the different groups depicted as means with SEM ($n = 5$; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; two-way ANOVA with Tukey's post test). The dashed line indicates the end-point criterion for euthanasia. (B) log10-transformed copy numbers of viral DNA in lung homogenates collected on day six post infection and normalized to total DNA content. Bars represent the means + SEM ($n = 5$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; one-way ANOVA with Tukey's post test). (C) Intracellular cytokine staining of restimulated splenocytes on day six post infection. Bars represent the mean with SEM ($^*p < 0.05$; $^{**}p < 0.01$; one-way ANOVA with Tukey's post test). (D) Env-specific IgG1 and IgG2a responses as determined by ELISA. Shown are mean values + SEM ($n = 5$; $^*p < 0.05$; $^{****}p < 0.0001$ vs. naive; one-way ANOVA with Tukey's post test; $^+p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$ vs. IgG2a paired two-tailed t test).

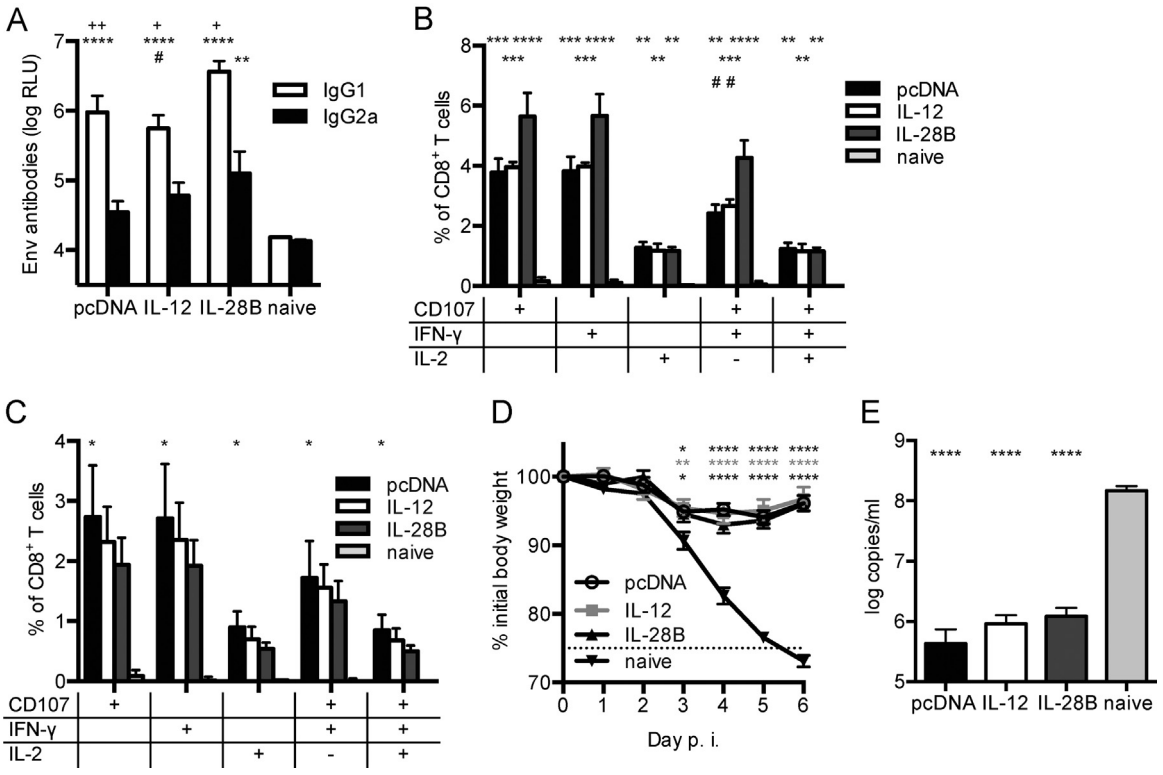

Fig. 2. Impact of genetic adjuvants on a bivalent HIV-1 DNA vaccine. Groups of mice were immunized twice against HIV-1 Env and Gag by intramuscular DNA electroporation either with IL-12 or IL-28B as genetic adjuvant or pcDNA as control (10 μg of each plasmid). Two weeks after the boost immunization four animals per group were sacrificed for intracellular cytokine staining. Three weeks later, humoral immune responses against Env in remaining animals were determined by ELISA. Subsequently, the animals were intranasally challenged with 5×10^4 PFU of rVV-Gag. (A) Env-specific humoral immune responses five weeks after the second DNA immunization including genetic adjuvants expressed as mean values with SEM ($n = 5$–6; $^{**}p < 0.01$; $^{****}p < 0.0001$ vs. naïve; $^{\#}p < 0.05$ vs. IL-28B; one-way ANOVA with Tukey's post test; $^{+}p < 0.05$; $^{++}p < 0.01$ vs. IgG2a paired two-tailed t test). (B and C) Intracellular cytokine staining of Env- (B) or Gag-specific (C) splenocytes analyzed two weeks after the boost immunization. Bars represent the mean with SEM ($n = 4$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ vs. naive; $^{\#}p < 0.05$ vs. IL-28B; one-way ANOVA with Tukey's post test). (D) Body weight curves for the different groups challenged five weeks after the boost immunization and depicted as means with SEM ($n = 5$–6; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$ vs. naïve; two-way ANOVA with Tukey's post test). The dashed line indicates the end-point criterion for euthanasia. (E) log10-transformed copy numbers of viral DNA in lung homogenates collected on day six post infection and normalized to total DNA content. Bars represent the means + SEM ($n = 5$–6; $^{****}p < 0.0001$ vs. naïve; one-way ANOVA with Tukey's post test).

mediator of antiviral protection [18–20]. Therefore, we analyzed if genetic adjuvants could improve the humoral immune response or further enhance CTL-mediated protection.

2.2. Impact of genetic adjuvants on vaccine-induced immune responses

Since the T_H2-associated IgG1 subclass is not considered adequate for antiviral protection, we chose the prototypic T_H1 cytokine IL-12 and the antiviral cytokine IL-28B [21] and co-applied respective expression plasmids with the mixed Env and Gag-Pol DNA vaccine (Fig. S1B). No significant differences in humoral immune responses between immunized groups were observed two weeks after the booster immunization (data not shown). However, five weeks after the second DNA immunization, the IL-28B co-immunized animals showed a trend toward increased Env-specific IgG1 responses compared to the mock-adjuvanted control group, while the opposite was true for the IL-12 group (Fig. 2A). Consequently, IgG1 levels were significantly different ($p = 0.0209$) between the two groups. While both adjuvanted groups also showed a trend toward higher Env-specific IgG2a responses as compared to immunized control animals, IgG1 was still the predominant subclass.

Two weeks after the second immunization, a subset of animals was sacrificed to verify the induction of cellular immune responses. All immunized animals showed significant $CD8^+$ T cell responses as determined by CD107, IFN-γ and IL-2 expression ($p < 0.05$). While IL-12 co-application did not affect Env-specific $CD8^+$ T cell responses, IL-28B led to a substantial increase in $CD107^+$ and IFN-γ^+ cells (Fig. 2B). Again, most of the responding $CD8^+$ T cells expressed both activation markers together and this subset was significantly expanded ($p < 0.05$) in the IL-28B-adjuvanted group. However, no differences were observed in the polyfunctional population also expressing IL-2, which accounted for about a quarter of all responding $CD8^+$ T cells.

Gag-specific $CD8^+$ T responses were generally lower than those directed against Env (Fig. 2C). In addition, co-application of genetic adjuvants seemed to reduce the cellular immune responses. Consequently, only in the mock-adjuvanted group Gag-specific $CD8^+$ T cell responses reached significant levels ($p < 0.05$). Again, two-thirds of the responding $CD8^+$ T cells expressed CD107 and IFN-γ together, and about one third expressed all three markers concomitantly.

Five weeks after the second immunization, remaining animals were challenged with rVV-Gag. None of the immunized animals demonstrated significant weight loss, while the naïve animals started to lose weight by day three post infection (Fig. 2D). In line with the previous experiment, the viral loads in the lung on day six post infection were also significantly reduced compared to naïve animals ($p < 0.0001$). Again, there was no statistically significant difference between the immunized groups (Fig. 2E).

In conclusion, the DNA prime-boost immunization induced polyfunctional and protective $CD8^+$ T cell responses against both vaccine antigens, while the humoral immune response against Env

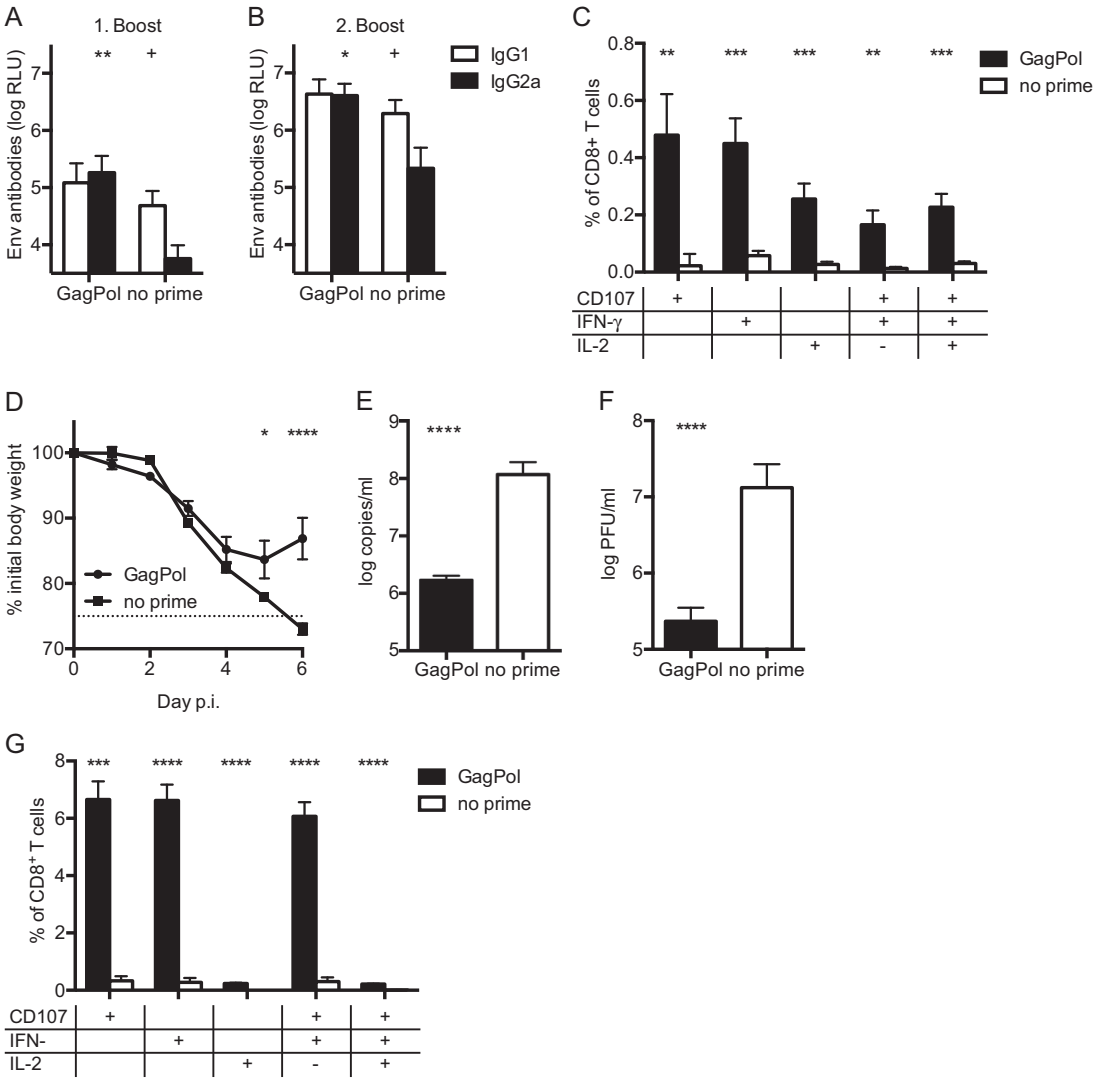

Fig. 3. The intrastructural help immunization induces functional antibody and protective CD8$^+$ T cell responses. Mice were immunized with 20 μg of HgpSyn by intramuscular DNA electroporation and boosted with VLPs containing Gag and Env five and eight weeks later. Two weeks after each VLP immunization humoral immune responses were determined by ELISA. Two weeks after the second VLP immunization and 6 days after the rVV-Gag challenge intracellular cytokine staining of splenocytes was performed. Five weeks after the second VLP immunization the animals were intranasally challenged with 5×10^4 PFU of rVV-Gag. (A and B) Env-specific humoral immune responses after the first or second VLP immunization, respectively. Results are expressed as mean values with SEM ($n = 4$; $^*p < 0.05$; $^{**}p < 0.01$ vs. naive; unpaired two-tailed t test; $^+p < 0.05$; vs. IgG2a paired two-tailed t test). (C) Intracellular cytokine staining of restimulated splenocytes analyzed two weeks after the second boost immunization. Bars represent the mean with SEM ($n = 3$–6; $^{**}p < 0.01$; $^{***}p < 0.001$; unpaired two-tailed t test). (D) Body weight curves for the two groups challenged five weeks after the second boost immunization and depicted as means with SEM ($n = 4$; $^*p < 0.05$; $^{****}p < 0.0001$; two-way ANOVA with Tukey's post test). The dashed line indicates the end-point criterion for euthanasia. (E) log10-transformed copy numbers of viral DNA normalized to total DNA content or (F) infectious rVV-Gag particles in lung homogenates collected on day six post infection. Bars represent the means + SEM ($n = 4$; $^{***}p < 0.001$; $^{****}p < 0.0001$; one-way ANOVA with Tukey's post test). (G) Intracellular cytokine staining of restimulated splenocytes analyzed six days after the rVV-Gag challenge. Bars represent the mean with SEM ($n = 4$; $^{**}p < 0.01$; $^{***}p < 0.001$; unpaired two-tailed t test).

was biased toward an unfavorable subclass. Considering the relative importance of Env-specific antibody responses for protection against HIV-1, we set out for an immunization approach able to induce higher IgG2a responses against Env, while maintaining a reasonable level of Gag-specific CTLs.

2.3. An intrastructural help immunization induces functional antibody and T cell responses

GagPol-specific T helper cells are able to modulate the humoral immune response against Env during VLP booster immunizations [17,22]. The underlying mechanism was referred to as intrastructural help (ISH) [17,23–26]. To analyze if such an immunization approach also induces functional CD8$^+$ T cell responses, we immunized mice by intramuscular DNA electroporation against GagPol. Five and eight weeks later, immunized and naïve mice received VLP booster immunizations (Fig. S1C).

First, we verified that the immunization induced the expected balanced humoral immune response against Env. Two weeks after the first VLP immunization, the GagPol-primed animals developed significantly stronger Env-specific IgG2a responses ($p = 0.0063$) compared to animals that only received VLPs (Fig. 3A). The minor impact on IgG1 led to an overall IgG2a-dominated humoral immune response against Env. In contrast, animals that received only the VLP immunization developed a similar IgG1 dominance, like DNA-immunized animals in previous experiments. Therefore, the bias in the Env-specific antibody response is not an artifact of the DNA immunization.

The second VLP immunization boosted Env-specific humoral immune responses in both groups. While IgG1 responses were similar in both groups, the GagPol-primed animals still exhibited

significantly higher IgG2a levels ($p = 0.0136$) against Env (Fig. 3B). Such a selective increase in Env-specific IgG2a is associated with enhanced functionality, as we have previously shown [22].

Analysis of Gag-specific $CD8^+$ T cells two weeks after the second VLP boost revealed lower frequencies of CD107, IFN-γ or IL-2 producing cells in DNA-primed mice compared with animals that received two DNA immunizations (Fig. 3C vs. Fig. 2C). However, the proportion of IL-2-secreting cells was increased, demonstrating their homeostatic potential. With no detectable Gag-specific T cells in unprimed animals, these results suggest that the VLPs only had a minor impact on $CD8^+$ T cell responses.

Five weeks after the second VLP immunization, the animals were challenged with rVV-Gag, which led to substantial weight loss (Fig. 3D). However, all DNA-immunized animals recovered from the weight loss, while all unprimed mice had to be euthanized due to the severity of the weight loss. This equates to a survival rate of 100%. In line with the significantly higher body weights, GagPol-primed animals had significantly reduced lung viral loads ($p < 0.0001$), as determined by QPCR and titration (Fig. 3E and F). In contrast, animals that received only the VLP immunizations had comparable rVV-Gag titers as naïve animals in the previous experiment (Fig. 2E).

Analysis of Gag-specific $CD8^+$ T cells after the challenge revealed substantial CD107 and IFN-γ recall responses in the DNA primed group with almost all responding T cells expressing both markers together (Fig. 3G). As expected for effector T cells, almost no IL-2 expression was detectable. However, the polyfunctional $CD8^+$ T cell population expressing CD107, IFN-γ and IL-2 together was still significantly larger ($p < 0.0001$) in the DNA primed animals compared to the group that only received the VLP immunizations. Therefore, our immunization approach based on ISH induced improved humoral immune responses against HIV-1 that were complemented by functional $CD8^+$ T cells.

3. Discussion

A prophylactic HIV-1 vaccine will have to induce protective humoral and cellular immune responses to be most effective. We report here on a vaccination approach capable of inducing HIV-1 Env antibody responses predicted to be highly functional as well as cytotoxic T cells against the more conserved Gag protein.

Although broadly neutralizing antibodies against HIV-1 are probably the most potent mediators of protection, their induction by vaccination has not been achieved yet [3–6]. However, highly functional binding antibodies against Env are associated with spontaneous control of the HIV-1 infection [27] and may suffice to confer protection after vaccination [5,8,9]. Unfortunately, Env-based antigens tend to induce antibodies with poor functionality, at least if administered repeatedly [8,9]. In line with previous reports [28–30], we also observed a predominant induction of the less functional IgG1 subclass in mice immunized against Env either by intramuscular DNA electroporation or with VLPs. Although not reported yet, it is tempting to speculate that a poor functionality of the Env-specific antibody response contributed to the failure of the recent HVTN505 trial, which employed a DNA/Ad5 vaccine expressing HIV-1 Gag, Pol, Nef and Env [6].

Co-administration of an IL-12 or IL-28B expression plasmid at the dose level tested was not able to overcome the IgG1 bias in the antibody response against Env. HIV-1 Env has been reported to induce IL-10 expression [31], which in turn could counteract the effects of IL-12. In addition, IL-28B belongs to the IFN-λ cytokine family that signals through a heterodimeric receptor containing the IL-10Rβ subunit. Although these cytokines are recognized for their antiviral activity, they may also exhibit functional similarity to IL-10 [21]. Therefore, these factors may have contributed to the failure of the adjuvants to overcome the IgG1 predominance.

In contrast, an immunization regimen based on intrastructural help induced a balanced Env-specific antibody response, as we have recently reported [22]. Here, GagPol-specific T helper cells were able to significantly increase Env-specific IgG2a levels after the VLP booster immunizations. The potential of IgG2a to trigger Fc-dependent effector functions through its cognate Fc receptor is well established and has been shown to be important for protection from viral infections [10,32–34]. Furthermore, murine IgG2a resembles the functional profile of human IgG3, which was identified as the major determinant of protection in RV144 [5,8,9]. While a direct proof is still missing, their association with control of viral replication and with vaccine efficacy strongly argues for an important role of polyfunctional antibodies for protection from HIV-1 acquisition [7,27]. Thus, an immunization based on intrastructural help is a promising approach for the development of a prophylactic HIV-1 vaccine.

The sheer number of variants of HIV-1 [35] poses a significant impediment to achieving protection against infection by humoral immune responses alone, as exemplified by the sieve effect in RV144 [5,36]. $CD8^+$ T cells play an important role in the control of an HIV-1 infection [37–39]. In addition to their potential to kill infected cells, $CD8^+$ T cells can mediate a plethora of antiviral activities via soluble factors. Among these are the $CD8^+$ cell anti-HIV factor (CAF), which was described early to contribute to HIV inhibition [40], nuclear proteins [41], β-chemokines [42] or antiviral cytokines, such as IFN-γ or TNF-α. Furthermore, polyfunctional T cells defined by their ability to produce several cytokines after antigen-stimulation have been associated with protection from disease progression [43].

The induction of HIV-specific $CD8^+$ T cells, along with functional antibodies, would provide another mechanism for inhibition of the target pathogen. Therefore, we performed intracellular cytokine stainings and analyzed the protection from a lethal intranasal challenge with a recombinant vaccinia virus in immunized mice, an established surrogate model of T cell mediated protection [44,45]. The DNA/DNA vaccine regimens provided excellent protection and this protection was associated with vaccine-induced and anamnestic polyfunctional $CD8^+$ T cell responses against HIV-1 Gag.

While several studies reported on the beneficial effects of incorporating a genetic adjuvant, such as IL-12 or IL-28B, within a genetic vaccine [46–48], these neither enhanced T cell responses nor protection under the conditions tested. Possible reasons include the aforementioned counteracting effects of IL-10, potentially induced by the Env component of the vaccine regimen [31], the need for further optimization of dose and administration conditions, or the striking immunogenicity of our DNA electroporation, which may have been too strong to be further enhanced by an adjuvant, at least in the mouse model.

However, a T cell-based vaccine will probably not suffice to protect from HIV-1 infection, as the highly immunogenic vaccine regimen of the Step study suggests [16,49]. Therefore we addressed whether our new vaccination regimen based on intrastructural help could not only overcome the bias in the humoral response to Env, but still induce protective T cell responses. Indeed, in addition to the balanced humoral immune response against Env, the DNA-primed animals had significantly stronger Gag-specific $CD8^+$ T cell responses and exhibited significantly reduced viral loads compared to the group immunized with VLPs only. Although the etiopathology was more severe than in previous DNA prime boost experiments, none of these animals succumbed to infection. Therefore, the protection was still remarkable, particularly when the significantly longer time between DNA immunization and challenge is considered (13 vs. 5 weeks).

The assumption that the induction of cellular and humoral immune responses would provide superior protection is backed up by recent studies in the macaque model [50,51]. While Barouch et al. also reported on the induction of cellular immune responses, they ascribe the notable efficacy of their Ad26 prime recombinant HIV-1 Env boost vaccine to the polyfunctional antibody response induced [50]. Lakhashe et al. on the other hand used GagPol VLPs in combination with recombinantly produced HIV-1 Tat and Env for immunization and identified neutralizing antibodies and IFN-γ ELISPOT responses as correlates of protection [51]. Since our intrastructural help vaccine regimen also induced IFN-γ^+ T cells and antibody responses with increased functionality, we are optimistic to achieve substantial protection in a relevant challenge model and respective studies are under way.

In conclusion, our immunization approach based on intrastructural help not only significantly enhances the Env-specific humoral immune response, but also generates Gag-specific $CD8^+$ T cells that protect against viral challenge at a mucosal surface. Therefore, combined immunization regimens integrating modalities to achieve intrastructural help represent a promising approach that should be further considered for the development a prophylactic HIV-1 vaccine.

4. Materials and methods

4.1. Plasmids

The codon optimized HIV-1 expression plasmids HgpSyn [52] for GagPol or pConBgp140GC/D [17,53] for Env that carries the cytoplasmic domain of VSV-G, were used for VLP production and as DNA vaccines. The murine IL-28B ORF was PCR amplified from cDNA of an influenza-infected mouse lung and cloned into the pVax plasmid (Invitrogen). The murine IL-12 expression plasmid pORF-mIL-12(p40:p35) was obtained from Invivogen. pcDNA3.1(+) (Invitrogen) was used to normalize total DNA amounts. Plasmids were prepared with NucleoBond Xtra Maxi EF or the PC10000 EF Kits (Macherey–Nagel).

4.2. VLP preparation

VLPs were prepared as described before [22]. Briefly, 293T (ATCC CRL-3216) cells were transfected with HgpSyn and pConBgp140GC/D using polyethylenimine. Two days later VLPs were purified from the supernatants by ultracentrifugation through a 20% sucrose cushion for 2.5 h at 90,000 × *g* and 4 °C. Purified VLPs were resuspended in PBS.

4.3. Mice and immunizations

Six to eight weeks old BALB/cJRj mice (Janvier) were housed at the animal facility of the medical faculty, Ruhr University Bochum and treated in accordance with the national law and institutional guidelines. DNA immunizations were performed under anesthesia with 100 mg/kg body weight ketamine (CP-Pharma) and 15 mg/kg body weight xylazine (Bayer). The TriGrid electrode array (Ichor Medical Inc.) with 2.5 mm electrode spacing bearing the centered injection needle was inserted into the shaved hind legs of mice. A volume of 50 μl PBS containing equal amounts of the different expression plasmids (2.0–15 μg in total) was injected intramuscularly in each hind leg immediately followed by the local application of electrical signals of 63 V amplitude and 40 mS total duration.

The VLPs were diluted in sterile PBS. All animals received 400 ng of Env per immunization distributed to both hind footpads.

4.4. ELISA

Env-specific antibodies were measured by ELISA as previously described [22]. In brief, ELISA plates (Greiner Bio One) were coated with antigen at 4 °C over night. After blocking with 5% skimmed milk powder, sera were added in blocking buffer. Bound IgG1 or IgG2a were detected with the respective HRP-conjugated antibodies (X56 and R19-15; BD Biosciences) using an ECL substrate in an Orion-96 microplate reader (Berthold). Humoral immune responses are expressed as log10 transformed relative light units (RLU).

4.5. Intracellular cytokine staining

Env- or Gag-specific $CD8^+$ T cell responses were determined by intracellular cytokine staining (ICS). Briefly, single cell suspensions of splenocytes were prepared using 70 μm cell strainer (BD Biosciences). After red blood cell lysis, 10^6 splenocytes were stimulated with 5 μg/ml of the MHC class I restricted peptide IHIGPGRAFYT (Env) or AMQMLKETI (Gag) in the presence of anti CD107a FITC or AlexaFluor488 (37.51; BD Bioscience or eBioscience, respectively) and 2 μM Monensin for 6 h at 37 °C in a humidified 5% CO_2 atmosphere. Surface staining was performed with anti-mouse CD8 PerCP or Pacific Blue (53–6.7; BD Bioscience) and Fixable Viability Dye eFluor® 780 (both eBioscience). After fixation with 2% paraformaldehyde cells were permeabilized with 0.5% saponin in the presence of Fc-block (eBioscience) and subsequently stained with anti-mouse IFN-γ PE (XMG1.2; eBioscience) and anti-mouse IL-2 APC (JES6-5H4; BD Bioscience). Data were acquired on a FACS Canto II (BD Bioscience) and analyzed with FlowJo (Tree Star).

4.6. Vaccinia virus challenge and qPCR

Animals received 5×10^4 PFU of recombinant vaccinia virus expressing HIV-1 Gag by intranasal application under anesthesia and body weights were monitored. Six days post infection animals were sacrificed, their lungs harvested and homogenized with 2 ml of PBS by gentleMACS (Miltenyi Biotec). After centrifugation, DNA was purified from supernatants using the QIAamp DNA blood kit (Qiagen). Vaccinia virus copy numbers were determined by qPCR using the QuantiTect Probe PCR Kit (Qiagen) with SybrGreen (Molecular Probes) and the oligonucleotides GACACTCTGGCAGCCGAAAT as forward and CTGGCGGCTAGAATGGCATA as reverse primer. The supernatant was also used to titrate infectious virus on CV-1 cells as previously described [45].

4.7. Statistical analysis

Unpaired and paired two-tailed *t* tests and one-way or two-way ANOVA with Tukey's post tests were performed as indicated using GraphPad Prism software.

Author contributions

MSgB, KÜ and MT planned the experiments and analyzed the data. MSgB and TN performed the experiments. DH provided the electroporation device. MSgB, DH, KÜ and MT drafted the manuscript. All authors contributed to the refinement of the manuscript and approved the final version.

Conflict of interest

D. Hannaman is employee of the company Ichor Medical Systems. The authors have no additional financial interests.

Acknowledgements

This work was funded by Transregional Collaborative Research Grants TRR-60 and TRR-130 from the Deutsche Forschungsgemeinschaft. Michael Storcksdieck genannt Bonsmann was supported by the Ruhr-University Research School.

Codon-optimized HIV gag-pol sequences were kindly provided by Ralf Wagner and Geneart. We thank Christine Trumpfheller for providing the recombinant vaccinia virus. The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pConBgp160-opt (Cat#11402) from Dr. Beatrice Hahn; HIV-1 IIIB rgp120 (CHO), NIH Cat #11784.

References

[1] Plotkin SA. Vaccines: correlates of vaccine-induced immunity. Clin Infect Dis 2008;47:401–9, http://dx.doi.org/10.1086/589862.

[2] Plotkin SA. Correlates of protection induced by vaccination. Clin Vaccine Immunol 2010;17:1055–65, http://dx.doi.org/10.1128/CVI.00131-10.

[3] Flynn NM, Forthal DN, Harro CD, Judson FN, Mayer KH, Para MF, et al. Placebo-controlled phase 3 trial of a recombinant glycoprotein 120 vaccine to prevent HIV-1 infection. J Infect Dis 2005;191:654–65, http://dx.doi.org/10.1086/428404.

[4] Pitisuttithum P, Gilbert P, Gurwith M, Heyward W, Martin M, van Griensven F, et al. Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. J Infect Dis 2006;194:1661–71, http://dx.doi.org/10.1086/508748.

[5] Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, et al. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N Engl J Med 2009;361:2209–20, http://dx.doi.org/10.1056/NEJMoa0908492.

[6] Hammer SM, Sobieszczyk ME, Janes H, Karuna ST, Mulligan MJ, Grove D, et al. Efficacy trial of a DNA/rAd5 HIV-1 preventive vaccine. N Engl J Med 2013;369:2083–92, http://dx.doi.org/10.1056/NEJMoa1310566.

[7] Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S, Tomaras GD, Alam SM, et al. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N Engl J Med 2012;366:1275–86, http://dx.doi.org/10.1056/NEJMoa1113425.

[8] Chung AW, Ghebremichael M, Robinson H, Brown E, Choi I, Lane S, et al. Polyfunctional Fc-effector profiles mediated by IgG subclass selection distinguish RV144 and VAX003 vaccines. Sci Transl Med 2014;6:228ra38-8, http://dx.doi.org/10.1126/scitranslmed.3007736.

[9] Yates NL, Liao HX, Fong Y, deCamp A, Vandergrift NA, Williams WT, et al. Vaccine-induced Env V1–V2 IgG3 correlates with lower HIV-1 infection risk and declines soon after vaccination. Sci Transl Med 2014;6:228ra39-9, http://dx.doi.org/10.1126/scitranslmed.3007730.

[10] Bournazos S, Klein F, Pietzsch J, Seaman MS, Nussenzweig MC, Ravetch JV. Broadly neutralizing anti-HIV-1 antibodies require Fc effector functions for in vivo activity. Cell 2014;158:1243–53, http://dx.doi.org/10.1016/j.cell.2014.08.023.

[11] Letvin NL. Progress and obstacles in the development of an AIDS vaccine. Nat Rev Immunol 2006;6:930–9, http://dx.doi.org/10.1038/nri1959.

[12] Stephenson KE, Li H, Walker BD, Michael NL, Barouch DH. Gag-specific cellular immunity determines in vitro viral inhibition and in vivo virologic control following simian immunodeficiency virus challenges of vaccinated rhesus monkeys. J Virol 2012;86:9583–9, http://dx.doi.org/10.1128/JVI.00996-12.

[13] Mudd PA, Martins MA, Ericsen AJ, Tully DC, Power KA, Bean AT, et al. Vaccine-induced CD8+ T cells control AIDS virus replication. Nature 2012;491:129–33, http://dx.doi.org/10.1038/nature11443.

[14] Shiver JW, Davies ME, Yasutomi Y, Perry HC, Freed DC, Letvin NL, et al. Anti-HIV Env immunities elicited by nucleic acid vaccines. Vaccine 1997;15:884–7, http://dx.doi.org/10.1016/s0264-410x(96)00251-4.

[15] Letvin NL, Mascola JR, Sun Y, Gorgone DA, Buzby AP, Xu L, et al. Preserved CD4+ central memory T cells and survival in vaccinated SIV-challenged monkeys. Science (New York, NY) 2006;312:1530–3, http://dx.doi.org/10.1126/science.1124226.

[16] Buchbinder SP, Mehrotra DV, Duerr A, Fitzgerald DW, Mogg R, Li D, et al. Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial. Lancet 2008;372:1881–93, http://dx.doi.org/10.1016/S0140-6736(08)61591-3.

[17] Nabi G, Genannt Bonsmann MS, Tenbusch M, Gardt O, Barouch DH, Temchura V, et al. GagPol-specific CD4+ T-cells increase the antibody response to Env by intrastructural help. Retrovirology 2013;10:117, http://dx.doi.org/10.1186/1742-4690-10-117.

[18] Markine-Goriaynoff D, Coutelier J-P. Increased efficacy of the immunoglobulin G2a subclass in antibody-mediated protection against lactate dehydrogenase-elevating virus-induced polioencephalomyelitis revealed with switch mutants. J Virol 2002;76:432–5, http://dx.doi.org/10.1128/jvi.76.1.432-435.2002.

[19] Ishizaka ST, Piacente P, Silva J, Mishkin EM. IgG subtype is correlated with efficiency of passive protection and effector function of anti-herpes simplex virus glycoprotein D monoclonal antibodies. J Infect Dis 1995;172:1108–11, http://dx.doi.org/10.1093/infdis/172.4.1108.

[20] Schlesinger JJ, Foltzer M, Chapman S. The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis in mice. Virology 1993;192:132–41, http://dx.doi.org/10.1006/viro.1993.1015.

[21] Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Publ Group 2003;4:63–8, http://dx.doi.org/10.1038/ni873.

[22] Storcksdieck genannt Bonsmann M, Niezold T, Temchura V, Pissani F, Ehrhardt K, Brown EP. Enhancing the quality of antibodies to HIV-1 envelope by GagPol-specific Th cells. J Immunol 2015;195:4861–72, http://dx.doi.org/10.4049/jimmunol.1501377.

[23] Russell SM, Liew FY. T cells primed by influenza virion internal components can cooperate in the antibody response to haemagglutinin. Nature 1979;280:147–8, http://dx.doi.org/10.1038/280147a0.

[24] Scherle PA, Gerhard W. Functional analysis of influenza-specific helper T cell clones in vivo. T cells specific for internal viral proteins provide cognate help for B cell responses to hemagglutinin. J Exp Med 1986;164:1114–28, http://dx.doi.org/10.1084/jem.164.4.1114.

[25] Dietzschold B, Wang HH, Rupprecht CE, Celis E, Tollis M, Ertl H, et al. Induction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein. Proc Natl Acad Sci U S A 1987;84:9165–9, http://dx.doi.org/10.1073/pnas.84.24.9165.

[26] Milich DR, McLachlan A, Thornton GB, Hughes JL. Antibody production to the nucleocapsid and envelope of the hepatitis B virus primed by a single synthetic T cell site. Nature 1987;329:547–9, http://dx.doi.org/10.1038/329547a0.

[27] Ackerman ME, Mikhailova A, Brown EP, Dowell KG, Walker BD, Bailey-Kellogg C, et al. Polyfunctional HIV-specific antibody responses are associated with spontaneous HIV control. PLoS Pathog 2016;12:e1005315, http://dx.doi.org/10.1371/journal.ppat.1005315.

[28] Daly LM, Johnson PA, Donnelly G, Nicolson C, Robertson J, Mills KHG. Innate IL-10 promotes the induction of Th2 responses with plasmid DNA expressing HIV gp120. Vaccine 2005;23:963–74, http://dx.doi.org/10.1016/j.vaccine.2004.03.072.

[29] Banerjee K, Andjelic S, Klasse PJ, Kang Y, Sanders RW, Michael E, et al. Enzymatic removal of mannose moieties can increase the immune response to HIV-1 gp120 in vivo. Virology 2009;389:108–21, http://dx.doi.org/10.1016/j.virol.2009.04.001.

[30] Geall AJ, Verma A, Otten GR, Shaw CA, Hekele A, Banerjee K, et al. Nonviral delivery of self-amplifying RNA vaccines. Proc Natl Acad Sci U S A 2012;109:14604–9, http://dx.doi.org/10.1073/pnas.1209367109.

[31] Pietrella D, Kozel TR, Monari C, Bistoni F, Vecchiarelli A. Interleukin-12 counterbalances the deleterious effect of human immunodeficiency virus type 1 envelope glycoprotein gp120 on the immune response to *Cryptococcus neoformans*. J Infect Dis 2001;183:51–8, http://dx.doi.org/10.1086/317648.

[32] Nimmerjahn F, Ravetch JV. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science 2005;310:1510–2, http://dx.doi.org/10.1126/science.1118948.

[33] Nimmerjahn F, Lux A, Albert H, Woigk M, Lehmann C, Dudziak D, et al. FcyRIV deletion reveals its central role for IgG2a and IgG2b activity in vivo. Proc Natl Acad Sci U S A 2010;107:19396–401, http://dx.doi.org/10.1073/pnas.1014515107.

[34] DiLillo DJ, Tan GS, Palese P, Ravetch JV. Broadly neutralizing hemagglutinin stalk-specific antibodies require FcyR interactions for protection against influenza virus in vivo. Nat Med 2014;20:143–51, http://dx.doi.org/10.1038/nm.3443.

[35] Malim MH, Emerman M. HIV-1 sequence variation: drift, shift, and attenuation. Cell 2001;104:469–72, http://dx.doi.org/10.1016/s0092-8674(01)00234-3.

[36] Rolland M, Edlefsen PT, Larsen BB, Tovanabutra S, Sanders-Buell E, Hertz T, et al. Increased HIV-1 vaccine efficacy against viruses with genetic signatures in Env V2. Nature 2012;490:417–20, http://dx.doi.org/10.1038/nature11519.

[37] Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 1994;68:4650–5.

[38] Goonetilleke N, Liu MKP, Salazar-Gonzalez JF, Ferrari G, Giorgi E, Ganusov VV, et al. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. J Exp Med 2009;206:1253–72, http://dx.doi.org/10.1084/jem.20090365.

[39] Radebe M, Gounder K, Mokgoro M, Ndhlovu ZM, Mncube Z, Mkhize L, et al. Broad and persistent Gag-specific CD8+ T-cell responses are associated with viral control but rarely drive viral escape during primary HIV-1 infection. Aids 2015;29:23–33, http://dx.doi.org/10.1097/QAD.0000000000000508.

[40] Levy JA. The search for the CD8 cell anti-HIV factor (CAF). Trends Immunol 2003;24:628–32, http://dx.doi.org/10.1016/j.it.2003.10.005.

[41] Sperandio S, Barat C, Cabrita MA, Gargaun A, Berezovski MV, Tremblay MJ, et al. TOE1 is an inhibitor of HIV-1 replication with

cell-penetrating capability. Proc Natl Acad Sci U S A 2015;112:E3392–401, http://dx.doi.org/10.1073/pnas.1500857112.

[42] Mackewicz CE, Barker E, Levy JA, Cocchi F, DeVico AL, Garzino-Demo A, et al. Role of β-chemokines in suppressing HIV replication. Science 1996;274:1393–403, http://dx.doi.org/10.1126/science.274.5291.1393.

[43] Heeney JL, Plotkin SA. Immunological correlates of protection from HIV infection and disease. Nat Immunol 2006;7:1281–4, http://dx.doi.org/10.1038/ni1206-1281.

[44] Nchinda G, Amadu D, Trumpfheller C, Mizenina O, Uberla K, Steinman RM. Dendritic cell targeted HIV gag protein vaccine provides help to a DNA vaccine including mobilization of protective CD8+ T cells. Proc Natl Acad Sci U S A 2010;107:4281–6, http://dx.doi.org/10.1073/pnas.1000621107.

[45] Tenbusch M, Nchinda G, Storcksdieck genannt Bonsmann M, Temchura V, Uberla K. Targeting the antigen encoded by adenoviral vectors to the DEC205 receptor modulates the cellular and humoral immune response. Int Immunol 2013;25:247–58, http://dx.doi.org/10.1093/intimm/dxs112.

[46] Lindsay RW, Ouellette I, Arendt HE, Martinez J, Destefano J, Lopez M, et al. SIV antigen-specific effects on immune responses induced by vaccination with DNA electroporation and plasmid IL-12. Vaccine 2013;31:4749–58, http://dx.doi.org/10.1016/j.vaccine.2013.08.011.

[47] Morrow MP, Pankhong P, Laddy DJ, Schoenly KA, Yan J, Cisper N, et al. Comparative ability of IL-12 and IL-28B to regulate Treg populations and enhance adaptive cellular immunity. Blood 2009;113:5868–77, http://dx.doi.org/10.1182/blood-2008-11-190520.

[48] Hirao LA, Wu L, Khan AS, Hokey DA, Yan J, Dai A, et al. Combined effects of IL-12 and electroporation enhances the potency of DNA vaccination in macaques. Vaccine 2008;26:3112–20, http://dx.doi.org/10.1016/j.vaccine.2008.02.036.

[49] McElrath MJ, De Rosa SC, Moodie Z, Dubey S, Kierstead L, Janes H, et al. HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. Lancet 2008;372:1894–905, http://dx.doi.org/10.1016/S0140-6736(08)61592-5.

[50] Barouch DH, Alter G, Broge T, Linde C, Ackerman ME, Brown EP, et al. Protective efficacy of adenovirus-protein vaccines against SIV challenges in rhesus monkeys. Science (New York, NY) 2015;349:320–4, http://dx.doi.org/10.1126/science.aab3886.

[51] Lakhashe SK, Wang W, Siddappa NB, Hemashettar G, Polacino P, Hu S-L, et al. Vaccination against heterologous R5 clade C SHIV: prevention of infection and correlates of protection. PLOS ONE 2011;6:e22010, http://dx.doi.org/10.1371/journal.pone.0022010.

[52] Wagner R, Graf M, Bieler K, Wolf H, Grunwald T, Foley P, et al. Rev-independent expression of synthetic gag-pol genes of human immunodeficiency virus type 1 and simian immunodeficiency virus: implications for the safety of lentiviral vectors. Hum Gene Ther 2000;11:2403–13, http://dx.doi.org/10.1089/104303400750038507.

[53] Kothe DL, Decker JM, Li Y, Weng Z, Bibollet-Ruche F, Zammit KP, et al. Antigenicity and immunogenicity of HIV-1 consensus subtype B envelope glycoproteins. Virology 2007;360:218–34, http://dx.doi.org/10.1016/j.virol.2006.10.017.