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RESEARCH

Relevance of the Gag antigen for developing vaccine candidates against HIV-1

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ABSTRACT

Gag is one of the most conserved HIV-1 proteins. Invariable regions of this antigen have proven to be critical for viral fitness and Gag-specific T-cell responses have been associated with reduced viral load. Because of that, the whole Gag antigen or their specific epitopes are usually included in vaccine candidates against the HIV-1. This review explores the use of Gag or some of its fragments as antigens in HIV-1 vaccines clinical trials, focusing on the characteristics of each formulation, the immunization protocols and the main results obtained with vaccine candidates. The different types of vaccine candidates tested are reviewed, according to the vaccine technology used: viral vectored-vaccines based on the vesicular stomatitis virus vectors and replication deficient adenovirus vectors, DNA vaccines alone or in prime boost regimes with recombinant modified vaccinia virus Ankara vectors, virus-like particle technology and synthetic peptides. The analysis shown demonstrates the rationale for the inclusion of Gag-based antigens in the candidates to be developed, either preventive or therapeutic, against HIV-1.

Keywords: HIV-1 vaccines, p24, prophylactic HIV-1 vaccines, therapeutic HIV-1 vaccine, vaccine technology

RESUMEN

Relevancia del antígeno Gag para el desarrollo de candidatos vacunales contra el VIH-1. Gag es una de las proteínas más conservadas del VIH-1. Se ha demostrado que las regiones invariables de este antígeno son críticas para la capacidad replicativa viral y la respuesta de células T específicas contra Gag se ha asociado con una menor carga viral. Por ello, el antígeno Gag completo del VIH-1 o sus epitopos específicos se han incluido regularmente en los candidatos vacunales contra este virus. En esta revisión se explora el uso de Gag o algunos de sus fragmentos como antígenos en los candidatos vacunales contra el VIH-1 que han sido evaluados en ensayos clínicos. Los candidatos vacunales analizados, según la tecnología vacunal en la cual se basan, son: vectores virales vacunales basados en el virus de la estomatitis vesicular y en adenovirus de replicación deficiente, vacunas de ADN solas o mediante estrategias de sensibilización y potenciación con virus Vaccinia Ankara recombinantes modificados, la tecnología de partículas similares a virus, y las vacunas basadas en péptidos sintéticos. El análisis presentado demuestra la racionalidad de la inclusión de antígenos derivados de la proteína Gag en todos los candidatos vacunales preventivos o terapéuticos a desarrollados contra el VIH-1.

Palabras clave: Vacunas contra el VIH-1, p24, vacunas profilácticas contra el VIH-1, vacunas terapéuticas contra el VIH-1, tecnologías vacunales

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Introduction

The human immunodeficiency virus (HIV) is the etiological agent causing the acquired immunodeficiency syndrome (AIDS) [1, 2]. According to reports of the Joint United Nations Program on HIV/AIDS (UNAIDS) on 2016, up to 36.7 million people were estimated living with HIV and 35 million people died since the beginning of the HIV/AIDS epidemic from AIDS-related illnesses [3]. Early optimism about immunological approaches for prevention and treatment of HIV infection was replaced by combined antiretroviral therapy (cART) to slow down the disease [4]. Unfortunately, there is still no preventive or therapeutic vaccine introduced. cART dramatically improved the clinical course of the infection and the life expectancy of HIV infected patients [5]. However, the associated side effects and long-term duration leads to the emergence of drug-resistant viruses [6]. This further emphasizes the need for therapeutic alternatives to lifelong cART treatment in HIV-infected patients [7].

Two closely related but distinct types of HIV cause AIDS, HIV-1 and HIV-2, the first preponderant for the pandemic worldwide. HIV is an enveloped retrovirus which viral genome encodes nine proteins divided into three classes: major structural proteins Gag, Pol and Env; regulatory proteins Tat and Rev; and accessory proteins Vif, Vpr, Vpu and Nef [8]. Notably, Gag is the most conserved viral protein among the HIV M group circulating subtypes. Its precursor p55 polyprotein is cleaved into p17 (MAtrix), p24 (CApsid), p7 (NucleoCapsid), and p6 proteins by the viral protease prior to or during virus budding [9, 10].

Due to both, the structural determinants of the viral particle and the roles played by its resulting peptides in HIV immunopathogenesis, attention has being put on Gag as to include it in HIV vaccine candidates. Contradictorily, many vaccine candidates have been developed to fight against HIV/AIDS, but none of them have yet displayed any protective effect in human clinical trials with the exception of the RV144 [11]. Therefore, in this review we summarized the rationale for the inclusion Gag protein or some of their

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proteolytic-resulting peptides in HIV vaccines candidates already tested in clinical trials. Despite the lack of effective vaccines against HIV, the role of Gag in HIV immunopathogenesis was analyzed. Moreover, vaccine candidate design, immunization schedules and the main results attained were also addressed, together with the antigen delivery strategies used.

Role of Gag antigens in HIV immunopathogenesis

Upon infection, HIV-1 preferentially replicates inside CD4+ T cells, including monocytes and macrophages to a lesser extent [12] Once viral replication reaches some threshold at the primary mucosal infection site, infection propagates to the draining lymph nodes, where a high number of activated CD4+ T cells get further infected. Then, the virus spreads throughout the body to other lymphoid tissues, mainly gut-associated lymphoid tissue (GALT) where approximately 80 % of activated CD4+CCR5+ memory T cells are irreversibly lost by virus infection or apoptosis [13, 14]. This process lasts for 1 to 3 weeks, with a massive amount of virions produced resembling the parental virus.

Every virion bears a viral capsid of more than 2000 molecules of the p24 antigen which encapsidates the HIV genome, together with the p17 protein matrix with guarantees the right orientation of the HIV envelope proteins in the mature virus [15]. Due to the high replication rate, the concentration of p24 subunits in the blood stream leads to the early production of p24-specific antibodies. Despite, anti-Gag antibodies neither neutralize viral particles nor induce antibody-dependent cell-mediated cytotoxicity (ADCC) or cell-mediated virus inhibition (ADCVI) [16, 17].

The p24 antigen has the most conserved amino acid sequence among all HIV-1 Gag products, in 10 000 full-length Gag sequences compared across eight major HIV-1 subtypes [18]. Some mutations in the Gag p24 conserved region has been found as decreasing HIV replication capacity and compromising viral fitness [7]. Conversely, other mutations in the highly variable Gag p17 region increased the viral replication capacity [19]. Either the case, natural variations found in the p17 amino acid sequence among HIV subtypes did not interfere on its biological function. A particular single amino acid position in the conserved motif within the major p17 antibody binding site in subtypes A, B and G isolates, was associated to child protection from vertical transmission, suggesting a protective role of the antibody response against that epitope [20].

During HIV infection, the number of CD4+ T helper cells also decreases in the periphery [21]. After the early viremia which promotes T cell responses mostly against Env and Nef proteins, a second wave of T cell response is triggered against Gag p24, which has been found essential for maintaining viral load at a set point [22]. In fact, a polyfunctional IFN-γ/IL-2 anti-Gag p24-secreting T cell response is developed during the first six months after HIV infection (acute infection), which correlates with a lower viral load and a low viral set point [23].

In the absence of cART the loss of CD4+ T cells further drop down the number of CTL lymphocytes, resulting in uncontrolled viral replication and progression to AIDS. This CD4+ Th loss erodes the effectiveness of the immune response both during the acute and chronic viral infection periods [24]. Significantly, the p24 capsid antigen concentration in blood is a useful marker to predict CD4+ T cell decline and clinical progression at early and late stages of infection [25]. It is also useful to monitor the effectiveness of cART both in adults and children [26, 27]. In suppressed viremia, p24 can be detected in the bloodstream and its concentration negatively correlates with CD4+ T cell number and positively with the levels of activated CD8+ T cell subsets [9].

There were experimental evidences on the strong correlation of anti-Gag IFN-γ-secreting CD4+ T cells with controlled viremia in different cohorts of HIV infected subjects. Robust and sustained p24-specific proliferative CD4+ T cell responses with significant levels of IFN-γ secretion were associated with controlled viremia and established non-progressor status in long-term non-progressors [28, 29].

Furthermore, in untreated chronically infected patients, viral load inversely correlates with both lymphoproliferative CD4+ response to HIV p24 [30] and Gag p24/p17-specific CTL response [31, 32]. Strong p24-specific CD4+ T cell responses were also associated with efficient viral control in primary HIV-1 infection [33].

In a study analyzing CD4+ T cell responses to the entire HIV proteome in 93 subjects at different stages of infection, IFN-γ+ CD4+ T cells developed against Gag p24 and p17 immunodominant epitopes strongly associated with lower levels of viremia [34]. Additionally, responses to immunodominant peptide sequences of two Gag p24 and one p17 epitopes were related to spontaneous HIV control [34].

The magnitude and the quality of anti-HIV-1 cellular immune responses are relevant to efficiently control viral replication. But Kannanganat et al. demonstrated that polyfunctional Gag-specific CD4+ T cells producing various (IL-2, IFN-γ and TNF-α) rather than a single cytokine were more efficient to control viral replication [35, 36].

Currently, the primary goal of many vaccine candidates against HIV-1 is to elicit strong CD8+ T cell responses, for controlling HIV replication and to preserve the global count of CD4+ T lymphocytes. In this line, Edwards *et al*. tested this hypothesis by stimulating PBMC from 27 chronically infected patients, most of them without cART, with 20-mer peptide pools from the HIV-1 antigens Gag, Env, Pol and Nef [37]. Although all patients secreted IFN-γ against at least one specific HIV peptide, the higher frequency of responders was detected with Gag or p24 peptide pools. The magnitude and breadth of the global T-cell responses inversely correlated with plasma viral load and directly with absolute CD4+ T-cell counts. These higher responses substantially decreased or disappear after CD8 + T cell depletion, indicating that most of the HIV-1-specific responses measured after stimulation with the 20-mer peptides were specific for CD8+ T-cell [37]. In this regard, these evidences emphasize the relevant role of p24 and p17 in virion assembly and the detection of an immunodominant T cell response against them along all stages of the HIV infection [37, 38].

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Approaches for developing Gag-based vaccine candidates

Recombinant vaccines comprise protein antigens natural or modified from their respective pathogens, which are encoded in plasmids, bacteria or viral vectors, expressed and purified from experimental cellular systems and administered for the induction of immunity. Technologies applied for developing Gag-based vaccine candidates against HIV-1 are discussed below and summarized in Table.

Viral vectors

Among them, viral vectors have been widely used against diseases caused by intracellular pathogens, due to its advantage as to directly deliver the antigen within cells of vaccinees and without previous protein purification. This enables their presentation on MHC-I molecules to effector T cells for activating the specific immune response. This strategy has antigen-dependent advantages such as their ability to mimic a natural viral infection and their capacity of inducing CD4+ and CD8+ T cell responses. In the field of HIV, some approaches have been widely used using viral vectors to develop vaccine candidates [39]. Two relevant examples will be discussed in the following, encoding for Gag as recombinant antigen.

VSV Indiana/HIV Gag vaccine

A vaccine candidate named rVSVN4CT1gag1 was tested, comprising a highly attenuated recombinant vesicular stomatitis virus (rVSV) vector which expressed a subtype B HIV-1 Gag gene in the first position of the vector's genome [40]. Its safety and immunogenicity was assessed in a randomized, double-blind, placebo-controlled dose-escalated phase Ia clinical trial (ID HVTN090) conducted in USA. The study, developed by Merck, included 60 healthy adults, 18-to-50 years-old, who reported low behavioral risk for HIV infection. The vaccination protocol included two intramuscular immunizations (weeks 0 and 8) at five-dose level (4.6×10^3 p.f.u.-3.4×10^7 p.f.u.) of rVSVN4CT1gag1. The use of this homologous prime-boost strategy induced a modest anti-Gag p24 antibody response. At the same time, 33 % of vaccinees (n = 38) exhibited HIV-1-specific T-cell responses at the highest dose, specifically IFN-γ- and/or IL-2-secreting CD4+ T cells after overnight stimulation with a consensus B Gag peptide pool. The magnitude of those anti-Gag CD4+ T cell responses correlated with VSV neutralizing antibodies titers induced in all the vaccinees at the end of immunization [41]. This trial demonstrated the safety and immunogenicity of an attenuated replication-competent gag-expressing rVSV-based vaccine as a promising candidate for future HIV vaccine development.

Replication deficient Ad5 vectored vaccine candidates

One of the most promising immunogens tested in humans for the induction of HIV-specific T cell responses was the replication-deficient Adenovirus type 5-vectored (Ad5) vaccine candidate expressing clade B HIV-1 gag [42]. That monovalent variant was named MRKAd5 gag and evaluated in a phase I randomized, double-blind, placebo controlled trial (ID V520-007). This study, conducted by Merck, included 360 healthy HIV-negative subjects from five geographic regions (North and South America, the Caribbean, South Africa and South-east Asia). Volunteers were 18-to-50 years-old and classified as low-risk for HIV. Preexisting Ad5 immunity was not an exclusion factor. The primary end point of this trial was to assess the safety, tolerability, and immunogenicity of a three-dose regimen of the vaccine

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Table. Examples of vaccine candidates tested using the HIV-1 Gag antigen or its fragments, as part of different vaccine technologies for prophylactic or therapeutic vaccines*

Study type	Technology	Candidate	Trial ID	Company	Phase	Volunteers serological state	Volunteers	Immunization route	Adjuvant	cArt
Prophylactic	Viral vector	VSVN4CT1gag1	HVTN 090	Merck	Phase I	Seronegative	60	i.m.	None	No
	Viral vector	MRKAD5	ID V520-007	Merck	Phase I	Seronegative	360	i.m.	None	No
	Viral vector	MRKAD5	ID ACTG 5197	Merck	Phase I	Seropositive	114	i.m.	None	No
	DNA vaccine	HIV-1 gag p37 DNA	HVTN 060 and 063 HVTN	Merck	Phase I	Seronegative	264	i.m.	IL-12 and IL-15 DNA	No
	DNA (IAVI 001) Viral vector (IAVI 003) Heterologous prime boost (IAVI 005)	pTHr.HIVA DNA and MVA.HIVA	IAVI 001, IAVI 003 and IAVI 005	Bristol-Myers Squibb Company	Phase IIa	Seronegative & seropositive	26	(IAVI 001) i.m. (IAVI 003 and IAVI 005) i.d.	None	Yes
	DNA vaccine	pTHr.HIVA	IAVI 006	Bristol-Myers Squibb Company	Phase I	Seronegative	119	i.m.	None	Yes
Therapeutic	VLP	P17/p24:Ty	AVEG 019	British Biotech Pharmaceuticals Limited	Phase IIb	Seropositive	74	i.m.	Aluminum hydroxide	Yes
	Synthetic peptides	Vacc-4x	NCT00659789	Bionor Pharma	Phase I	Seropositive	11	i.d.	rhGM-CSF	Yes (9) No (2)
			NCT01473810	Bionor Pharma	Phase II	Seropositive	24	i.n.	Endocine	Yes
			NCT02092116	Bionor Pharma	Phase Ia/IIb	Seropositive	270 17	i.n. i.d.	rhGM-CSF	Yes

* i.d.: intradermal; i.m.: intramuscular; i.n.: intranasal.

(weeks 1, 4 and 26) at 1×10^9 and 1×10^{10} viral particles (vp) per dose. A secondary objective was to compare immune responses to the vaccine as a function of baseline Ad5 titers and participants' geographic region. Of them, 296 were vaccinated and 64 received at least one dose of placebo, all randomized. Immunogenicity was measured by IFN-γ ELISPOT assay using 15-mer Gag peptides.

No serious vaccine-related adverse events occurred in any treatment group. ELISPOT responses were significantly higher for recipients of both active vaccine doses compared to placebo in all regions except southern Africa. Only data of 14 African subjects were available. The vaccine was generally well tolerated in all the subjects and no vaccine-related serious adverse events were reported, but some of the events were put in consideration. Local adverse events appeared to be dose-dependent, but were not significantly affected by baseline Ad5 titers. There was an observed trend toward higher rates of fever in subjects with low baseline Ad5 titers at the 1×10^{10} vp dose. Despite, the small size of the sample for low and high Ad5 titers' groups limited the possible conclusions on the higher frequency of systemic adverse events in subjects with low preexisting immunity to Ad5.

Moreover, the effect of baseline Ad5 titers on ELISPOT responses differed according to region. Although in North America the group of subjects with baseline Ad5 titers above 200 had a distinctive lower ELISPOT response rate, this did not hold true for participants from Asia. These findings could be related with the storage conditions of PBMCs during transportation and also HLA distribution. Furthermore, seroconversion was directly related to vaccine dose and inversely to baseline Ad5 titer. Approximately 17 % of study participants had positive whole virus (Ad5)-based immunoassay results at week 78, but they were negative for HIV PCR tests. Among those ELISA-positive subjects, ELISA positivity persisted through week 156 for participants receiving the 1×10^{10} vp dose [42].

Regarding the protection against infection, four vaccinees became HIV infected, higher than in the placebo group but at the same infection rate among placebo recipients. The authors conclude that the Ad5 vector was well tolerated and immunogenic in diverse global populations and, recommended it as a possible good candidate for other cell-mediated vaccine strategies.

The other example antigen was the one tested in the STEP clinical trial [43]. It was based on the immunization with a trivalent replication-deficient Adenovirus type 5 vaccine vector (Ad5) expressing three different clade B HIV-1 *gag*, *pol*, and *nef* genes. In the case of the *gag* gene, it was the same previously included in the MRKAD5 vector. The trial was conducted by Merck Research Labs and by the HVTN in Australia, the Caribbean and North and South America. STEP included a subpopulation of volunteers at increased risk of HIV infection [43], its results regarded as a significant issue for the continuation of the MRKAD5 vaccine strategy just with the *gag* gene.

The MRKAD5 construct was also tested in a therapeutic setting [44]. One hundred fourteen participants were randomized (2:1 ratio) in a double blinded phase I study to receive the vaccine or placebo. The HIV-infected volunteers were stratified by the highest known pre-therapy HIV-1 RNA level (Groups I, II and III: $< 30{,}000$, $\geq 30{,}000$ RNA copies or unknown, respectively). An analytical treatment interruption (ATI) procedure was applied on week 16, further monitoring plasma HIV-1 RNA levels and CD4 cell counts [44]. RNA levels above 500 copies/mL or CD4+ cell counts below 500/mm^3 during vaccination were considered as interruption criteria, and antiretroviral therapy could be resumed at their discretion. Virus-specific cellular immunity was assessed by PBMC IFN-γ secretion upon *in vitro* incubation for 18 h with or without Gag, Nef, or one of two Pol peptide pools. CD4+ or CD8+ T cells' IFN-γ secretion was evaluated by 4-color scattered intracellular cytokine flow cytometry. Values were expressed as the number of IFN-γ producing cells/10^6 lymphocytes after peptide stimulation. HIV-specific proliferation capacity was also tested on CD4 or CD8+ T cells once stimulated with an inactivated HIV-1 or p24 antigens. It was assessed by carboxyfluoresceinsuccinimidyl ester (CFSE) staining prior to the stimulation and quantitation of dye dilution by flow cytometry after 7 days.

Finally, 113 participants received all 3 injections and 110 entered the ATI. During the ATI, 3 participants met protocol specified criteria recommending therapy restart. The primary goal of the study was to evaluate the effect of vaccination on viral rebound kinetics during ATI. The second co-primary endpoint was determination of the 'ATI viral set point', defined as the mean of the log10 plasma HIV-1 RNA levels at 12 and 16 weeks following ATI, with one of two values when the other was missing [44].

Overall, vaccination was regarded as generally safe and well tolerated in all the patients. HIV-1 specific cellular immune responses were found, despite a modest viral rebound kinetics following ATI. The Gag-specific IFN-γ-secreting CD4+ T cell frequencies were higher than for CD8+ T cells, the first strongly correlating with controlled viral replication during ATI. Nevertheless, these responses were neither correlated to an effect on infected cells, nor demonstrated as a surrogate of immune responsiveness, or indirectly increased the HIV-1 specific cellular immunity through other cellular effectors. In case that vaccination would have induced HIV-1 specific effectors cells, potent and specific enough, they would not be able to migrate to HIV-1 replication sites in lymphoid organs. Ultimately, the authors recommended attaining a substantially higher immunogenicity threshold in future studies prior to testing ATI responses. In summary, the favorable trends in viral rebound kinetics were not enough as expected as to achieve a possible long-term therapeutic effect.

DNA vaccines

DNA vaccination is based on the delivery of a plasmid or DNA-based vector expressing the gene of interest for inducing an immune response [45]. Despite the limited and poorly immunogenic results of this technology in humans, different approaches have been searched for with HIV DNA vaccines to increase the immune response. One of them included the heterologous prime-boost, with DNA vaccine priming followed by a boost with a recombinant viral vector or HIV recombinant proteins [45].

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HIV-1 gag DNA/ Interleukin vaccine

A HIV-1 *gag* p37 DNA vaccine was derived from strain HXB2 [46]. It was engineered for RNA optimization to achieve a high level of Rev-independent-expression, and including *IL-12* or *IL-15* genes as vaccine adjuvants. Two plasmid vectors were assessed as adjuvants, with a dual promoter cassette expressing the two chains of the IL-12 gene, or IL-15. Plasmid formulations were tested in two multicenter, randomized, placebo-controlled, double-blind Phase I clinical trials for safety, reactogenicity and tolerability. The HVTN 060 (including IL-12) trials was conducted in the US and Brazil (063) and the HVTN 063 (including IL-15) in Thailand (060), both by Merck. Study participants were randomized to receive gag pDNA alone (1500 μg) or the same *gag* pDNA + dose escalations of 100, 500 and 1500 μg of IL-12 pDNA (HVTN 060) and pDNA IL-15 (HVTN 063). Thirty individuals received two additional vaccinations with gag pDNA alone and 30 volunteers received two immunizations with gag pDNA + 1500 μg of IL-12 pDNA (HVTN 060) and IL-15 pDNA (HVTN 063). Shots were intramuscularly administered at 0, 1 and 3 months, followed by boosting doses on months 6 and 9. The *ex vivo* T cell responses were assessed in subjects cryopreserved PBMCs using a validated IFN-γ ELISpot assay with a panel of two consensus Clade B Gag peptide pools. Additionally, the antibody levels against p55 were evaluated with a validated ELISA at a 1/100 serum dilution.

Vaccines were well tolerated, with no statistically-significant differences found between placebo and the vaccine group regarding adverse events and no correlation between severity and doses.

No severe adverse events were related to vaccination, indicating that cytokine production was limited to the injection site and cause no undesired side effects. Cellular immunogenicity following three and four vaccinations were regarded as poor. After the third vaccination, there were 0/10 responders in the gag DNA + IL-12 DNA (100 μg) group and 4/9 (44 %) responders in the gag DNA + IL-12 (500 μg) group. For the gag pDNA + IL-12 1500 μg group and the *gag* pDNA + IL-15 dose groups, was observed a complete lack of response. Following the fourth vaccination dose, the *gag* DNA alone response rate was 2/23 subjects (8.7 %); for *gag* pDNA + IL-15 1500 μg were 3/26 responders (11.5 %) and in subjects receiving three gag pDNA + IL-15 vaccinations followed by vaccination with *gag* DNA + IL-12, 3.6 % the rate of response was 1/28 (0.6 %). No participant developed a vaccine-induced anti-gag response. Other clinical trials have resulted in a lack of anti-gag antibody and low frequency and magnitude cellular responses after DNA vaccination [47], probably indicating intrinsic differences of the human response to HIV-1 Gag with this technology. In summary, these data indicated that IL-12 and IL-15 were safe, but they offered little advantage to increase the cellular immune responses at least using naked DNA delivery. Recent studies have proven the efficiency of electroporation as a DNA delivery method in animal models [48] and a small human trial showed HIV-1 DNA delivered via electroporation was safer and more immunogenic than DNA delivered via standard vaccination [49].

DNA prime and recombinant modified vaccinia virus Ankara boost

Two HIV-1 vaccine candidates combining a pTHr. HIVA DNA vaccine prime followed by a boost with a modified vaccinia virus Ankara (MVA)-vectored HIV vaccine named MVA.HIVA [50] were assayed. Both candidates expressed the HIVA immunogen, derived from consensus HIV-1 clade A Gag p24/ p17 sequences and a string of clade A CTL epitopes. They were aimed primarily to elicit cell-mediated immune responses. Preclinical studies in mice and macaques demonstrated good immunogenicity for both of them [50]. With the primary end point of addressing vaccine safety, DNA and MVA candidates were evaluated on their own and in a prime-boost regimen in three combined phase I prophylactic clinical trials (ID IAVI 001, 003 and 005) [51]. Healthy HIV-1 uninfected individuals, 18 to 60 years old, were recruited in the United Kingdom and the cohort was randomized in either pTHr.HIVA or MVA. HIVA. Volunteers in the pTHr.HIVA DNA arm were randomly distributed to receive 100 μg (n = 6) or 500 μg (n = 12) of plasmid DNA in a double-blind schedule. Subjects received two immunizations at days 0 and 21 into alternate deltoid muscles. The MVA.HIVA arm was an open-label study, including eight volunteers that were immunized intra-dermally with 5×10^7 p.f.u. of MVA.HIVA, on days 0 and 21. A third part of the study was a prime-boost schedule including nine volunteers from the DNA arm. The subjects were boosted using the same dose of MVA, timing and route as in the MVA-alone trial at 9-14 months after their last DNA inoculation. T-cell responses to intramuscular pTHr.HIVA DNA immunization were measured by interferon γ enzyme-linked immunospot (IFN-γ ELISPOT) assay on fresh whole PBMCs against four overlapping peptide pools of Gag domain and three pools of CTL epitopes included in the HIVA immunogen. The immunization with MVA gave small reactions at the site of intradermal injection that persisted up to 14 days. Overall, the DNA and MVA vaccines alone and the combination of DNA prime-MVA boost proved to be safe and immunogenic in humans.

Regarding the CTL response in the DNA arm, due to the small number of volunteers, no differences were observed between the two doses. A total of 14 (78 %) volunteers responded to the DNA vaccination, four (28 %) of them receiving 100 μg and 10 (72 %) 500 μg, with T-cell responses classified as weak and transient. No differences between both dosages levels were observed, the responses regarded as low. Surprisingly, HIV-specific responses were detected at one year after vaccination in seven volunteers. The authors pointed out that these unexpected late responses need to be confirmed in future trials. Positive T-cell responses in MVA.HIVA arm were detected in seven out of eight (88 %) volunteers, five of them (63 %) reporting HIV-specific responses still a year after vaccination.

In case of the prime-boost schedule, eight out of nine (89 %) volunteers developed positive cellular responses, with MVA-induced responses stronger and more frequently detected than those elicited by DNA vaccines. This effect was independent of the small

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number of volunteers tested, and with very similar cellular responses as MVA alone.

A second multi-arm, placebo-controlled phase I/IIa trial (ID IAVI 006) was conducted in the United Kingdom including 119 healthy volunteers at low risk of infection [52]. The volunteers were randomized in a 1:1:1 ratio to receive priming with 2 mL solution containing the vaccine (0.5 or 2 mg dose) or placebo, intramuscularly. Volunteers received either dose of pTHr.HIVA DNA intramuscularly twice, followed by 4- or 16-week intervals before two intradermal doses of MVA.HIVA. Either vaccine or placebo was delivered into alternating deltoid muscles. The end point of this trial was to induce HIV-1 specific T CD8+ lymphocytes combining pTHr.HIVA and MVA.HIVA vaccines in a prime-boost regimen. The prime-boost strategy was applied in a factorial trial design, using 0.5 mg or 2 mg of pTHr.HIVA DNA vaccine or Placebo followed by two intradermal booster vaccinations with 5×10^7 MVA.HIVA or Placebo at weeks 8 and 12 (early boost) or weeks 20 and 24 (late boost). HIV-1 specific T-cell responses were measured by IFN-γ ELISPOT assay in cells stimulated with overlapping peptides of HIV-1 clade A Gag protein and CTL vaccine epitopes.

HIV-1 specific T-cell responses, measured by ELISPOT, were observed in 12 out of 119 (10 %) of volunteers, ten of them observed only after MVA vaccination. Two of them (20 %) received placebo at priming and eight (80 %) received pTHr.HIVA DNA at priming. A similar number of responders were found in early and late boost subjects. In two volunteers, ELISPOT positive responses were observed following pTHr.HIVA vaccination and persisted after boosting with MVA (weeks 20 and 24). The trial IAVI-006 showed a lower positive response rate compared to other studies using the same antigens. It possibly resulted from a lack of consensus in the field for defining cut off values for a positive HIV-1 specific T-cell response. Ultimately, assay's sensitivity was lower compared to criteria adopted by other groups. Hence, the immunization with pTHr.HIVA and MVA.HIVA clade A vaccines proved to be safe and well tolerated and no serious adverse events were reported [52].

Virus-like particles vaccines

Virus-like particles (VLPs) are multimeric protein structures assembled from viral structural antigens that includes repetitive high-density epitopes surfaces [53]. VLPs T- and B-cell epitopes are capable to induce strong cellular and humoral responses, further displaying a potent adjuvant activity, thereby enhancing immunogenicity against weak antigens [53].

In the case of HIV clinical trials, an example of VLPs vaccination approach is the p17/p24:Ty HIV-1 vaccine candidate, developed in the early 90's by the British Biotech Pharmaceuticals Limited company [54]. The VLPs produced expressed a TYA:p17/p24 fusion gene in yeast and included a portion of the HIV-1IIIB Gag sequence, specifically the C-terminus of Gag p17 and the N-terminus of Gag p24 proteins. The purified antigen was adjuvanted in aluminum hydroxide. It was tested for safety, toxicity and immunological response a phase II double-blind placebo-controlled therapeutic clinical trial (ID AVEG 019), with the effect of vaccination on CD4 cell count and viral load as trial endpoints. The study enrolled 74 asymptomatic HIV-1-infected subjects, 18-60 years-old, with a positive anti-p24 antibody response. Several doses of 100, 500, or 1000 µg of p24-VLP were intramuscularly injected into the deltoid muscles in experimental groups, and evaluated in comparison with a placebo control group (alum in PBS) on weeks 0, 4, 8, 12, 16, and 20. Sixty-three volunteers were followed up until week 48, with immunogenicity assessment for p24, p17 and Ty specific-antibody responses by ELISA.

The vaccine candidate was proven safe, showing minor adverse events in all the patients, including the placebo group. Moreover, it was immunogenic, with a four-fold increase in p24 antibody levels equivalent among participants in all the treatment groups. Some of the increased effects developed within the follow up. Otherwise, the increased antibody responses did not correlate with the CD4 cell counts or p24 antibody titers at baseline. Variations seen in p17 antibody levels were not associated to the immunization with p24-VLPs. Particularly, responses to the yeast Ty antigen showed to be influenced by the antigen dosage and the baseline CD4+ cell count of the volunteers. Specifically in the 1000 µg group, all subjects but one developed an antibody response to Ty. Otherwise, repeated immunization with p24-VLP did not induce significant differences in CD4+ cell counts between groups. Additionally, viral load was measured in a cohort of patients randomly selected from the placebo group (n = 4) and the group receiving 1000 µg of p24-VLP (n = 10). No statistically significant difference were found among the groups. Hence, the repeated immunization with p24-VLP did not induce significant differences in the CD4+ cell counts among groups, with no detectable effect on markers specific for disease progression [54].

Afterwards, a study into the long-term effects of the p17/p24:Ty phase II clinical trial was conducted in a multicenter cohort study enrolling 56 participants, aimed to assess the effect of therapeutic vaccinations on clinical outcomes and to add information to the safety database. The end point was to evaluate the effect of the p24-VLP vaccination on disease progression and death, comparing the rates of CD4+ cell counts decline between vaccinees and the placebo group. The use of ART (either duo or triple ART, according to the intention-to-treat) and age were included as potential confounders. Results suggested that therapeutic vaccination with p24-VLP had no long-term effects on disease progression or death, or clinical deterioration of subjects [55].

These evidences suggest that antiretroviral therapy improves T-cell responses. Therefore, the effect of combined therapy with p24-VLP and zidovudine (ZDV) was further assessed. A double dummy, double-blind randomized placebo-controlled Phase II trial of the therapeutic vaccine p24-VLP in absence or presence of ZDV was conducted [56]. ZDV-naive individuals were randomized to one of the three groups: group A-200 mg of ZDV, three times daily and six immunizations every four weeks with placebo vaccine; group B-200 mg of ZDV three times daily plus six immunizations with p24-VLP in alum monthly;

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Group C-placebo pills three times daily and six immunizations with p24-VLP in alum monthly. The primary endpoint was p24 antibody titers on weeks 24 and 52, and secondary were safety, CD4+ lymphocyte counts, viral load, T-cell proliferative responses to p24, cutaneous DTH responses to recall antigens and disease progression. An eight-fold increase in p24 antibody endpoint titer was set as clinically relevant, and up to 61 patients received vaccinations. Proliferative assays were performed on a subgroup of 30 patients using PBMCs on weeks -2, 0, 12, 24, 36 and 48.

The serum antibody production against p24 and Ty antigens was assessed by ELISA and the effects of treatment on T-cell function in vivo were measured by DTH at weeks -2, 4, 12, 24 and 48. The total DTH score was calculated as the sum of the mean diameters of each positive antigen response (positive response defined as an induration area with mean diameter higher than 2 mm). Normal response was set as with diameter higher than 10 mm of total induration, hypoergic in the range 2-10 mm, and anergic if not greater than 2 mm to any antigen. The levels of CD3+ CD4+, CD3+ CD8+ and CD3+ CD38+ cells were measured as markers of disease progression and immune activation by flow cytometry in PBMCs samples. Samples were collected at the screening visit and at weeks -2, 0, 1, 4, 8, 12, 16, 20, 21, and 22. Despite the induction of antibody and proliferative responses on weeks 24 and 52, no significant differences were detected among groups for antibody responses against p24, CD4+ or CD8+ cell counts, viral load, T-cell responses to p24, p17, the recall antigen or the mitogen, or markers of immune activation. There was a tendency to improve total DTH response as observed in the ZDV-alone group at week four, but it did not achieve statistical significance. The lack of efficacy of the trial was consistent with results of previous studies exploring the efficacy of therapeutic immunization with recombinant proteins derived from envelope proteins of HIV. The candidate was found safe, despite the occurrence of four serious adverse events, three of them regarded as unrelated to treatment and one as possibly related. In general, the immunization was well tolerated [56].

Synthetic peptides

Because viral proteins contain a number of epitopes which do not induce a protective immune response, synthetic peptides comprising immunorelevant epitopes have been included in vaccination strategies. Vaccines based on synthetic peptides are considered safe and cost effective. Nevertheless, the small sizes of peptides make them weak immunogens and they require the use of carrier molecules and adjuvants [57].

One such peptide candidate was Vacc-4x, a peptide-based therapeutic vaccine developed by Bionor Company [58], comprising Gag p24 short soluble 24-27 amino acids-long peptides. It was included in an open phase I clinical trial aimed to evaluate its safety and immunogenicity at different doses [58]. No control groups were included and patients were enrolled having CD4+ cell count > 250×10^6/L with an unchanged cART treatment for the last 20 weeks or without treatment. Exclusion criteria were CD4+ cell counts below 150×10^6/L or a plasma HIV-1 RNA increase over 10 fold lasting more than 2 weeks. Finally, 11 patients were included, nine of them receiving ART. The peptides were lyophilized and reconstituted in sterile distilled water immediately before injection. The protocol of the trial included immunization and DTH test. All patients received doses of Vacc-4x including 0.8 mg of peptides at each immunization time-dose, 0.4 mg in form of immunogen (0.1 mg per peptide) and 0.4 mg of the same peptides were used to assess the DTH response. The vaccine was administered intradermally with recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) for optimal uptake of the peptides by the antigen-presenting cells (APCs). The immunization schedule comprised three immunization periods with 12 repeat inoculations: six administrations on weeks 1, 2, 3, 4, 6 and 10, followed by two booster periods (single immunizations on weeks 14, 15 and 16 and weeks 24, 25 and 26, respectively).

From week 6 on, the vaccination schedule was dependent of the DTH response at the two previous time points. Volunteers with positive DTH tests over 100 mm^2 indurations did not receive any further vaccination with Vacc-4x until a lower DTH response were recorded, resulting in different peptide amounts administered among patients. Each immunization include two 100-µL volumes of the vaccine peptide solution; 30 µg of rhGM-CSF was injected intradermally in the abdomen and, 15 min later, 100 µL of the vaccine were also injected in the same site. The DTH test included a 100-µL portion of Vacc-4x peptide solution injected at the abdominal collateral site of the immunization. The DTH skin reaction was evaluated two days after injection and diameters over 5 mm were considered positive. Serum samples and peripheral blood were collected. The antibody response to Vacc-4x was assessed by a magnetic particle ELISA, and the CD8+ T cell response by ELISPOT assay on patients' PBMCs samples, preceded by HLA class I typing [58].

Overall, the vaccine was safe and well tolerated, and no serious adverse events were reported. Nevertheless, all patients reported at least one adverse event considered to have a possible relationship to treatment. For the nine patients under ART, no major changes in CD4+ and CD8+ counts were found. The immunization did not increase plasma viremia and there were no changes in humoral responses for any of the immunized patients. Five patients reported adverse effects like fatigue and vertigo, mainly after receiving either immunization or the DTH test. Painful injection was the most frequently reported adverse events. No patient had a positive DTH response after the first immunization, but all patients had a positive DTH reaction during the study. Regarding de ELISPOT response, three patients responded to at least one of the peptides and one of them showed a four-fold increase in the response against one of the peptides. The three patients responding to ELISPOT assay did not had a particular HLA-A or HLA-B profile compared with the rest of volunteers. The authors mentioned limitations such as the small number of volunteers and the heterogeneity of the samples, also referring that the amount of peptides used in the formulation could be considered low. In our opinion, two important limitations of this study were the use of

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the intradermal-abdominal route, which might be very difficult to implement in a vaccination campaign, and the exclusion of a placebo group.

In 2014, the results of another Vacc-4x clinical trial were published [59]. A single-blinded, randomized trial was conducted, including 24 HIV-infected patients over 18 years-old. Safety and immunogenicity after intranasal administration were primary endpoints at different vaccine doses, and the vaccine-specific immune regulation mediated by cytokines that potently inhibits Th1 responses as secondary endpoint. The subjects were infected for more than one year, on effective ART for at least six months with viral load lower than 50 copies/mL, CD4+ T cells counts above 400 cells/mL and a CD4+ nadir higher than 200/mL. Exclusion criteria were AIDS-defining illnesses and malignancies.

Vacc-4x was designed to mainly induce systemic cellular immune responses, further studying if it would be capable of eliciting nasal and rectal humoral responses. Patients were randomly allocated in two groups to receive either Vacc-4x with Endocine as adjuvant or adjuvant alone [59]. Endocine is an adjuvant based on entirely endogenous lipids, developed by Eurocine Vaccines, and used for mucosal immune response stimulation when mixed with the antigen and administered as drops by the intranasal route. In this trial, 18 patients were immunized with low, medium or high levels of Endocine, (LD, 80 µg Vacc-4x), (MD, 400µg Vacc-4x) or (HD, 1200 µg Vacc-4x), respectively. Six patients received adjuvant only. All patients were given 150 µL in each nostril. A total of four doses were administered at weekly intervals and nasal and rectal mucosal secretions were collected on weeks one and eight. The vaccine-specific IgA and IgG antibodies in mucosal secretions and serum were evaluated by ELISA. A Vacc-4x DTH was performed at the end of the study (week 8) by intradermal injection of 400 µg Vacc-4x in 100 mL sterile water.

A positive test was defined as a palpable skin infiltrate with an induration area higher than 10 mm^2. T cell proliferation and regulation was performed using fresh PBMCs pulse-labeled with carboxyfluorescein-succinimidyl ester (CSFE). No serious adverse event related to the vaccine was reported. Four weeks after the last vaccine or adjuvant dose, the DTH skin test performed to evaluated cellular immune responses *in vivo* evidenced a dose-dependent Vacc-4x DTH induration. DTH induration tended to be larger for the Vacc-4x group compared to the adjuvant group. No significant differences were observed in Vacc-4x-specific proliferative T cell responses among any of the four groups. Vacc-4x IgA and IgG antibody responses were detected in rectal mucosal samples in 91 and 64 % of volunteers, respectively, correlating with the anti-p24 antibody levels. Nasal Vacc-4x IgA and IgG antibody levels were above the cut-off in 78 and 61 % of volunteers, respectively. During the study period, a significant increase in rectal mucosal Vacc-4x IgA and IgG antibodies was seen only among LD patients. The LD group was the only one where rectal Vacc-4x IgA antibodies increased significantly compared to the adjuvant group.

Nevertheless, an increase in nasal IgA antibodies was observed in the two higher dose groups compared to the adjuvant group. In serum, Vacc-4x IgG antibody levels increased significantly only in the HD group compared to the adjuvant group. Negative correlations were found between systemic proliferative T cell responses and nasal Ig antibody levels. The results showed that intranasal administration of Vacc-4x adjuvated with Endocine was safe. Regardless the limited numbers of patients receiving each dose, dose-dependent differences in both cellular and humoral vaccine specific responses were observed. The authors proposed that the clinical significance of these findings should be addressed in larger cohorts [59].

Another phase 1B/2A single-arm vaccine trial involving Vacc-4x vaccine candidate was conducted, named REDUC [60]. Its primary endpoint was to evaluated the effect on the size of the latent HIV-1 reservoir of the combination of the vaccine candidate Vacc-4x and rhGM-CSF as local adjuvant followed by treatment with the latency-reversing agent romidepsin. Up to 17 patients out of 270 interviewed were enrolled and immunized, aged $\geq$ 18 years old and an HIV-1 successful treatment with plasma RNA loads lower than 50 copies/mL the preceding year and CD4+ counts 500 cells/µL. Exclusion criteria were: CD4+ counts below 200 cells/µL within the past 2 years, active hepatitis B or C infections, and clinically significant cardiac disease. The volunteers receive six intradermal immunizations at week 0, 1, 2, 3, 11 and 12 with 0.1 mL of 12 mg/mL Vacc-4x and 0.1 mL of 0.6 mg/mL rhGM-CSF. After the final immunization, the patients were treated weekly for 3 weeks with 5 mg/m^2 intravenous romidepsin infusions. Patients were under ART until an analytical treatment interruption (ATI) followed the immunization and activation phase. ART were resume after two consecutive viral load measurements higher than 1000 copies/mL or CD4 T-cell counts below 350 cells/µL. Changes in total and integrated HIV-1 DNA and in the frequency of infectious units per million (IUPM) of resting memory CD4+ T cells were the main outcomes of the study.

Overall, the vaccine candidate plus rhGM-CSF was shown safe and tolerable enough, with mild adverse events that did not modify the regimen under study [60]. Furthermore, total HIV-1 DNA at screening was assessed six weeks before and six weeks after romidepsin treatment, together with the integrated HIV-1 DNA at baseline before romidepsin treatment and 8 weeks after romidepsin treatment. All volunteers showed detectable total HIV-1 DNA, 15 out of 17 having integrated HIV-1 DNA and six of 17 detectable IUPM of resting CD4+ cells at baseline. A significant mean reduction of 39.7 % was observed in total HIV-1 DNA up to 6 weeks after romidepsin treatment and 14 out of 16 tested individuals decreased in total HIV-1 DNA until day 161. Integrated HIV-1 DNA decreased from baseline eight weeks after romidepsin treatment without statistical significance. The mean of IUPM of CD4+ cells decreased by 38.0 % in six participants.

The direct effect of romidepsin on chromatin was measured through histone H3 acetylation assessment in lymphocytes by flow cytometry. In all 17 participants, H3 histone acetylation increased rapidly within hours of each romidepsin administration and then

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decreased 3 to 7 days after infusion. Viral transcriptional activity significantly decreased from baseline to day 105 after vaccination (cell-associated unspliced HIV-1 RNA). There were also significant increases in cell-associated unspliced HIV-1 RNA about 30 min after each romidepsin infusion. Overall, this dual intervention significantly reduced the HIV-1 reservoir, significantly decreasing total HIV-1 DNA and replication competent virus in patients with assessable data. Despite the promising results of this combined intervention, it did not prolong time to viral rebound during ART interruption. The authors declared that further optimization should be required to achieve a significant impact on the latent reservoir, resulting in clinically measurable benefits for people living with HIV-1 [60].

The continuing REDUC B followed in 2017, as a single-arm phase IB/2A trial aimed to evaluate the immune response following Vacc-4x and rhGM-CSF immunization [61]. Correlation between the immune responses and the HIV reservoir measurements reported in the previous REDUC trial (previously described) was set as endpoint. This time, participants were on cART and received six Vacc-4x intradermal immunizations, using rhGM-CSF as local adjuvant. It was followed by three weekly intravenous infusions of romidepsin. Seventeen patients completed all the immunization protocol.

The frequency of HIV-infected cells was measured by total and integrated HIV DNA quantification, the latent reservoir by quantitative outgrowth assay, and the cellular response by IFN-γ ELISPOT and T-cell proliferation using overlapping p24 15-mer peptide antigens (1 μg/mL). ELISPOT or T-cell proliferation 'responders' were defined as having a negative response to the peptides at baseline and positive response post-baseline, or more than a two-fold increase from baseline to a post-baseline time-point.. Intracellular cytokine staining (ICS) and viral inhibition were assessed both using thawed PBMCs. T-cell memory regulatory T-cells and natural killer subsets were assessed by flow cytometry.

REDUC B was the first clinical trial to show reductions in total DNA, integrated DNA and latent reservoir when combining immune-based therapy with latency reversal as part of a 'shock and kill' approach [61]. The reduction of total HIV DNA did not get any effect in the delaying of viral rebound and the percentage of NK cells increased following the vaccination but without any correlation with the HIV reservoir measurements. A non-significant and reversible reduction of CD8+ T-cell proliferation during romidepsin treatment was observed, consistent with other reports. Moreover, a transient increase in the percentage of CD4+/CD39+ Treg cells was attained during this phase. Regarding the ELISPOT assay, only one participant could be defined as a Vacc-4x 'responder', while six participants could be defined as 'responders' considering CD8+ T-cell proliferation assay, and a higher inhibitory activity post-immunization was found. The authors remarked that the clinical trial had limitations such as the small size of the sample, the absence of the control groups required, and the fact that HIV reservoirs were only measured in PBMCs. They further recommended caution with the interpretation of results, since immunogenicity was modest. Larger placebo-controlled trials must be carried out to explore the potential of this particular antigenic combination.

Conclusions

Currently, the correlates of protection for HIV infection are still unknown and researchers try to find the best antigen/adjuvant/dose/schedule for innovative vaccine design. The lack of good HIV animals models makes difficult to know whether the preclinical results will be predictive of vaccine-induced protection in humans, since such information will only result from human trials. Despite the failure, innumerable lessons have been learned. In this context, the inclusion of Gag-p24 epitopes should be considered in any strategy to induce a Th1 response with induction of CTL to control the HIV viral load. The p24 and p17 proteins appear to be the signature antigens in gag-based HIV vaccines, including their roles during virion assembly. Neither p6 nor p7 Gag products have been associated with effective anti-HIV cellular response. This could be influenced by the very limited number of T cell epitopes reported within them and their lower amounts inside mature viruses in comparison with p24 or p17 antigens. Hence, despite the absence of any remarkable results in the abomentioned vaccine trials, Gag proteins or their peptides could be regarded as central for any vaccine strategy to contain viral replication or achieve viral eradication. So far, researchers worldwide are committed to develop new candidates through novel technological approaches, with Gag antigens contributing to it.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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